Modulation of the Permeability of Human Endothelial Cell Monolayers

STELLINGEN

1 Gezien de verschillen in de regulatie van de permeabiliteit tussen humaan en dierlijk endotheel *in vitro* is het noodzakelijk nader uit te zoeken of hier *in vivo* verschillen dan wel *in vitro* artefacten aan ten grondslag liggen.

2 De conclusie van Mizuno-Yagyu et al. ten aanzien van de aanwezigheid van tight junctions tussen endotheelcellen op grond van weerstandsmetingen is op z'n minst voorbarig te noemen.
Mizuno-Yagyu et al., Biochem Pharmacol 1987, 36: 3809-3813.

3 De relatief hoge waarde voor de basale passage-snelheid van macromoleculen door endotheel-monolagen *in vitro* is mogelijk niet het gevolg van een niet intakte cel-monolaag maar van het ontbreken van een intakte basaal membraan *in vitro*.

4 Bij een *in vitro* onderzoek naar de rol van very low density lipoproteinen in het proces van arteriosclerose dient meer rekening te worden gehouden met de verschillen tussen preparaten als gevolg van de heterogeniteit van dit lipoprotein-deeltje bij verschillende donoren.
Mulder et al., Gaubius Instituut, in preparation.

5 De bevinding dat humane endotheelcellen, 24 uur na stimulatie met hoge concentraties TNF, "urokinase-type plasminogen activator" specifiek naar de basolaterale zijde van het endotheel uitscheiden, geeft aan dat de barrière functie van het endotheel ook in deze omstandigheid nog nagenoeg intakt is.
Van Hinsbergh et al., Blood 1990, 75: 1991-1998.

6 De recente vondst van een punt-mutatie in de androgeen-receptor van de humane prostaat kanker cellijn LNCaP geeft niet alleen antwoord op veel onverwachte resultaten met betrekking tot de receptor-specificiteit van deze cellijn, maar geeft bovendien de mogelijkheid de werking van anti-androgenen nader te bestuderen.
Trapman et al., J Ster Bioch 1990, in press.

7 Bij het gebruik van wiskundige modellen voor het berekenen van biochemische processen of ethologische parameters dient er altijd rekening mee gehouden te worden dat onder gestimuleerde condities het model niet meer geschikt is om berekeningen mee uit te voeren. Een correctie op het model is meestal noodzakelijk maar wordt helaas vaak achterwege gelaten.
Haccou et al., Behavioural Processes 1988, 17: 145-166.

8 Vrijwel ieder wetenschappelijk onderzoek heeft na vier jaar meer nieuwe vragen opgeroepen, dan het oude heeft beantwoord. Hoewel dit verschijnsel inherent is aan wetenschap en het bovendien weer werk oplevert voor nieuwe aio's geeft het de vertrekkende promovendi het gevoel dat hun werk niet "af" is.

9 Het afschaffen van stellingen weerspiegelt niet alleen een verminderd universeel karakter der universiteiten maar leidt ook tot een verminderde leesbaarheid van het proefschrift bij ontvangst door de gemiddelde lezer(es).

10 Het anti-AIDS-middel van Professor Buck is net als Buckler bier: de belangrijkste stof is in onvoldoende mate aanwezig.

11 Voor zowel roeien als kanoën geldt dat je moet kunnen "roeien met de riemen die je hebt".

12 Hoewel het schrijven van een proefschrift gepaard gaat met stress en stress tot de risicofactoren voor hart- en vaatziekten behoort, mag nog niet geconcludeerd worden dat promoveren slecht is voor hart en vaten.

Leiden, november 1990 Erna Langeler

Modulation of the Permeability of Human Endothelial Cell Monolayers

Proefschrift
ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden
op gezag van de Rector Magnificus Dr. J.J.M. Beenakker,
hoogleraar in de faculteit der Wiskunde en Natuurwetenschappen,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 29 november 1990
te klokke 15.15 uur

door

ERNA GERDA LANGELER

geboren te Zutphen in 1962

Promotiecommisie:

Promotoren:	Prof. dr. P. Brakman Prof. dr. W.C. Hülsmann (Erasmus Universiteit Rotterdam)
Copromotor:	Dr. V.W.M. van Hinsbergh (Gaubius Instituut TNO)
Referent:	Dr. Ph.G. de Groot (Rijksuniversiteit Utrecht)
Overige leden:	Prof. dr. M.R. Daha Prof. dr. R. van Furth Prof. dr. J.F. Jongkind (Erasmus Universiteit Rotterdam) Prof. dr. G.J. van der Vusse (Rijksuniversiteit Maastricht)

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Aan iedereen, die de afgelopen vier jaar heeft meegedacht, meegeholpen of meegeleefd.

CHAPTER 1

GENERAL INTRODUCTION

Introduction

This chapter presents some background information about the study entitled "Modulation of the Permeability of Human Endothelial Cell Monolayers". In the first part of this chapter various functions and types of endothelial cells will be summarized. One of the functions of the endothelial cells is the control of the passage of fluid, solutes and macromolecules through the endothelium into the tissues. Various hypotheses about the mechanisms underlying this passage process are discussed in the second part of this chapter. Under pathological conditions (e.g. inflammation) vascular permeability can increase. This happens when chemical mediators, released from storage cells in the tissue, specifically activate the endothelium and, within seconds, completely change the permeability characteristics of the microvascular membrane. Furthermore, various (vascular) diseases such as arteriosclerosis, cancer and pulmonary diseases are associated with an increased endothelial permeability. The relation between these diseases and endothelial permeability is discussed in part three. Subsequently, various options for the study of endothelial permeability are summarized (1.4).

1.1 ENDOTHELIAL CELLS

1.1.1 Functions of Endothelial Cells

The endothelial lining of the blood vessels is one cell layer thick and is strategically located at the interface between the vascular tissues and the circulating blood. It has been estimated that the total amount of endothelial cells in the human body is about 720 gram, while the total capillary surface covered by endothelial cells is about 600 m^2 (Wolinsky 1980). In the last 20 years, it has become clear that endothelial cells are involved in many (patho-) physiological processes. One important function of the endothelium is to prevent leakage of fluid and blood components into the tissues, in order to maintain the circulating volume. In this function, the endothelium selectively

and actively controls the passage of macromolecules. Among other functions, endothelial cells prevent the coagulation of the blood (Dahlback 1986, Esmon 1987, Jaffe 1987) and also produce factors that are involved in the regulation of fibrinolysis (Levin and Loskutoff 1982, Emeis and Kooistra 1986, van Hinsbergh 1988). They metabolize and respond to vasoactive substances (Majno et al 1969, Heltianu et al 1982, Rotrosen and Gallin 1986, Haddock et al 1987) and play a role in the regulation of the vascular tone (Yanagisawa et al 1988, Vanhoutte and Miller 1989). They also respond to cytokines and growth factors (Cotran 1987, Mantovani and Dejana 1989), synthesize important regulatory mediators (Wallis and Harlan 1986) and are the prime cell type involved in angiogenesis (Folkman et al 1982, Sprugel et al 1987, Bussolino et al 1989).

1.1.2 Types of Endothelial Cells and their Junctions

Endothelial cells are present in all tissues in the human body and, depending on the particular function of this tissue, the integrity of the endothelial monolayer differs. Three main types of endothelial cells can be recognized. Firstly, in the brain, endothelial cells form a very tight monolayer. Through this type of monolayer, intercellular passage is virtually absent; passage occurs via a vesicular transport, which can be regulated. The second type of endothelium is the continuous, non-fenestrated endothelium. This endothelial type represents the continuous endothelia, such as in muscle capillaries and in large blood vessels and diaphragmed endothelia, which are encountered in organs, such as the visceral bed and the lungs. The cells form a one thin layer in which cells make close contact to each other, while large perforations are rare. Thirdly, in organs like liver, adrenals and bone narrow large fenestrae (400 Å) in the endothelial monolayer exist, while in the spleen other discontinuities (larger than 1000 Å) between the endothelial cells are present. Central to the interrelationships between the endothelial cells in respect to the different functions are the interendothelial junctions: tight and gap junctions (McNutt and Weinstein 1973) (figure 1).

Tight junctions are structures, located at the interface between the cells. The major biological function of tight junctions are: a) to form a barrier to paracellular permeability (Schneeberger and Lynch 1984) b) to maintain cellular apical-basal polarity (Schneeberger and Lynch 1984) and c) to aid in intercellular adhesion (McGuire and

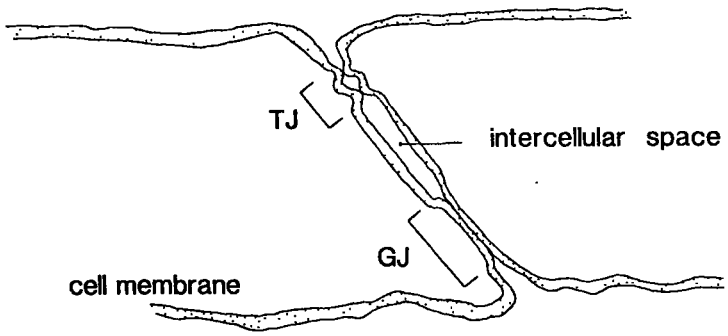

figure 1 Schematic view of two adjacent cells, these cells are connected by one tight junction (TJ) and one gap junction (GJ).

Twietmeyer 1985). The variable distribution of tight junctions throughout the vasculature seems to be correlated with the variable permeability characteristics of different types of endothelia (Wissig 1979). In brain microvascular endothelium, which is a very tight endothelium, extensive junctional networks are present (Crone and Olesen 1982). On the other hand: the continuous endothelium of muscle capillaries is less tight. This lesser degree of tightness has been attributed to discontinuities in the tight junctional networks (Tani et al 1977).
Gap junctions provide adjacent cells with a direct pathway for exchange of ions and small molecules (Larsen 1983). Although information about the structure and composition of gap junctions is available, little is known about the biological role of gap junctions in endothelial cells. No evidence for a relationship between endothelial permeability and gap junctions has been described. Gap junctions are large and frequent in arteries and arterioles (Simionescu et al 1975, Hüttner et al 1973), smaller and less frequent in veins and muscle venules (Schneeberger and Karnovsky 1976) and rare in postcapillary venules and capillaries (Hüttner et al 1973).

1.2 ENDOTHELIAL PERMEABILITY IN "NORMAL" BLOOD VESSELS

1.2.1 Historical Overview

In 1896, Starling has already formed the hypothesis that the direction and rate of fluid transfer between plasma and tissue fluid is not only dependent on hydrostatic and osmotic factors across the membrane, but also on the physical properties of the

capillary membrane. Later on, Landis (1927) and Pappenheimer and Soto-Rivera (1948) verified this hypothesis. In 1951, Pappenheimer et al postulated that hydrodynamic flow becomes more important for large plasma proteins than for small molecules in the exchange process. Subsequently, Grotte described in 1956 a relationship between the molecular weight of various dextrans and the passage rate of these molecules. From these studies, the "pore theory" was postulated. Molecules can pass the endothelium through large and small pores. Today, there seems little doubt that the interendothelial clefts are the structural equivalent of the small pores (Crone and Levitt 1984). The identification of the large pores has been hampered by their extreme rarity. Possible candidates have been indicated: widened endothelial junctions, transendothelial channels created by fusion of plasmalemmal invaginations, shuttling of free vesicles and fenestrae. On the basis of Grotte's results (1956), it has been calculated that one large pore for every 30.000-40.000 small pores was needed to explain the size dependent selectivity of the endothelium to macromolecules. From lymph protein fluxes, a ratio of 438:1 was calculated (Taylor and Granger 1984). The "pore theory" is based on the presumption that transport of large weight plasma proteins occurs by interendothelial junctions. Many attempts have been undertaken to measure the (theoretical) pore diameter. Although values vary for different types of endothelial cells, small pores appeared to be smaller than 100 Å, while large pores are in the order of several hundred Å.

Permeability studies have been carried out in whole organ models (Renkin 1959, Crone 1963, reviews: Taylor and Granger 1984, Renkin 1985). From these classical studies, it was concluded that the endothelium is a single passive, semi-permeable membrane. Later on, permeability studies were performed with single capillary techniques (reviews: Gore 1982, Curry et al 1983, Crone and Levitt 1984, Michel 1984, Crone et al 1989). These experiments established the pore theory (Zweifach and Intaglietta 1968). In addition, from these kind of studies, Curry and Michel (1980) postulated a fiber matrix model. The entry of solutes into the junctional pathway and the diffusion of solute within the junctional pathway are regulated by the size and distribution of a network of fibrous molecules within the wide part of the junction. The interaction between albumin molecules and the side chains of proteogyclan aggregates (both present in the fiber matrix) is important and requires positively-charged arginine groups of the albumin molecule (review: Curry 1986).

Probably, the lysine groups of the albumin are also involved, because Schneeberger et al (1990) have shown that after modification of the albumin molecule with cyclohexane-dione (blockade of the arginine residues) or by succinylation (modification of the lysine residues) the passage of ferritin through the endothelium in vivo increases, suggesting that the modified albumin molecules have lost their ability to keep the monolayer intact.

In the mid sixties, it became clear that the endothelium is not always a passive element in the exchange of solutes. Majno et al (1961, 1967) and later on, Persson and Svensjö (1985) showed that, especially under pathological conditions, endothelial cells may actively regulate vascular permeability.

Once, the possibility to culture human endothelial cells (Jaffe et al 1973, Gimbrone et al 1973) existed, the knowledge of the endothelial cell was further enlarged. In vitro, growth and specific functions of the endothelial cells could be studied. After the discovery and use of an endothelial cell growth factor for bovine endothelial cells (Gospodarovicz et al 1976) and for human endothelial cells (Maciag et al 1979), it became possible to subculture endothelial cells for a prolonged period of time, and to keep them for prolonged periods as a healthy monolayer. With the use of the growth factor, it became possible to culture endothelial monolayers, which form a barrier for the passage of macromolecules, with characteristics approximating the in vivo situation. This has previously been done with animal endothelial cells, but also appears possible for human endothelial cells, as described in this thesis.

1.2.2 Transport Pathways

As described above, the endothelial cells form the inner lining of the blood vessels, through which macromolecules can be transported. In fenestrated vascular beds it is generally accepted that the fenestrae represent the major route of macromolecular transfer (Bundgaard et al 1983). In the study described in this thesis, only relatively tight endothelia have been studied. Macromolecules can exchange across these endothelial linings either by an intracellular route: by direct diffusion across the cell (figure 2, route a) or by transendothelial channels formed by temporary or permanent connections between the two sets of vesicular invaginations (route b), or by an intercellular route: leaks between the endothelial cells (route c) (Renkin 1977, Renkin 1988). It has also been suggested by Scow et al (1980) that small lipophilic

agents like cholesterol, phospholipids from chylomicrons and free fatty acids may cross capillary endothelium by lateral movement in cell membranes (figure 2, route d).

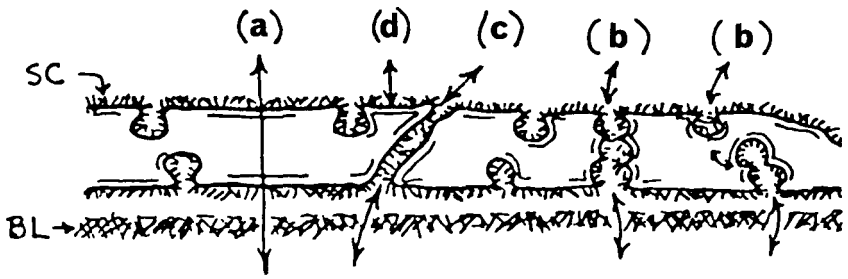

figure 2 : Various transport pathways across a nonfenestrated endothelial monolayer: (a): direct diffusion of small molecules through the membrane, (b): transport via vesicles, (c): intercellular transport and (d): lateral movement across the membrane. BL: Basal Lamina, SC: Cell Surface Coat. (From: Renkin 1977).

There is some debate about the mechanism by which macromolecules may cross the endothelium. Several investigators believe that the significant number of molecules is transported via vesicles (Clementi and Palade 1969, Stein et al 1973, Vasile et al 1983), while others think that this pathway is hardly involved in the process. In the latter opinion, vesicles do not exist freely within the cytoplasm, but represent invaginations of the plasma membrane (Bundgaard et al 1983, Frokjaer-Jensen 1984). From electron microscopical studies, in which fixed cells are used, no definitive conclusions can be made. The appearance of particles in vesicles is not enough to support the theory that vesicles are involved in the passage process, because it is not clear that the particles are taken up at one side of the cell (endocytosis), transported through the cell (transcytosis) and extruded at the other side of the side of the membrane (exocytosis). The presence of particles in the intercellular space is difficult to establish using microscopical techniques.

In the case of the passage of ions, Hodson and Wigham (1987) calculated that because of the low electrical resistance across the endothelium, 99% of these particles have to be transported by the paracellular route.

In contrast to the disagreement about the transport mechanism of macromolecules in normal endothelium, it is generally accepted that in pathological situations, in which the permeability is increased, especially the intercellular passage has been increased.

1.2.3 Modulation of Endothelial Permeability

A highly selective endothelial monolayer of a continuous microvascular bed can be transformed within seconds into a less selective filter containing numerous large pores. The level of the increase in permeability depends on the type of microvascular bed. The reaction is predominantly present in the postcapillary venules, and commonly absent in larger veins, capillaries and arterioles (Grega et al 1988). The clinical manifestations of the microvascular changes are local flare, swelling, heat and pain.

The change may be induced by infectious, traumatic, chemical or immunological stimuli but is not usually caused by the stimulus directly, but by specific chemical mediators released from storage cells or synthesized "de novo" at the inflammatory site. Classical mediators are histamine (derived from mast cells), bradykinin (formed enzymatically in plasma) and basophile leucocytes (which can be activated by other mediators). More recently, many metabolites of arachidonic acid (thromboxanes and prostaglandins) have been implicated as participants in inflammatory reactions (Johnson et al 1985).

The release of inflammatory mediators leads to a manifold increase in both the influx of fluid and solutes into the tissues and in the normally very low macromolecular permeability. Leakage of albumin causes edema and increased lymph formation. These effects have been well documented at the level of the whole organ (Grega et al 1979, Parker et al 1984, Svensjö et al 1978) as well as in single microvessels (Curry et al 1986, Huxley et al 1987).

Electron microscopical studies show that mediator-mediated stimulation of vascular permeability causes changes in endothelial cell shape and formation of large inter-endothelial gaps (Majno and Palade 1961, Majno et al 1969, Fox et al 1980). The first studies about the underlying cellular actions linking mediator stimulation and changes of endothelial cell shape pointed to the importance of Ca^{++} as second messenger for the increases of the endothelial permeability (Haraldsson et al 1986, Lückhoff and Busse 1986, Rotrosen and Gallin 1986).

In addition, the involvement of the cytoskeleton in endothelial permeability has been described (Shasby et al 1982, Schnittler et al 1989, Wysolsmerski and Lagunoff 1990). However, at the start of this study the underlying mechanism was still unknown.

1.3 ENDOTHELIAL PERMEABILITY IN VASCULAR DISEASES

1.3.1 Endothelial permeability in various vascular diseases

Permeability to plasma components is strikingly enhanced in various situations. The most common example is the mosquito bite. As a reaction on the locally released histamine, the postcapillary venule endothelium becomes leaky, and fluid and plasma components penetrate into the tissue.

In various diseases, such as inflammation, cancer, idiopathic edema, pulmonary diseases and arteriosclerosis, an increased endothelial permeability may play a crucial role. In addition to well known inflammatory mediators, new very active biological mediators have recently been discovered, such as the so-called vascular permeability factor, which is secreted by tumour cells and causes normal blood vessels to leak plasma proteins (Senger et al 1983, Keck et al 1989).

In addition, in the normal vasculature, in which endothelial cells show no visible alteration, the influx of macromolecules can be locally increased (Stemerman et al 1986). This suggests that the function of endothelial cells can locally be in disorder; however, the biochemical background of this process is poorly understood.

1.3.2 Endothelial Permeability in the Lung

Bronchial airway disease is associated with increases in macromolecular permeability (Persson et al 1982, Persson 1987). In many species, there is a richly-developed capillary-venular barrier situated just beneath the epithelium. Although two barriers are involved, exposure to an inflammatory stimulus produces a change in the permeability of the endothelial-epithelial barrier. In allergic airway disease, plasma excudation may be an important factor in the pathogenesis of the disease. The physical and physiological aspects of an increased microvascular and epithelial bronchial permeability suggest the involvement of this mechanism in several facets of asthma pathology.

Cancer patients being treated with interleukin-2 (IL-2) have great problems with the vascular leakage syndrome (Lotze et al 1986). The pathogenesis of this toxic effect is unknown. It has been suggested by Cotran et al (1987) that IL-2 induces the production of other cytokines as tumor necrosis factor, interferon-gamma and interleukin 1. These substances may activate the endothelium, through which more substances can be transported. On the other hand, experiments from Damle and

Dayle (1989) showed that a direct cell-to-cell contact between IL-2 activated lymphocytes and endothelial cells is necessary and sufficient to cause increased endothelial permeability in an in vitro model system.

1.3.3 Endothelial Permeability and Atherosclerosis

One of the underlying causes of many vascular problems are atherosclerotic plaques, which are rich in cholesterol, fibrin, leucocytes, monocytes-derived macrophages and smooth muscle cells. A relation between the initiation of atherosclerosis and endothelial permeability has already been postulated as early as 1856. In this year, Virchow described the hypothesis that plasma components, which passively pass through the endothelium, accumulate in the subendothelial space, where they can form an atheroma. Another theory about the initiation of atherosclerosis, the "encrustation theory" often ascribed to Rokitansky, hypothesised that small thrombi, composed of platelets, fibrin and leukocytes, growth over foci of endothelial injury and are responsible for plaque formation (Duguid 1949). The most recent, generally accepted hypothesis about the formation of atherosclerosis, is a composition of three components: the "lipid infiltration model" (Roach et al 1976, Watanabe 1980, Sorlie et al 1981), the "response to injury theory" in which Virchow's ideas are incorporated (Ross and Harker 1976, Ross 1986) and a model in which the smooth muscle proliferation in the intima (especially induced by platelet derived growth factor, Ross and Vogel 1978) induced by other factors than endothelial damage, is the crucial initial event for atherosclerosis. In the first component of the hypothesis, it has been suggested that an elevated plasma cholesterol concentration, particularly that present in the LDL fraction, promotes atherosclerosis. The second one suggests that repeated episodes of endothelial damage caused by e.g. hypertension, smoking or immune injury lead to the development of atheromata. The third aspect of the theory about the initiation of atherosclerosis describes the mechanisms by which smooth muscle proliferation occurs (particularly the role of growth factors and mutagens and smooth muscle injury). In the combined model, the three hypothesises are taken together, in this model an elevated LDL concentration is one of the causes of an endothelial injury, which can result in smooth muscle proliferation.

Several in vivo studies support the idea that an elevated serum cholesterol level may contribute to an increased endothelial permeability and may subsequently result in an

increased accumulation of macromolecules in the subendothelium. Studies from Stefanovich and Gore (1971) and Stemerman (1981) have shown an increased permeation of material into the vessel wall in animals with elevated levels of serum cholesterol, induced by an inborn error of LDL metabolism or a high fat diet. In addition, Schwenke and Carew (1986 a,b) showed a rapid, marked and focal increase in the concentration of intact LDL within the arterial wall of cholesterol-fed rabbits. If the influx of material is larger than the efflux, substances can accumulate in the subendothelial space (Nordestgaard et al 1990). A series of pathological changes occurs, leading to development of atherosclerosis.

Furthermore, the involvement of endothelial injury in the atherosclerotic process, has been shown in studies of Davies et al (1988). In these studies it was shown that the endothelial surface of the right coronary artery from patients with atherosclerosis, showed widespread focal abnormalities of the endothelial cells ranging from adhesion and migration of monocytes, on and deep into the surface, as well as adhesion of platelets to the exposed subendothelial tissue. After regeneration of the endothelium, the influx of macromolecules as measured for peroxidase, remain high for an extended period (Webster et al 1974).

The source of most of the cholesteryl ester present in the atherosclerotic lesions is most likely plasma lipoproteins as suggested by the work of several investigators (Newman and Zilversmit 1962, Zilversmit 1968, Dayton and Hashimoto 1970). So, accumulation of the lipoproteins depends on the balance between influx and efflux of lipoproteins through the arterial endothelium.

Three observations further support the idea that an elevated plasma LDL-concentration (and thus an elevated endothelial permeability) may contibute to an increased plaque formation. Firstly, an elevated plasma LDL-cholesterol level has been identified as an important risk factor for heart and vessel diseases and has been correlated with the formation of atherosclerosis (Goldstein and Brown 1977, Steinberg 1983, Tyroler 1987). Secondly, the influx of LDL in the unaffected vessel wall is linearly correlated with the plasma concentration of LDL (Stender and Hjelms 1988) and thirdly, intact LDL has been identified in the atherosclerotic lesions of several species, including humans (Hollander et al 1979, Nicoll et al 1981).

However, from all these studies, it is not clear whether, an increased endothelial permeability is the cause or the consequence of formation of an atherosclerotic

plaque. If atherosclerotic plaques are formed, it is clear, that at that moment, LDL and other molecules enter at increased rates into these parts of the arterial wall (Bremmelgaard et al 1986).
In vitro studies have suggested that if oxidative modification were to occur in the arterial wall in vivo, it might contribute to the atherosclerotic process in several ways (Morel et al 1984, Quinn et al 1987, Parthasarathy et al 1987). Their oxidative products may effect the endothelium and increase its permeability (Hennig and Boissonneault 1987, Hennig and Watkins 1989). Recently, studies from Carew (1989) have shown that oxidative modification of LDL indeed occurs in vivo; both malondialdehyde conjugated-LDL (MDA-LDL) (Haberland et al 1988) and oxidized forms of LDL are demonstrated in the atherosclerotic lesions (Goldstein et al 1981).

1.4 VARIOUS MODELS TO STUDY ENDOTHELIAL PERMEABILITY

1.4.1 Introduction

Endothelial permeability can be studied in various models. From in vivo or ex vivo experiments usable results have been obtained. In vivo experiments in humans (Nicoll et al 1981, Stender and Hjelms 1987) showed that the influx of LDL and HDL is much larger in atherosclerotic plaques than in normal areas of the vessel wall. From animal experiments of Mason et al (1977) and Curry (review: 1986) it has become clear that both the presence of calcium ions and plasma proteins are necessary to keep an intact endothelial barrier. Histamine, leukotrienes, platelet activating factor, immune complexes, bradykinin, complement fragments and free radicals are among the many inflammatory mediators known to induce visible leakage of macromolecules from animal postcapillary venules (review: Olesen 1989). In the mid seventies, agents were discovered which inhibited mediator-stimulated formation of venular leakage sites and edema formation. They include the β-adrenergic stimulants and glucocorticoids (see discussion chapter 4). Some of these components have particular applications in patients whose vascular permeability has been increased by toxic, mechanic or other injury. Although, in vivo experiments have many advantages, these experiments also have some limitations: the endothelium is relatively inaccessible, no general lymph-pool is present, and effects on other cell types or other general parameters may mask or worsen the effect on the endothelium. Furthermore, especially in the case of human subjects many experiments are prohibited for ethical

reasons. Therefore, an in vitro model would be useful to obtain important, additional information about the endothelial permeability characteristics.

1.4.2. Basal Requirements of In Vitro Endothelial Monolayers

Introduction

From in vivo experiments certain information is available about the passage of macromolecules through the vascular endothelium and in vitro models should reflect these known characteristics. They include a transendothelial electrical resistance (TEER), a dependency of the molecular mass on the passage rate, the presence of plasma proteins and calcium ions to keep the endothelial monolayer intact and tight junctions between adjacent cells.

Transendothelial electrical resistance

The transendothelial electrical resistance (TEER) has been measured by various authors. Although the component in the endothelial monolayer responsible for the measured value has yet to be identified, it is generally accepted as a value for the intactness of the endothelial monolayer. The value is possibly related to the integrity of the tight junctions between the adjacent cells as suggested by the correlation between the passage of small ions through and the TEER across the monolayer (Olesen and Crone 1983, Navab et al 1986). In table 1 some published values for in vivo observations are listed. The resistance across various types of endothelial cells is low in comparison with the electrical resistance across brain endothelial monolayers and also low in comparison with monolayers of epithelial cells (Powell 1981). This discrepancy is probably caused by differences in the complexity of the tight junctions between the various kind of cells.

Sieve characteristics

In vivo studies have shown that large molecules penetrate more slowly into the arterial wall than small molecules. This phenomenon was studied for both variously sized dextrans (Grotte 1956, Carter et al 1974) and variously sized lipoproteins (Stender and Zilversmit 1981, Stender and Hjelms 1988). Very large lipoproteins such as very low density lipoproteins and chylomicrons can hardly enter the arterial wall (Nordestgaard et al 1988a,b, 1989). The molecular sieve characteristics of the

endothelial monolayer seem to be dependent on the integrity of the monolayer. Treatment of the endothelial cells with histamine (which increases the permeability) results in a smaller difference between the passage rate of large molecules versus small molecules (Carter et al 1974). It has been suggested by Curry (review 1986) that the molecular selectivity of the endothelial monolayer is caused by the size and distribution of a network of fibrous molecules (especially albumin) on the cell surface and within the cellular junctions.

table 1 Electrical resistances across endothelial monolayers in single capillaries.

first author	species	tissue	electrical resistance ($Ohm.cm^2$)
Crone (1982)		brain	1000-2000
Crone (1982)	frog	muscle	23-36
Olesen (1983)	frog	mesenteric	1-3
Olesen (1984)	frog	skin	24-70
Olesen (1985)	frog	connective tissue	
		-arterial	18
		-venous	3.5
O'Donnell (1986)	rabbit	aorta	10

Dependency of plasma proteins and calcium ions

As mentioned earlier, the integrity of the endothelial monolayer in vivo is dependent on the presence of plasma proteins (especially intact albumin) (Levick and Michel 1973, Mason et al 1977, Michel et al 1985, Schneeberger et al 1990) and on the presence of calcium ions (Chambers and Zweifach 1940, Busch et al 1989). The glycocalyx and the cellular junctions appear to be especially sensitive to the removal of these components (Curry and Michel 1980, Schneeberger and Hamelin 1984).

Tight junctions

The type of endothelial cells, as used in this study, is characterized in vivo by intercellular junctions; both tight and gap junctions are found (Simionescu et al 1976, Schneeberger and Lynch 1984)

1.4.3 In Vitro Models

In 1981 the first report was published on the culture of bovine aorta endothelial cells on porous polycarbonate membranes (Taylor et al). The passage of both albumin and neutrophils through these monolayers was largely restricted as compared with membranes without cells. In the same year McCall et al (1981) described a system for the culturing of porcine aorta endothelial cells on porous PTFE-membranes. In this report it was shown that high concentrations of cells have to be seeded on the membranes to obtain a confluent monolayer. The passage of both fluid and albumin under "normal" conditions through these monolayers was described. One year later, Shasby et al (1982) described the importance of the cytoskeleton in the maintenance of the porcine pulmonary endothelial barrier function, using a diffusion model. Later on, Baetscher and Brune (1983) described a model to study porcine endothelial permeability under hydrostatic pressure. In 1985, the first experiments were published in which the influence of biological agents on animal endothelial permeability was studied (Hennig et al, Shasby et al). In 1986, Killackey et al described an in vitro model in which human umbilical vein endothelial cells were cultured on micro-carriers. The cells prevented the staining of the microcarriers with Evans blue dye. After exposure of the cells to histamine or thrombin (but not bradykinin) the staining increases.

1.5 AIM OF THIS STUDY

At the start of this study, in 1986, several models had been described for the study of animal endothelial permeability. However, no in vitro model was available to study human arterial endothelial permeability. The first goal of this study was therefore:

-To set up a model to study the permeability characteristics of human arterial endothelial cell monolayers.

The characteristics of the human endothelial monolayers were compared with in vivo observations on electrical resistance, morphology (electron microscopy, tight junctions), molecular sieve characteristics, dependency of plasma proteins and of calcium ions.

In the atherosclerotic plaque of the arterial wall, the passage of lipoproteins has been increased. Before evaluating the underlying mechanism, it was necessary:

-To study the passage process of low density lipoproteins.

This passage process has been described in chapter 3. It appeared to be a rather a-selective process. Therefore, as a marker protein, peroxidase has been used in further studies.

In vascular diseases many substances may interact with the arterial endothelium, and may activate it. So the third goal of this study was:

-To study the effects of various groups of substances (vasoactive- and inflammatory-substances, catacholamines, lipoproteins and fatty acids) on endothelial permeability.

and in addition:

-To explain the observed effects on the biochemical level.

In the first instance human umbilical artery endothelial cells have been used, and subsequently the observed effects have been checked with human aorta (sometimes carotid artery and vena cava) endothelial cells.

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CHAPTER 2

CHARACTERIZATION OF AN IN VITRO MODEL TO STUDY THE PERMEABILITY OF HUMAN ARTERIAL ENDOTHELIAL CELL MONOLAYERS

Erna G. Langeler and Victor W.M. van Hinsbergh

Gaubius Institute TNO, Leiden, the Netherlands

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ABSTRACT

A model has been developed to study the transport of fluid and macromolecules through human umbilical artery endothelial cell monolayers in vitro. Cells were cultured on fibronectin-coated polycarbonate filters and formed within a few days a tight monolayer, with an electrical resistance of 17 ± 4 Ohm.cm^2. The cells were connected by close cell contacts with tight junctions. The passage rate of horse-radish peroxidase (HRP) through these filters was 20-40 fold lower than through filters without an endothelial monolayer. The continuous presence of 10% human serum was needed to maintain the electrical resistance of the monolayer and its barrier function towards macromolecules. Chelation of extracellular calcium resulted in an increased permeability and a decreased electrical resistance of the monolayer. This process was reversible by re-addition of calcium ions to the cells. The permeation rate of dextrans of various molecular weights (9-480 kD) was inversely related to the molecular mass of the molecule. No difference was measured between the passage rate of dextran of 480 kD and dextran of 2,000 kD. Incubation of the endothelial cell monolayer with 2-deoxy-D-glucose resulted in a decreased permeability but it had no effect on electrical resistance. This suggests that the passage-process is energy-dependent.

Fluid permeation through the endothelial cell monolayer on filters was measured in a perfusion chamber under 20 mmHg hydrostatic pressure. It was decreased by the presence of serum proteins and responded reversibly on the chelation and re-addition of extracellular calcium ions.

INTRODUCTION

The endothelium is a metabolically very active monolayer of cells which lines the blood vessels, and thus represents the interface between blood and tissue. Except for liver, adrenals and bone narrow, in which the endothelial cells are fenestrated, the endothelium consists of a continuous lining of cells (1). One of its functions is the control of the influx of water, nutrients, and macromolecules into tissues (2,3). Particularly with respect to the influx of macromolecules, it has become clear that the endothelium acts not merely as a passive semipermeable membrane, but is actively involved in the passage process (4,5). Many pinocytotic vesicles are involved in the transcellular passage of macromolecules (2,6-8). This passage process has been

reported to be energy-dependent (9,10). On the other hand, in the microvasculature intercellular passage of macromolecules has also been demonstrated (4,5,11,12). Very little is known at present about the biochemical properties of the passage process.

An in vitro system of human endothelial cell monolayers cultured on filter membranes would be valuable to obtain information about the regulation of the passage process through these monolayers. Recently, several groups (10,13,14) have described in vitro models to study the passage of macromolecules through monolayers of animal endothelial cells in vitro. The cells of these monolayers are connected by tight junctions (13). Such endothelial cell monolayers have an electrical resistance of 10-15 Ohm.cm^2, a value that approximates to the electrical resistance of the endothelial cells of various vascular beds in vivo (15,16). These monolayers displayed molecular sieving characteristics (17,18) and were sensitive to removal of calcium from the medium by EGTA (19-21). So far, attempts to establish tight human endothelial cell monolayers have failed (22). Based on our experience with endothelial cells from human umbilical arteries, we investigated whether these cells could be a suitable source for a preparation of a tight monolayer of human endothelial cells. Here we report a procedure for the routine culture of tight human cell monolayer on polycarbonate filters and describe its characteristics, in relation to in vivo observations.

MATERIALS AND METHODS

Materials

Medium 199, supplemented with 20 mM Hepes was obtained from Flow Laboratories (Irvine, Scotland); Polytetrafluoroethylene (PTFE) filters supported by a polypropylene (PP) scrim were obtained from Gore (Putsbrunn, FRG); Transwell polycarbonate filters and all other tissue culture plastics were from Costar (Cambridge, MA). Collagenase-dispase and penicillin-streptomycin were purchased from Boehringer (Mannheim, FRG). A crude preparation of endothelial cell growth factor was prepared from bovine hypothalamus as previously described (23). Human fibronectin was a gift of J.A. van Mourik (Red Cross Central Blood Transfusion Laboratory, Amsterdam, The Netherlands). Human serum was prepared in our laboratory from fresh blood of healthy donors, pooled and stored at 4°C. It was not heat-inactivated

before use. Human plasma was prepared from blood that was anti-coagulated with 5 U/ml heparin. Newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands), dextrans, anthrone and horse-radish peroxidase (HRP) from Sigma Chemical Co. (St. Louis, MO).

Isolation and Culture of Endothelial Cells

Endothelial Cells from human umbilical arteries were isolated by 0.05% (w/v) collagenase-dispase treatment according to the method of Jaffe et al. (24). Cells were characterized as previously described (25). Cells were seeded on fibronectin-coated dishes and cultured in Medium 199 supplemented with 10% NBCS, 10% human serum, 150 μg/ml crude endothelial cell growth factor and 5 U/ml heparin. At confluency cells were released with trypsin/EDTA and transferred to new dishes with a split ratio of 1:3 for further propagation. For passage studies only cultures that had been passaged once or twice were used. The cells were seeded on Transwell polycarbonate filters (pore size 3 μm; exposed area 0.33 cm^2), which had been coated with 10 $\mu g/cm^2$ human fibronectin (30 minutes at room temperature). To obtain high density cultures confluent cells were detached from culture dishes and seeded on a filter surface that was two-fold smaller than the original dish surface. Four to 6 hours after seeding, non-attached cells were removed. Filters were incubated at 37°C under 5% CO_2/95% air in the same culture medium as given above, and the culture-medium was replaced every other day. Experiments were done 4-7 days after seeding the cells.

Passage Studies

The experimental design to study the passage of macromolecules through an endothelial monolayer is given in figure 1A. One hour before the start of an experiment, the medium was replaced by Medium 199 supplemented with 20% human serum and penicillin/streptomycin. At the start of an experiment, at the top of the endothelial cell monolayer (the upper compartment) a known amount of HRP in Medium 199 supplemented with 20% human serum was added, and at the other side (the lower compartment) Medium 199 with 20% human serum but without HRP was added. During the experiments, the filters were kept at 37°C. At several time-points

samples (50-100 μl) were taken from the lower compartment, and the same volume of Medium 199 with 20% human serum was replaced in this compartment to prevent fluid permeation due to hydrostatic pressure. The HRP concentration in the samples was determined spectrophotometrically after incubation with o-dianisidine and hydrogen peroxide. Passage rate is expressed in ng/h/well.

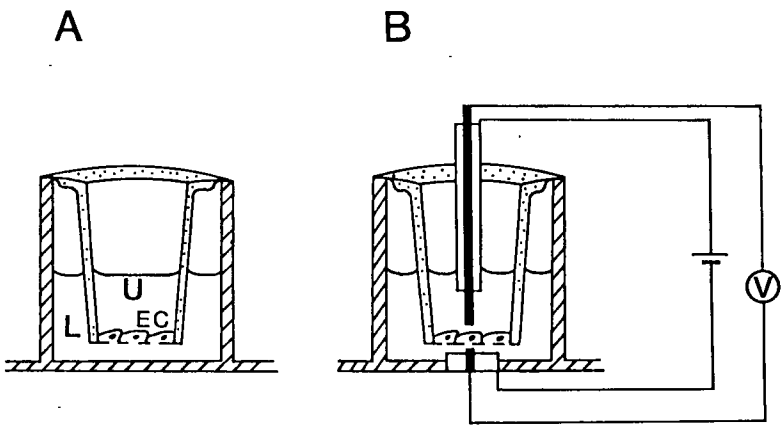

figure 1 A: Schematic view of the assay system for measuring the diffusion of macromolecules through monolayers of human umbilical artery endothelial cells. Polycarbonate filters were coated with fibronectin. Endothelial cells were seeded in a high density and cultured for 4-7 days to form a tight endothelial cell monolayer. During experiments, a known amount of HRP was added to the upper compartment (U); at several time-points a sample was taken from the lower compartment (L). Experimental details are given in Materials and Methods B: The trans-endothelial electrical resistance is measured in a special chamber. An alternating current (50 μA, 1 pulse/min) passes the monolayer by two source-electrodes (2 cm distance). Two other electrodes detect the potential difference across the monolayer (3 mm distance).

Passage studies of dextrans were done in a similar way. Samples containing dextrans were first dialysed against PBS to remove glucose from the medium. Subsequently, the dextran concentration was determined by the anthrone-method, as described by Scott and Melvin (26). The passage rates of all dextrans were determined through both fibronectin-coated, unseeded filters (P_F) and fibronectin-coated filters with an endothelial monolayer (P_{F+EC}). The permeability of the endothelial monolayer alone (P_{EC}) was calculated from

$$\frac{1}{P_{EC}} = \frac{1}{P_{F+EC}} - \frac{1}{P_F}$$

as described by Siflinger-Birnboim et al. (17).

Transendothelial Electrical Resistance

Transendothelial electrical resistance (TEER) was measured in a special chamber (figure 1B). An alternating current (50 μA, 1 pulse/min) was passed across the monolayer by 2 source electrodes (distance 2 cm) and the potential difference across the monolayer was measured by 2 detecting electrodes (distance 3 mm). Electrical resistance was calculated by Ohm's law and expressed in Ohm.cm^2. Electrical resistance of the filter without an endothelial monolayer (3.2 Ohm.cm^2) has been subtracted from all measured values.

Fluid Permeation Experiments under Hydrostatic Pressure

For fluid permeation experiments, a similar method was used to that described by Baetscher and Brune (21) with a few modifications. Polycarbonate filters (3 μm pores) or corona discharge treated PTFE/PP filters (0.4 μm pores) were mounted between two rings. The outer ring (Ø 25/20 mm) consisted of PTFE and the inner (Ø 20/15 mm) of perspex. The mounted filters were sterilized in 70% ethanol and rinsed in Medium 199; thereafter filters were coated with human fibronectin. Endothelial cells were seeded and cultured on these filters, as described above. Filters with an endothelial monolayer were placed between two chamber-halves, which were fixed together. Pressure was induced by a fluid column connected to the upper chamber-half (figure 2). Fluid that had been passed through the monolayer

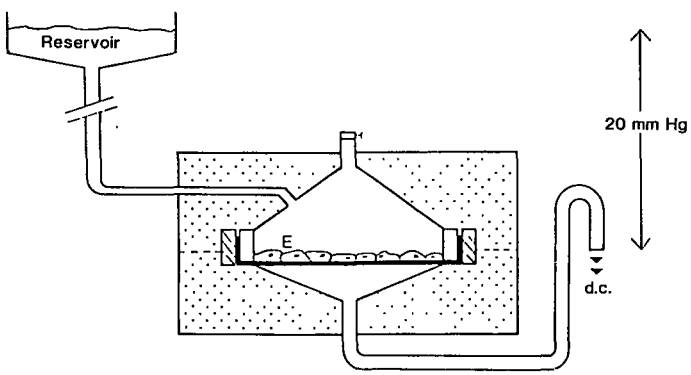

figure 2 Schematic view of the system for fluid permeation studies under hydrostatic pressure. Filters were mounted between two rings. Mounted filters with an endothelial monolayer (E) are placed in a special chamber. The upper half is connected to a reservoir containing Medium 199 supplemented with human serum. The drops that have passed through the monolayer are counted by a drop-counter (d.c.) The volume of the drops is determined by weighing.

was led through a small silicon tube, which ended at the same level as the endothelial monolayer. The drops were registered by a drop counter and the time intervals between the drops were recorded on a Kipp BD5 micrograph recorder. Calibration of the drop volume was performed by weighing the drops assuming a density of 1 g/ml. The rate of fluid permeation was expressed in microliters per minute. During the experiments, the temperature was kept at 37°C. Unless otherwise indicated, hydrostatic pressure was 20 mmHg.

RESULTS

When human umbilical artery endothelial cells were seeded in high density on fibronectin-coated polycarbonate filters, they formed a confluent monolayer. Three to 4 days after seeding, the cells had formed a tight monolayer with an electrical resistance of 17.0 ± 4.3 Ohm.cm^2 (*mean ± SD, 45 determinations, 10 cultures,* measured in 10% human serum). On electronmicroscopical examination, the cells were closely connected to each other by tight junctions (figure 3). Under our experimental conditions, the electrical resistance of the cell monolayers remained constant for another 4-7 days. All experiments were done between 4 and 7 days after seeding the cells.

figure 3 Thin section electron micrograph, showing a part of the endothelial monolayer, grown on a fibronectin-coated polycarbonate filter. Tight junctions are observed between the cells (indicated by arrows) (the bar represent 1 μm).

To evaluate the passage of macromolecules through the endothelial cell monolayer, we used horse-radish peroxidase (HRP) as a model protein. When HRP was added to the upper compartment of the diffusion assay system (figure 1A), a constant passage rate of HRP through the monolayer over a 24 hour period was found (figure 4). The passage of HRP increased linearly with the HRP concentration in the upper compartment (1-20 μg/ml) (data not shown). When 5 μg/ml HRP was added, the passage rate was 4.8 ± 2.7 ng/h/well (47 determinations, 14 cultures), while its passage through fibronectin-coated filters without endothelial cells was 20-40 fold higher (figure 4).

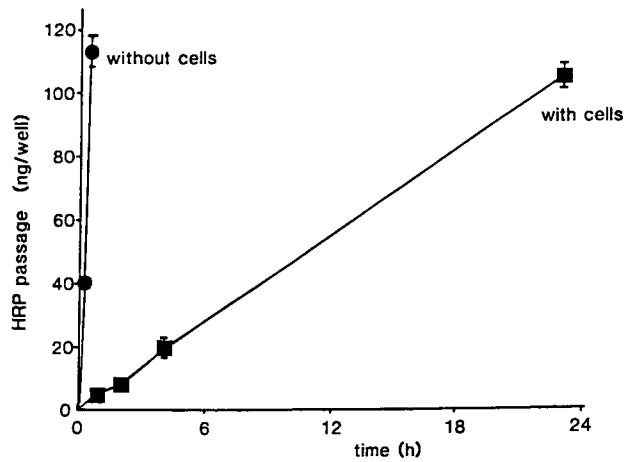

figure 4 Time course of horse-radish peroxidase (HRP) passage through fibronectin-coated filters covered with or without an endothelial monolayer. The passage assay was performed in Medium 199 with 20% human serum as described in figure 1A. Peroxidase (5 μg/ml) was added to the upper compartment at the start of the assay; peroxidase concentration in the lower compartment was determined at various time intervals.

Effect of Various Concentrations of Human Serum or Plasma on the Endothelial Monolayer

The presence of human serum proteins appeared to be necessary for the maintenance of the barrier function of the endothelial cells. When the serum concentration in the medium was decreased to 0 or 0.1%, the passage of peroxidase always increased (figure 5A). When the serum concentration was decreased to 1% the passage of peroxidase increased in the majority of the cell-monolayer tested (figure 5A). No differences between the peroxidase passage rates in 10, 20 or 50% human serum

were found (figure 5A). Similar effects of the presence of serum-proteins on HRP-passage were found with media that contained various concentrations of human plasma (figure 5B).

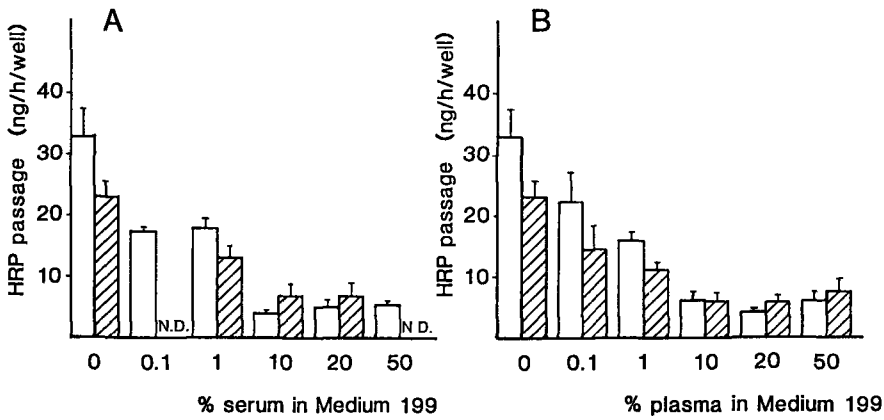

figure 5 Effect of various serum or plasma concentrations on the transport of peroxidase (HRP) through human umbilical artery endothelial cell monolayers. Cells were cultured in Medium 199 with 10% human serum and 10% newborn calf serum. Eighteen hours before the start of the experiment, cells were placed in Medium 199 with 20% human serum (A) or with 20% human plasma, anticoagulated by 5 U/ml heparin (B). Thirty minutes before the start of the experiment, cells were transferred to media with the various serum concentrations.
Passage of peroxidase was measured over a 2 hours-period. Values are means ± SD (n=3) for two different cell cultures (open and shaded bars).
N.D. = Not determined

The effects of various concentrations of human serum on the electrical resistance across the endothelial cell monolayer were also evaluated. After renewal of the medium with 10 or 20% serum for medium with the same percentage of serum, the electrical resistance decreased for a short time period (1-5 min) after which it recovered quickly (figure 6A). However, when the concentration of serum was lowered to 0.1 or 1 %, the electrical resistance of the monolayer decreased (figure 6B). A rapid restoration of the electrical resistance was usually observed when media supplemented with 0.1 or 1% serum was replaced by medium supplemented with 10% serum. No significant difference was found between the electrical resistances measured in 10 or 20% serum.

The resistance of fibronectin-coated filters without endothelial cells was not dependent on various concentrations of human serum (Medium 199: 3.1 Ohm.cm^2; Medium 199 with 1% serum: 3.15 Ohm.cm^2; Medium 199 with 10% serum: 3.2 Ohm.cm^2).

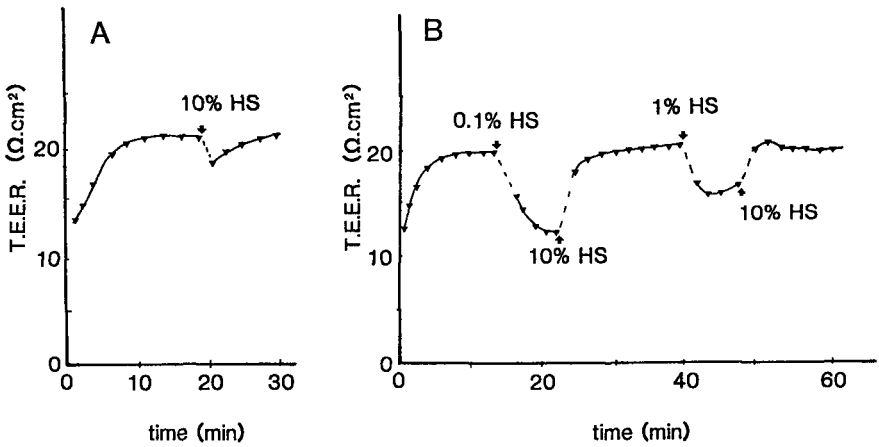

figure 6 Transendothelial electrical resistance (TEER) of the endothelial monolayer. TEER was measured in Medium 199-10% human serum. When the medium was replaced by new medium with 10% human serum (HS), the electrical resistance only decreases for a short period (A). However, when the medium was replaced by medium supplemented with a lower concentration of serum, the electrical resistance decreases and did not restore, until medium supplemented with 10% human serum was re-added (B).

In the second model (figure 2) confluent human endothelial cell monolayers were placed in a pressure chamber under 20 mmHg hydrostatic pressure and the fluid permeation was measured at 37°C. Figure 7 shows a representative experiment, in which the fluid permeation was followed over a 7.5 hour period. The decrease in fluid permeation, which is found at the start of every experiment, is probably due to recovery of the cells after mechanical stimulation during transfer of the monolayer into the pressure chamber. The fluid permeation was markedly influenced by the serum concentration (figure 7). No differences between serum and plasma were observed. When the low and high molecular weight fractions of serum were compared (each equivalent to 20% serum), only the high molecular fraction was comparable to 20% unfractionated serum (figure 7).

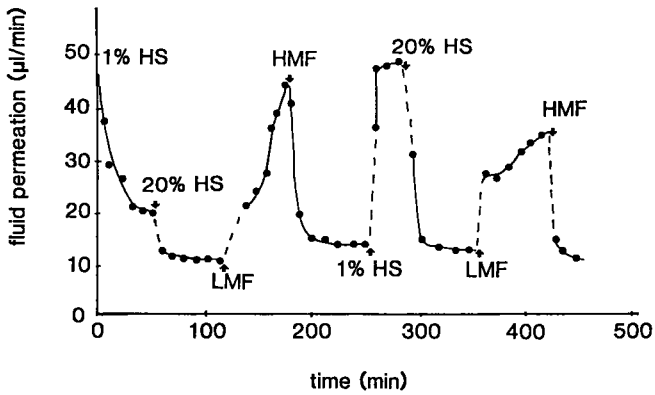

figure 7 Fluid permeation was measured through an endothelial monolayer under 20 mmHg hydrostatic pressure. The fluid permeation was dependent on the concentration of human serum (HS). The effect of serum was contributed by the high molecular fraction (HMF; molecules with a molecular weight larger than 10 kD) and not to the low molecular fraction (LMF; molecules with a molecular weight smaller than 10 kD). HMF and LMF were added in a concentration that was equivalent to their content in 20% human serum.

Effect of Extracellular Calcium on the Endothelial Monolayer

To evaluate the effect of extracellular calcium ions on the integrity of the endothelial cell monolayer in vitro, we studied the effect of EGTA on the HRP-passage and fluid permeation through the endothelial monolayer and on the electrical resistance across the monolayer. As can be seen in figure 8, HRP-passage increased when 5 mM EGTA was present in the passage-assay. The ratio of increase varied within six different cultures from 1.65 until 5.05. When cells were pre-incubated for 30 minutes with 5 mM EGTA but normal Ca-level was restored in the passage-assay, the passage-rate of HRP was not significantly different from its passage-rate through untreated endothelial cell monolayers. In a comparable form, EGTA effects the electrical resistance of the monolayer (figure 9) and the fluid permeation under hydrostatic pressure (figure 10). Both the electrical resistance and the fluid permeation through the endothelial monolayer decreased immediately after addition of 5 mM EGTA and reverted slowly to the initial values when the incubation medium was replaced by medium supplemented with normal Ca^{2+}-concentrations.

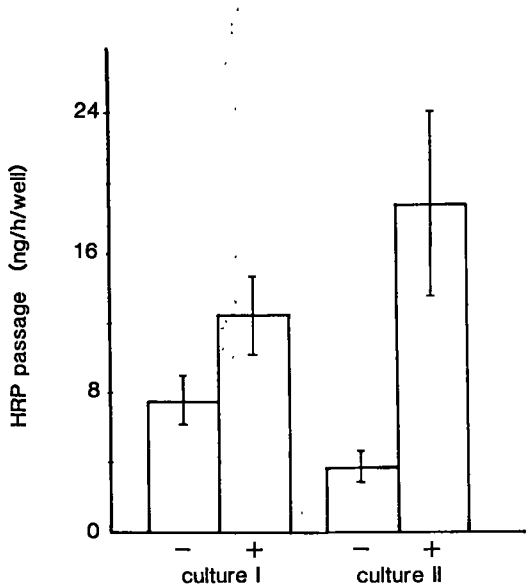

figure 8 Transport of peroxidase (HRP) through a monolayer of human umbilical artery endothelial cells is dependent on the extracellular Ca^{2+} concentration. Ca^{2+}-ions were removed from the medium by addition of 5 mM EGTA. Transport of peroxidase was measured over a 1-hour period in the absence (-) or presence (+) of EGTA. Two representative experiments are shown. Means ± SD of 4 monolayers.

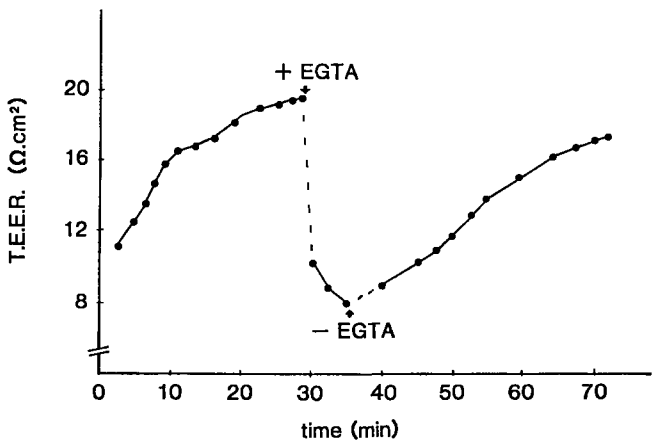

figure 9 The electrical resistance across the endothelial monolayer decreases immediately when Ca^{2+}-ions are removed from the medium by EGTA. The electrical resistance restores when the EGTA was removed from and Ca^{2+}-ions were re-added to the medium.

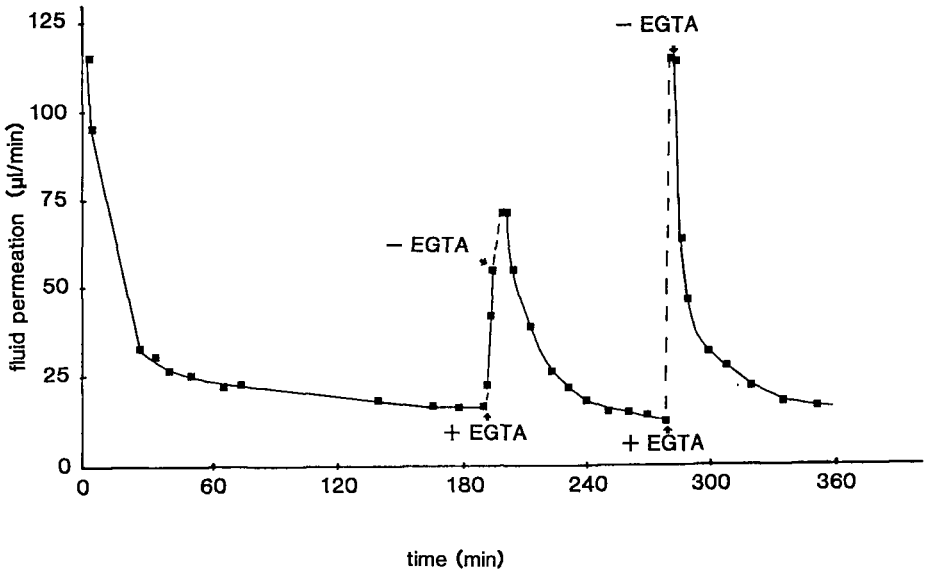

figure 10 Fluid permeation through an endothelial monolayer under 20 mmHg hydrostatic pressure is dependent on the extracellular Ca^{2+} concentration. The fluid permeation increases when EGTA was added to the medium, but restores to control level when the EGTA was removed from and Ca^{2+}-ions were re-added to the medium.

Molecular Sieving Characteristics of the Monolayers

To evaluate molecular sieving characteristics of the monolayer we studied the transport of dextrans with various molecular weights. The passage of all dextrans was linear in time and non-saturable in the studied range (up to 25 mg/ml) (data not shown). The passage rates of all dextrans through both fibronectin-coated unseeded filters and fibronectin-coated filters with an endothelial monolayer are presented in Table 1. The calculated passage rates of dextrans through the endothelial monolayer alone are given in table 1 and shown in figure 11. To control the integrity of the monolayers during the passage studies of dextrans we determined the passage rates of peroxidase in the presence of the dextrans. The passage rate of peroxidase was not dependent on the presence of low molecular weight dextrans (up to 150 kD) but in the presence of high molecular weight dextrans (480 kD and 2,000 kD) the passage rate of peroxidase decreased (figure 11).

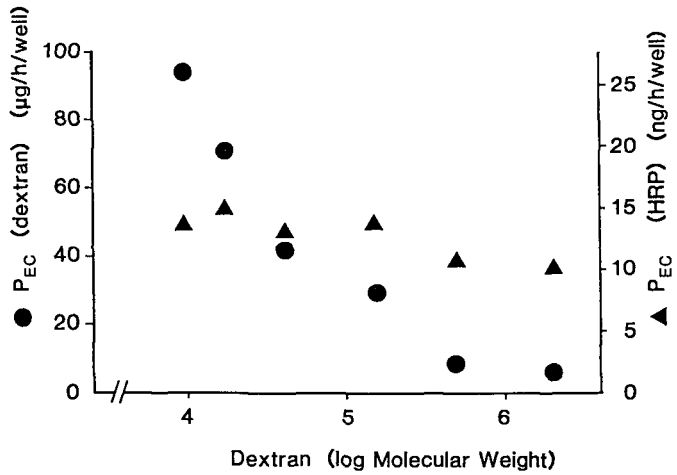

figure 11 Passage of dextrans with various molecular weights through a monolayer of human umbilical artery endothelial cells. Equal amounts of dextrans (●, 25 mg/ml) were introduced in the upper compartment. The passage rates of the dextrans through fibronectin-coated filters without an endothelial monolayer were determined over a 10 minutes period; through fibronectin-coated filters with an endothelial monolayer over a 2 hours period. Passage rates through the endothelial monolayer alone were calculated as described in Materials and Methods. In the same experiment the passage rates of peroxidase (40 kD, HRP, ▲, 5 µg/ml) was determined in the presence of the various dextrans. Passage rates of peroxidase through the endothelial monolayer alone were calculate in similar way.

table 1 Passage rates of different sized dextrans

MW	P_F (µg/h/well)	P_{F+EC} (µg/h/well)	P_{EC} (µg/h/well)
9,400	874.7 ± 178.9	86.2 ± 11.4	95.6
17,200	780.2 ± 77.4	65.3 ± 8.7	71.2
40,200	480.3 ± 39.4	39.1 ± 9.1	42.6
150,000	497.7 ± 90.8	27.9 ± 5.5	29.6
480,000	491.1 ± 86.7	8.4 ± 2.5	8.5
2,000,000	285.6 ± 57.1	6.0 ± 1.1	6.2

Passage rates of dextrans with various molecular weights (MW) were determined through fibronectin-coated filters (P_F) and through fibronectin-coated filters with an endothelial monolayer (P_{F+EC}). For experimental details see legends figure 11.
Values are means ± SD of six determinations (2 cultures). P_{EC} (the passage rate through an endothelial monolayer alone) is calculated from the equation given in Materials and Methods, using mean values for P_F and P_{F+EC}.

Effect of Metabolic Inhibitors on the Monolayer

To study whether the passage of HRP through an endothelial monolayer is an energy-dependent process, we evaluated the effects of metabolic inhibitors that interfered with the glucose metabolism or the oxidative phosphorylation. Addition of 10 mM 2-deoxy-D-glucose to the medium during transport studies resulted in a decrease of HRP-passage through the monolayer (figure 12), but its addition did not influence the TEER.

Addition of inhibitors of the oxidative phosphorylation (10 mM NaN_3 or 10 mM KCN) resulted in a loss of integrity of the endothelial cells, as indicated by an increased HRP-passage rate and a decreased electrical resistance (data not shown).

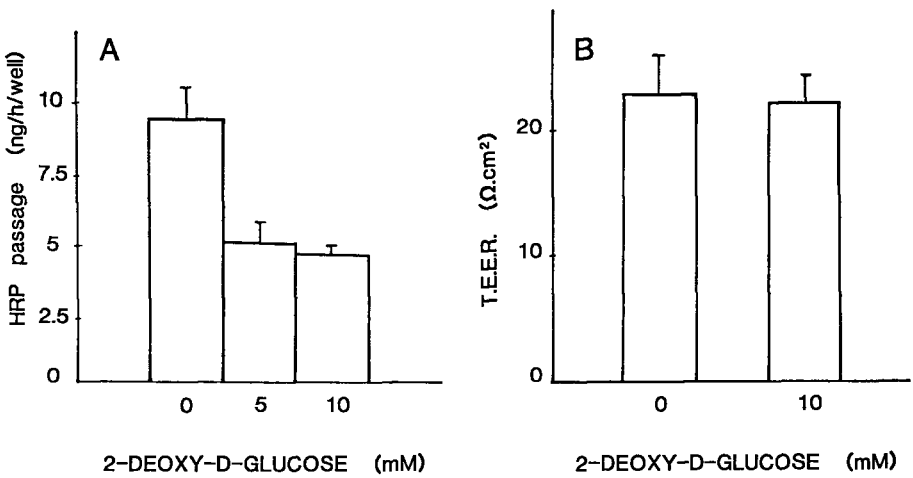

figure 12 Effects of 2-deoxy-D-glucose on the permeability of peroxidase (HRP) through (A) and the electrical resistance across (B) a monolayer of human umbilical artery endothelial cells. 2-deoxy-D-glucose was added to both the upper and lower compartment.

DISCUSSION

In this paper we have described a method for the routine culture of tight monolayers of endothelial cells from human umbilical arteries. The cultured endothelial cell monolayers showed similar characteristics to endothelial monolayers in vivo. The cells are connected by overlapping areas, in which one or more tight junctions are visible. Similar cell contacts are also found in in vitro monolayers of rabbit endothelial cells

(13) and in blood vessels in vivo (27). The endothelial cell tight junctions represent a special type of junction, that are less tight than those of epithelial cell layers (28). This probably explains why the electrical resistance of the endothelial cells in vivo (which varies from 13 to 40 Ohm.cm^2) in various capillary beds (15,16) is much less than that of epithelia (29). The cultured human endothelial cell monolayers have an electrical resistance of 17 Ohm.cm^2, which is similar to that reported from rabbit endothelial cell monolayers (13) and 1.5- to 2- fold higher than that reported from pig and bovine endothelial cells (10, 30). When our filters were covered by two layers of endothelial cells, one at the top and one at the lower surface of the filter, we found an electrical resistance of 30-40 Ohm.cm^2. Therefore, it is very unlikely that in our experiments the electrical resistance was overestimated by the development of a second monolayer of endothelial cells on the revers side of the filter. Navab et al (13) reported that the electrical resistance of rabbit endothelial cell monolayers decreased when the cells were kept for several days in serum-containing medium, whereas they remained intact in medium supplemented with lipoprotein-depleted serum. Hashida et al (10) found that transport of rhodamine labeled LDL was much lower in the absence of Foetal Calf Serum (FCS) than in the presence of FCS. In contrast to these observations, we found that the electrical resistance and passage of peroxidase through human endothelial cells did not change when the cells were maintained for a week in medium supplemented with 20% human serum or heparin-plasma. However, when human endothelial cells were transferred overnight into medium supplemented with 10 or 20% lipoprotein-depleted human serum, part of the cells detached and in the integrity was destroyed (unpublished results).

When during the assay of the peroxidase passage or electrical resistance the serum concentration in the medium was decreased to 0.1% or less, the passage of peroxidase increased and the electrical resistance decreased. This suggests that the endothelial cell monolayers need a minimal concentration of protein in their surrounding medium to maintain a proper barrier function towards macromolecules. Interestingly, in vivo studies on microvascular fluid permeation have shown that fluid efflux is influences by albumin or other proteins (31-34). This process depends on the interaction of the arginine-residues of the albumin molecules with endothelial cell components (35), and may reflect a filter function of the glycocalyx of the endothelial cell (36,37). It is not yet known whether the observed short term effects of proteins in our

models represent a similar phenomenon, or whether they mainly reflect a protection of the cells by serum proteins, or a combination of both.

Chambers and Zweifach (38) initially stated that calcium ions are necessary for the maintenance of normal capillary permeability. Also Shasby and Shasby (20) and Baetscher and Brune (21) have shown a direct effect of calcium chelation of the endothelial cell monolayer. Our report extends these observations for human umbilical artery endothelial cells in vitro. Addition of EGTA to the medium resulted in an increased permeability and a decreased electrical resistance across the monolayer. The electrical resistance restored after removal of EGTA, which indicates that the viability of the endothelial cells was not really affected by EGTA.

It is likely that in the absence of calcium ions, the Ca^{2+}-dependent connections between the cells are broken, leading to gap-formations between the cells. More fluid and macromolecules passes through these gaps, and the transendothelial ion flux (indicated by the electrical resistance) was decreased. It should be noted that in our model we observed an immediate effect of EGTA, while other authors (19,20,39) reported a lag time of about 20 minutes before an effect of EGTA became apparent. This may indicate that Ca^{2+} independent cell interactions were stronger in the other systems.

In figure 11 we showed that the passage rate of various dextran-molecules was dependent on their molecular size. This selectivity of molecular mass on permeation through an endothelial monolayer is in agreement with in vivo observations (40-42). Lowering the temperature (15°C) of the endothelial monolayer resulted in our system in a loss of integrity of the monolayer (unpublished results). However, addition of an inhibitor of the glucose-uptake and glycolysis (2-deoxy-d-glucose) to the culture medium resulted in a decreased passage of HRP. This suggests that the passage is an energy-dependent process. Similarly, Hashida et al. (10) reported that the passage of LDL through pig aorta endothelial cell monolayers in vitro decreases by addition of 2-deoxy-D-glucose and inhibitors of oxidative phosphorylation.

In summary, in this report we described an in vitro model to study the permeability of human endothelial cell monolayers. The studied characteristics of the monolayer were in close agreement with in vivo observations. This model enables us to study the passage of various plasma proteins, such as lipoproteins and coagulation factors, under physiological as well as under pathological conditions.

ACKNOWLEGDEMENTS

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CHAPTER 3

PASSAGE OF LOW DENSITY LIPOPROTEINS THROUGH MONOLAYERS OF HUMAN ARTERIAL ENDOTHELIAL CELLS

EFFECTS OF VASOACTIVE SUBSTANCES IN AN IN VITRO MODEL

Erna G. Langeler[1], Ineke Snelting-Havinga[2], Victor W.M. van Hinsbergh[1]

[1]Gaubius Institute TNO, Leiden, The Netherlands
[2]Dept. of Dermatology and Electron Microscopy, Leiden State University, Leiden, The Netherlands

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ABSTRACT

The endothelium controls the influx of lipoproteins into the arterial wall, a process that may be disturbed in arteriosclerotic blood vessels. We have used an in vitro model to investigate the characteristics of the passage of low density lipoproteins (LDL) through monolayers of human arterial endothelial cells. Umbilical artery, aorta, or carotid artery endothelial cells were cultured on polycarbonate filters, and formed a tight monolayer in which the cells were connected by tight junctions. Passage of ^{125}I-LDL through these monolayers proceeded linearly over a 24 hour period. It was threefold lower through monolayers of aorta or carotid artery cells than through monolayers of umbilical artery cells. The LDL passage process did not show saturation with LDL concentrations up to 800 μg/ml LDL-protein (i.e. 1.6 nmol/ml apolipoprotein B) between 2 to 4 hours after addition. However, during the first 30 to 60 minutes after addition of high concentrations of LDL, a reduction of the passage rate of both LDL and peroxidase, resulting in an apparent saturation of the passage process, was observed. The passage rate of the negatively charged acetylated LDL was twofold lower than that of native LDL. Addition of histamine to the endothelial monolayer resulted in a large, but transient, increase in permeability paralleled by a decrease in electrical resistance. The effects of histamine were mediated via an H1 receptor. Thrombin and Ca^{++}-ionophore also induced an increase in permeability of the monolayer, while bradykinin did not. The effects of histamine and thrombin were paralleled by a rapid and marked increase in cytoplasmatic Ca^{++} concentration of the endothelial cells, while bradykinin induced only a small increase. Although the cyclic adenosine 5'-monophosphate-elevating agent, forskolin, markedly decreased the basal rate of LDL passage through the endothelial cell monolayers, it did not change the relative increase in permeability induced by histamine. Thus, histamine induces small, but significant increases in the permeability of tight endothelial cell monolayers.

INTRODUCTION

Studies on the development of atherosclerotic lesions have indicated that the accumulation of lipid deposits in the arterial intima is related to the concentration of low density lipoproteins (LDL) in plasma (1,2). One of the factors that contributes to the accumulation of cholesterol in the vascular wall is the passage of lipoproteins

through the endothelial barrier. Several studies in humans (3-5) and rabbits (6) have indicated that the influx of LDL and other macromolecules is markedly increased in atherosclerotic regions as compared to unaffected arteries. Stemerman et al. (7,8) observed focal accumulation of peroxidase or LDL after infusion into normal and hypercholesterolemic rabbits, whereas no gaps between the endothelial cells were found. This suggests the existence of focal differences in lipoprotein influx, which may depend on (dys)regulation of endothelial cell function.

The mechanism of the passage of LDL through the endothelium is poorly understood. Morphological studies have pointed to a transcellular route of LDL through the endothelium of nonaffected rat arteries (9,10). An in vitro approach (11-15) may contribute to a better understanding of the regulation of the passage process. In studies using monolayers of bovine, pig, and rabbit aorta endothelial cells on porous membranes (12,14,15), the LDL passage has been reported to be a saturable process, which suggests that a specific transport or carrier process is involved in LDL passage. However, the clearance rate of LDL in vivo, which is expressed in nanoliters per amount of tissue per hour, did not differ in rabbits with a high or low plasma level of LDL (16). We have recently described a model to study the passage of fluid and macromolecules through monolayers of human arterial endothelial cells (17). This model enabled us to investigate the characteristics and modulation of the LDL passage process.

Vasoactive substances and cyclic adenosine 5'-monophosphate (cAMP)-increasing agents might be involved in the changes in the permeability characteristics of endothelial cell monolayers. Histamine and thrombin have large effects on the permeability of postcapillary venules in vivo (18,19), a process that may be mediated by endothelial cell contraction (20). Also, the passage of albumin through bovine aorta (21,22) and human umbilical vein (23) endothelial cells in vitro is changed after a stimulation by vasoactive substances. Since H1 receptors are present on human coronary arteries (24) and on guinea pig endothelial aorta cells (25), we wondered whether histamine could also have effects on the permeability characteristics of endothelial cell monolayers of human arteries. In this report we describe the effects of histamine, thrombin, bradykinin and calcium ionophore on LDL passage and intracellular calcium concentration of the endothelial cells.

The cAMP-increasing agents are able to alter reversibly the permeability of tight

junctions of epithelial cells (26). Prostaglandins E_1 and E_2, agents which increase the cAMP concentration in vascular cells (27), are able to decrease venular permeability in the hamster cheek pouch (28) and dextran transport through porcine arterial endothelial cells (29). In addition, Antonov et al. (30) suggested that the adenylate cyclase activator, forskolin, intensified the cell-cell contacts between human aorta endothelial cells. We studied the effects of forskolin and isobutyl methyl xanthine (IBMX) on permeability and showed that these agents by themselves decrease the permeability, but that, in the presence of these agents, histamine is still able to raise the permeability.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM Hepes was obtained from Flow Laboratories (Irvine, Scotland); all tissue culture plastics, including Transwells (diameter 0.65 cm; pore size 3 μm) were from Costar (Cambridge, MA). Penicillin/streptomycin was purchased from Boehringer (Mannheim, FRG). A crude preparation of endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. (31). Human serum was prepared in our laboratory from fresh blood of healthy donors, pooled (12 to 20 donors), and was stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY), heparin from Leo Pharmaceuticals Products (Weesp, The Netherlands), human serum albumin from the Red Cross Central Blood Transfusion Laboratory (Amsterdam, The Netherlands). Horse-radish peroxidase (HRP), histamine, bradykinin, A23187, pyrilamine, cimetidine and α-thrombin were obtained from Sigma Chemical Co. (St. Louis, MO) ^{125}I-iodine was purchased from Amersham (Amersham, UK). Forskolin was from Hoechst (La Jolla, CA) and isobutyl methyl xanthine (IBMX) from Janssen Chimica (Beerse, Belgium).

Isolation and Culture of Endothelial Cells

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al. (32). Endothelial cells from human aortas or carotid artery were isolated (33) and characterized as described previously (33,34). Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS,

150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/ streptomycin; this was kept at 37°C under 5% CO_2-95% air.
For passage studies, endothelial cells from umbilical artery (primary), aorta (third passage) or carotid artery (sixth passage) were released with trypsin ethylenediaminetetraacedic acid, were seeded on fibronectin-coated polycarbonate filters in the Transwell system and were cultured under the same circumstances as described above. For the assay of cytoplasmic calcium, cells were cultured on coverslips coated with glutaraldehyde cross-linked gelatin.

Preparation and Iodination of Low Density Lipoproteins

LDL was isolated from fresh serum prepared from the blood of healthy volunteers by gradient ultracentrifugation according to the method of Redgrave et al. (35). Iodination of LDL was performed by the ^{125}Iodine monochloride method described by Bilheimer et al. (36). After iodination, LDL was dialyzed against phosphate buffered saline (4 hours, 4 renewals). Subsequently, it was stabilized by the addition of 1% human serum albumin and again dialyzed against Dulbecco's modified Eagle's medium. Iodinated LDL was stored at 4°C. LDL concentrations are expressed in μg protein/ml and nmol apolipoprotein (apo) B/ml (briefly, nmol/ml), assuming a molecular weight of apo B of 512,000 D and 1 mol apo B per mole LDL. The specific activity ranged from 25 to 50 cpm/fmol. Protein concentration was determined by the method of Lowry et al. (37) with albumin as a standard. Lipoprotein-depleted serum (LPDS) was prepared from fresh serum by ultracentrifugation, was dialyzed against phosphate-buffered saline and Medium 199, and was stored at 4°C.

Methylation and Acetylation of ^{125}I-Low Density Lipoproteins

Methylation and acetylation of ^{125}I-LDL were performed as described by Weisgraber et al. (38) and Basu et al. (39), respectively. After methylation of the LDL particles, more than 98% of the terminal aminogroups were methylated, as determined with the aid of the trinitrobenzenesulfonic acid reaction. The iodinated LDL was not aggregated after acetylation, because only one peak (at identical elution volume as ^{125}I-LDL) was found after separation on a Sepharose CL-6B-column.

Passage of Different Macromolecules

Passage of ^{125}I-lipoproteins through human endothelial cell monolayers was studied with the Transwell system as earlier described (17). In short, confluent monolayers of human arterial endothelial cells cultured on fibronectin-coated polycarbonate filters (surface, 0.33 cm^2) were used. A known amount of the iodinated lipoprotein (usually 25 μg protein/ml, 49 pmol apoB/ml) in Medium 199 and 20% human serum was added on top of the endothelial cell monolayer (upper compartment, 150 μl). In the lower compartment (700 μl) 20% human serum in medium 199 was present. During the experiments monolayers were kept at 37°C. At several time points, samples (100 μl) were taken from the lower compartment and the same volume of Medium 199 with 20% human serum was re-added to prevent fluid exchange due to hydrostatic pressure. Trichloroacetic acid precipitation was performed in all samples to correct for degradation of the LDL particle. Subsequently, samples were counted in a gamma-counter. Passage rates are expressed in pmol/h/cm^2 or pmol/cm^2. The clearance rate (nl/h/cm^2) was calculated when the passage of different macromolecules was compared.

To control the integrity of the monolayers during experiments in which the passage rate of various lipoproteins was evaluated, peroxidase was added simultaneously with the lipoprotein in the uppercompartment. Passaged peroxidase in the sample of the lower compartment was measured spectrophotometrically (408 nm) after incubation with o-dianisidine and hydrogen peroxide.

Vasoactive substances and cAMP-increasing agents were added to both the upper and lower compartments when tested for their ability to influence the passage of macromolecules.

Transendothelial Electrical Resistance

The transendothelial electrical resistance (TEER) was measured as previously described (17). In short, an alternating current (50 μA) was passed across the monolayer (one pulse every minute). The measured electrical potential difference was used to calculate the electrical resistance by Ohm's law.

Assay of Cytoplasmatic Calcium Concentration

Confluent monolayers of endothelial cells grown on coverslips were loaded with 2

μmol/l fura-2/AM in serum-containing medium. After 30 to 60 minutes, cells were gently washed with Tyrode's buffer, and the coverslip was placed in a special cuvette in a Kontron SFM 25 fluorimeter. Fluorescence intensity was measured at 37°C over a period of 15 to 30 minutes. Cells were excitated alternately at 340 nm and 380 nm, while the emission was measured at 510 nm (40).

RESULTS

Passage of ^{125}I-Low Density Lipoproteins through Monolayers of Human Arterial Endothelial Cells in Vitro

Human umbilical artery endothelial cells seeded at high density on fibronectin-coated polycarbonate filters formed in 4 days a monolayer of closely apposed cells connected by tight junctions (17) and with an electrical resistance of 17 ± 4 Ohm.cm^2 (mean ± SD, 45 determinations). When ^{125}I-LDL (25 μg protein/ml; 49 pmol/ml) in Medium 199 with 20% human serum was added to the upper compartment, a linear passage of ^{125}I-LDL was observed over a 24 hour-period (figure 1). No difference was observed between the relative passage rate of 49 pmol/ml ^{125}I-LDL in the presence of 49 pmol/ml unlabeled LDL and the relative passage rate of 98 pmol/ml ^{125}I-LDL, suggesting that iodination of the LDL particle does not influence its passage rate.

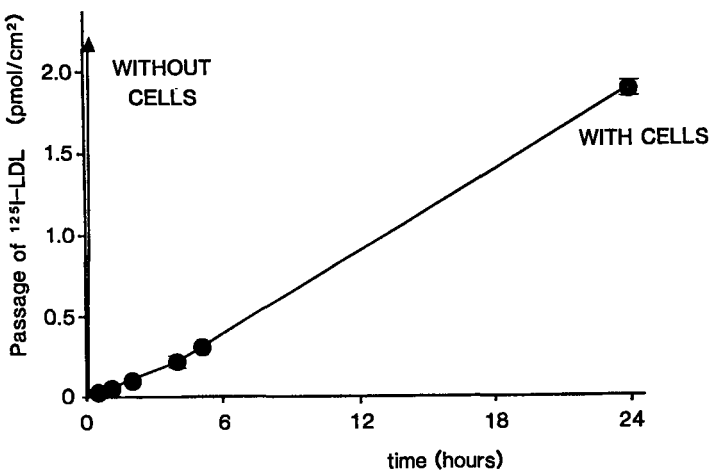

figure 1 Passage of ^{125}I-low density lipoproteins (LDL) (pmol/cm^2) through fibronectin-coated polycarbonate filters with or without a monolayer of human umbilical artery endothelial cells. At the start of the experiment ^{125}I-LDL (25 μg protein/ml, i.e., 49 pmol/ml) was added to the upper compartment and at several time-intervals, a sample was taken from the lower compartment as described in Materials and Methods. The filters were kept at 37°C in 5% CO_2-95% air. Values are means ± SD of a triplicate determination of a representative experiment.

The average passage rate of ^{125}I-LDL (49 pmol/ml) through monolayers of 29 different cultures was 88 ± 39 fmol/h/cm^2. This passage rate was 60- to 120- fold lower than through membranes that were coated with fibronectin only (figure 1).
To investigate whether the passage of LDL through monolayers of human endothelial cells was dependent on the LDL concentration, experiments were performed in Medium 199 supplemented with 1% human serum albumin, while to the upper compartment, a fixed amount of ^{125}I-LDL, various concentrations of unlabeled LDL, and a fixed amount of peroxidase were added. During the first 30 to 60 minutes after the addition of various concentrations LDL, a reduced rate of increase of the passage rate of LDL was observed (figure 2A). This phenomenon was accompanied with a reduced passaged rate of peroxidase in the presence of a high concentration of LDL (figure 2B). When the passage rates of the various LDL concentrations were determined between 2 and 4 hours after the addition, no saturation of the passage process was observed. The passage of peroxidase was thus not influenced by the presence of high LDL-concentrations (figure 2A and 2B).
The basal rate of LDL passage (49 μg pmol/ml ^{125}I-LDL) through monolayers of human aorta endothelial cells was 24.4 ± 8.4 fmol/h/cm^2 (mean ± SD of nine determinations of one culture). This value is about threefold lower than that obtained with monolayers of human umbilical artery endothelial cells. Nevertheless, the LDL passage process through these monolayers also showed an apparent saturation during the first 30 minutes after addition of the LDL (figure 2C), while no saturation was observed between 2 and 4 hours after the addition (figure 2C). The passage of peroxidase through these cells was comparable with its passage through human umbilical artery cells (figure 2B and 2D).

Comparison of the Passage of Various Modified Low Density Liopoproteins Particles
In table 1, the passage rates of various modified LDL particles through endothelial cell monolayers are compared. Methylation of the lysine residues of the LDL particle results in a modified LDL particle (Me-LDL), that has the same electrical charge as LDL, but has lost its ability to bind to the B,E receptor. The passage rate of Me-LDL is identical to that of LDL (table 1), indicating that the B,E-receptor is not involved in the passage process. When the surface charge of the LDL particle was more negative by acetylation of the lysine- and arginine-residues, the passage rate

decreased (table 1). The passage rate of peroxidase that was measured simultaneously with the different lipoproteins was not influenced by them.

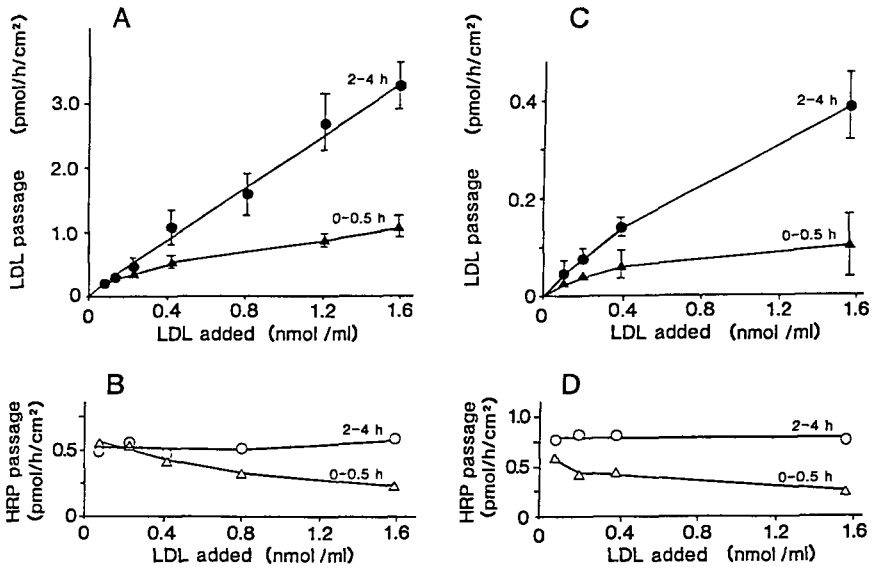

figure 2 Various concentrations low density lipoproteins (LDL) (49 pmol/ml of ^{125}I-LDL with different amounts of unlabeled LDL) and 125 pmol/ml peroxidase (HRP) were added on top of various monolayers of the same culture of human umbilical artery (A,B) or human aorta (C,D) endothelial cells. Passage rates of LDL (A,C) and peroxidase (B,D) were calculated both in the first 30-minutes period and between 2 and 4 hours after addition of the LDL and peroxidase to the monolayers. The values are expressed in pmol/h/cm^2 and represent the means ± SD of a triplicate determination of a representative experiment.

table 1 Comparison of the passage of native and modified low density lipoproteins through human endothelial cell monolayers.

Lipoprotein	Passage of lipoprotein	Passage of peroxidase
LDL	70 ± 10	N.D.
Me-LDL	67 ± 12	N.D.
LDL	89 ± 16	645 ± 165
Ac-LDL	48 ± 5	638 ± 105

Values (fmol/hr/cm^2) are the means ± range for two different experiments, each performed in triplicate. The passage rates of various lipoproteins (49 pmol/ml) and peroxidase (125 pmol/ml) through monolayers of human umbilical artery endothelial cells were simultaneously determined. Samples from the lower compartment were taken after a 1-hour incubation.
LDL = low density lipoprotein; Me-LDL = methylated LDL; Ac-LDL = acetylated LDL; N.D. = not determined.

Effect of Vasoactive Substances on Low Density Lipoproteins Passage

Addition of histamine (10^{-5} M) to the culture medium resulted in a rapid increase in the passage of ^{125}I-LDL (figure 3) and peroxidase (not shown). Upon examination with the electron microscope, the cell contacts remained intact in the control monolayers (figure 4A and 4B), while partial loss of cell contacts was regularly observed after stimulation with histamine (figure 4C and 4D). The effect of histamine on the passage rate of LDL was transient (figure 3). It was largest during the first 2 hours after the addition of histamine and decreased rapidly thereafter. At 4 to 6 hours after the addition of histamine, the passage rates of LDL and peroxidase were comparable to those in control monolayers. Thereafter, even a decrease of the passage rate was observed.

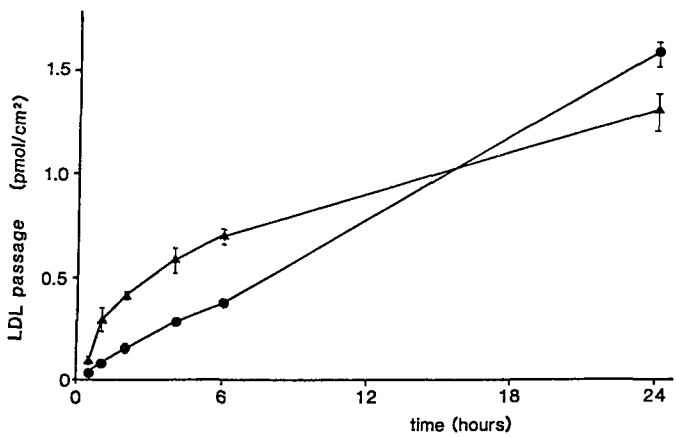

figure 3 The effect of histamine on the passage of ^{125}I-low density lipoproteins (LDL) through monolayers of human umbilical artery endothelial cells. Passage of ^{125}I-LDL was followed over a 24 hour period at 37°C in 5% CO_2-95% air, in the absence (●) or presence (▲) of 10^{-5} M histamine. At the start of the experiment, histamine was added to both upper and lower compartments of the assay system, while ^{125}I-LDL (49 pmol/ml) was added only to the upper compartment. At several time intervals samples were taken from the lower compartment as described in Materials and Methods.

When the effect of histamine on monolayers of human aorta or carotid artery endothelial cells was evaluated, similar results were obtained (table 2). In four independent experiments with monolayers from two different aortas and one carotid artery, a 2.1 ± 0.5- fold increase in LDL passage was observed after a 1-hour incubation with 10^{-5} M histamine (mean ± SD).

The increase in LDL passage by histamine appeared to be mediated by H1 receptors (table 3). It could be prevented by the simultaneous addition of the H1 antagonist

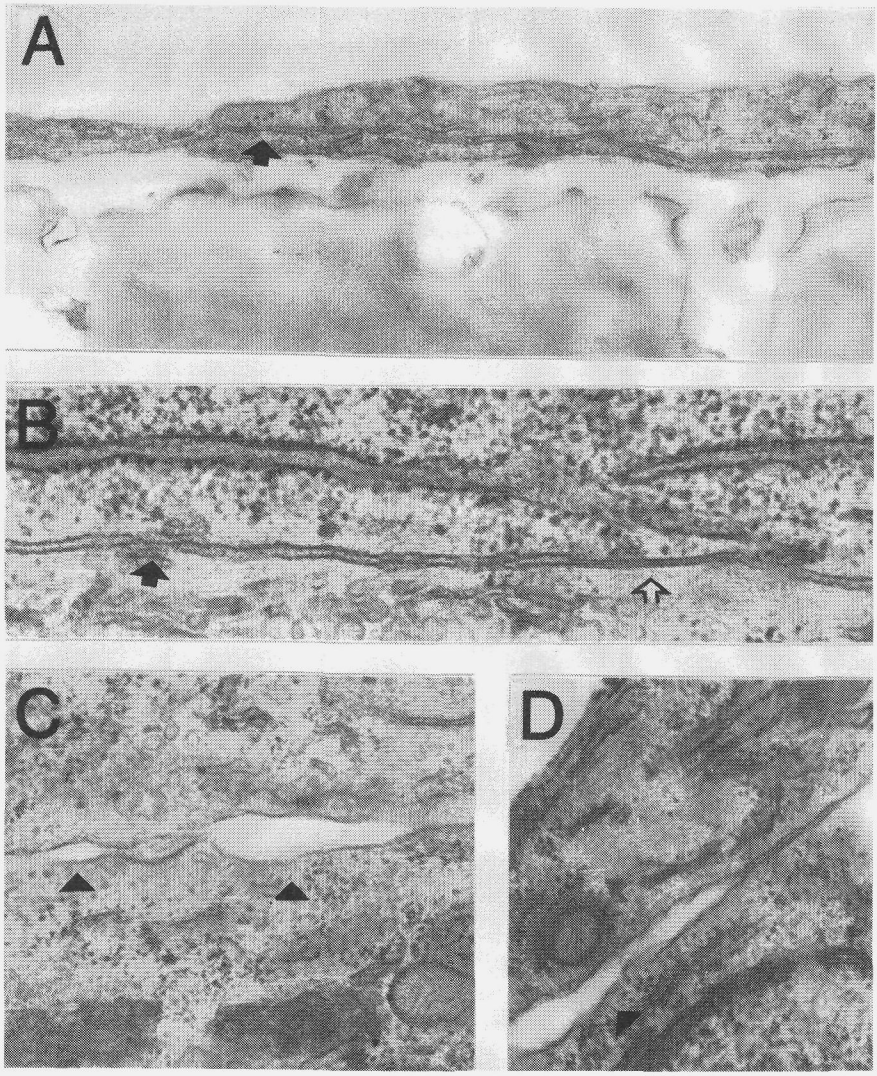

figure 4 Transmission electron micrographs of cell contacts between human umbilical artery endothelial cells cultured on fibronectin-coated polycarbonate filters. Thin section electron microscopy was performed as previously described (58). In control monolayers (A x 40,000, B x 75,000) tight junctions (closed arrows) and occasionally gap junctions (B, open arrows) were observed. After stimulation with histamine (10^{-5} M, 10 minutes), partial loss of cell contacts was observed (C,D x 50,000, arrowheads).

pyrilamine, whereas the addition of the H2 antagonist cimetidine did not prevent this (table 3). In contrast to histamine, the addition of bradykinin (up to 10^{-4} M) to the medium did not increase the passage of LDL. On the other hand, addition of α-thrombin (1 U/ml) or the calcium ionophore A23187 (10 μM) to the medium resulted in a threefold increased passage rate of LDL and a decrease in the electrical resistance of the monolayers.

Endothelial cells produce prostacyclin upon stimulation by histamine (41,42). Prostacyclin appears not to be involved in the histamine-induced increase of the LDL passage, because indomethacin (25 μM) and aspirin (25 μM) did not prevent the effect of histamine (not shown).

table 2 Effect of histamine on passage of ^{125}I-LDL and peroxidase and on electrical resistance across monolayers of human aorta and carotid artery endothelial cells.

Endothelial cells	Addition	LDL passage ($fmol/h/cm^2$)	Peroxidase passage ($fmol/h/cm^2$)	Electrical resistance ($Ohm.cm^2$)
Aorta	none	18 ± 5	908 ± 75	8.1 ± 0.3
	histamine	52 ± 7	1725 ± 158	6.1 ± 0.9
Carotid Artery	none	43 ± 5	345 ± 90	11.2 ± 0.4
	histamine	70 ± 4	495 ± 90	7.8 ± 0.8

Values represent the means ± SD (passage rates, triplicate determination) or the means ± range (electrical resistance, duplicate determination) of a representative experiment. Passage of ^{125}I-LDL (49 pmol/ml) and peroxidase (125 pmol/ml) through monolayers of human aorta and carotid artery endothelial cells were determined in the absence and presence of 10^{-5} M histamine after a 1-hour incubation. Electrical resistance was determined before and 10 minutes after addition of histamine to the incubation medium.
LDL = Low density lipoprotein

Effect of Vasoactive Substances on the Cytoplasmic Calcium Concentration of Endothelial Cells

An increase in the cytoplasmic calcium concentration underlies the release of several other endothelial cell products, such as endothelium-derived relaxing factor and von Willebrand factor (43-45), and may play a role in inducing endothelial cell contrac-

tion (20,46). By the fura-2 assay, we found a basal level of 0.11 ± 0.05 μM calcium ions in the endothelial cells. After addition of histamine, an immediate increase in cytoplasmic calcium concentration was observed, as detected fluorimetrically by an increase in fluorescence at excitation 340 nm and emission at 510 nm (figure 5A). Because the increase in fluorescence was accompanied by a decrease in fluorescence at excitation 380 nm, it is highly unlikely that the increase in fluorescence is the result of morphological changes of the cells. Pyrilamine, but not cimetidine, prevented the histamine-induced increase in cytoplasmatic calcium concentration (figure 5B). Addition of bradykinin had only a very small effect on the cytoplasmic calcium levels of our cells (figure 6). On the other hand, addition of α-thrombin resulted in a similar increase in cytoplasmic calcium ions and LDL passage, as did histamine (figure 6, table 3). As expected, a marked rise in the cellular calcium level was observed after the addition of the calcium ionophore ionomycine.

table 3 Effect of histamine and histamine antagonists, bradykinin, thrombin and A23187 on LDL passage and electrical resistance across human umbilical artery endothelial monolayers.

Addition	LDL passage	Electrical resistance
None	100	100
Histamine (10^{-5} M)	233 ± 66 (10)	68 ± 8 (8)
Histamine (10^{-5} M) + pyrilamine (10^{-6} M)	132 ± 14 (3)	95 ± 7 (5)
Histamine (10^{-5} M) + cimetidine (10^{-4} M)	180 ± 21 (3)	75 ± 2 (5)
Bradykinin (10^{-4} M)	92 ± 2 (3)	92 ± 1 (4)
Thrombin (1U/ml)	290 ± 76 (4)	65 ± 13 (4)
A23187 (10^{-5} M)	261 ± 26 (3)	54 ± 25 (4)

Values (% of control) represent the means ± SD of independent experiments (number in parentheses) performed with endothelial cells from different donors. Passage of ^{125}I-LDL (49 pmol/ml) and electrical resistance across human umbilical artery endothelial monolayers were determined in the absence or presence of histamine (with the H1 antagonist, pyrilamine, or the H2 antagonist, cimetidine), bradykinin, thrombin, and A23187. Passage of ^{125}I-LDL was determined in triplicate after a 1-hour incubation and is expressed as percentage of the value observed in control monolayers (determined in triplicate). Electrical resistance was determined before and 10 minutes after addition of the indicated substance(s) to the incubation medium.
LDL = Low density lipoprotein

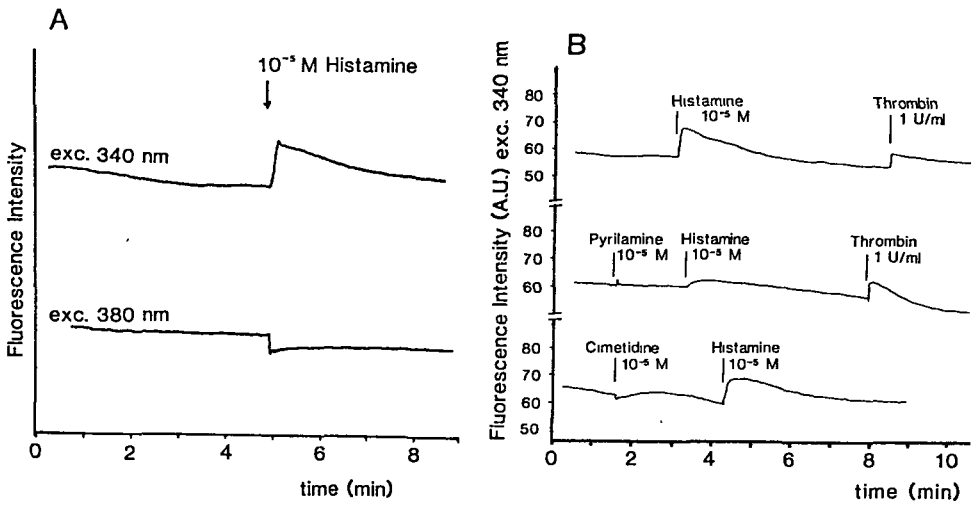

figure 5 A rapid increase in cytoplasmatic calcium concentration of human umbilical arterial endothelial cells was observed after stimulation of fura-2-loaded endothelial cells with 10^{-5} M histamine. A. A full record of the changes in fluorescence intensity at excitation wavelengths of 340 and 380 nm (emission wavelength of 510 nm) was made by combination of the data of four overlapping experiments with cells of the same batch. B. Changes in fluorescence intensity were measured at 340 nm excitation and 510 nm emission. The effect of histamine on cytosolic calcium concentration could be prevented by the H1 antagonist, pyrilamine, but not by the H2 antagonist, cimetidine.

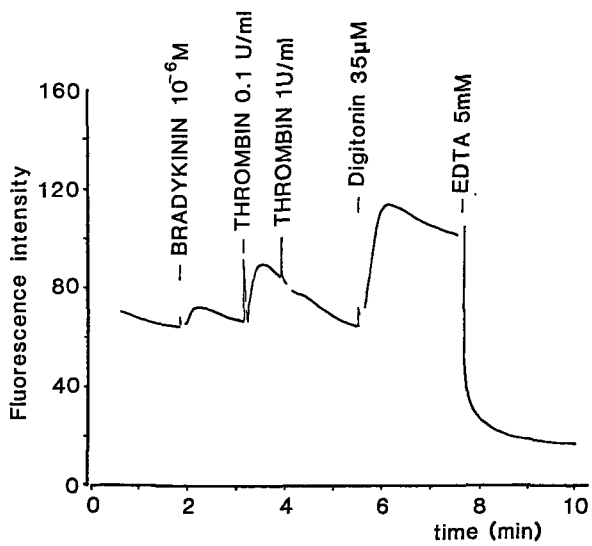

figure 6 Changes in cytoplasmatic calcium concentrations of human umbilical artery endothelial cells after stimulation of fura-2-loaded cells with bradykinin and thrombin. (excitation 340 nm, emission 510 nm).

Effect of cAMP Increasing Agents

Addition of forskolin or isobutyl methyl xanthine (IBMX), agents that increase the cellular cAMP level, increased the electrical resistance of the endothelial cell monolayers (not shown) and decreased the passage rate of LDL (figure 7). The effect of forskolin on the passage of LDL through the monolayers was instantaneous and remained constant over a 24 hour period. When endothelial cells were incubated with forskolin or IBMX, they still responded to histamine (figure 7). Interestingly, in both nontreated cells and cells that had been treated with forskolin or IBMX histamine induced a threefold increase in passage rate. Furthermore, in the presence of forskolin, again no saturation of LDL passage through human umbilical artery endothelial cell monolayers was observed at concentrations up to 800 μg/ml LDL protein (1.6 nmol/ml) (figure 8).

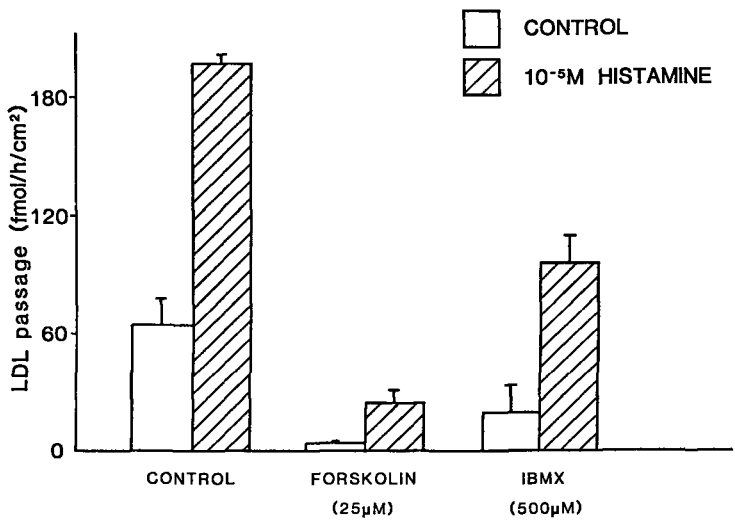

figure 7 Effects of 25 μM forskolin or 500 μM isobutyl methyl xanthine (IBMX) on the passage of ^{125}I-low density lipoproteins (LDL) (49 pmol/ml) through monolayers of human umbilical artery endothelial cells. In the presence of these substances, histamine (10^{-5} M) has a similar effect on the permeability of the monolayers as on control monolayers. Passage rates were determined 1 hour after addition of the substance(s) and histamine to the incubation medium.

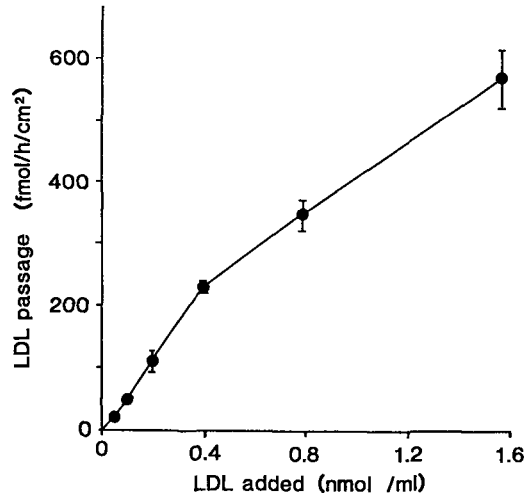

figure 8 The passage rate of various concentrations low density lipoproteins (LDL) (49 pmol/ml ^{125}I-LDL and different amounts of unlabeled LDL) through monolayers of human umbilical artery endothelial cells was determined in the presence of 25 μM forskolin. Passage rates were determined over a 4-hour incubation period. Values are expressed in fmol/h/cm^2 and represent the means ± SD of a triplicate determination.

DISCUSSION

The availability of an in vitro model to study the passage of macromolecules through monolayers of human arterial endothelial cells on porous membranes enabled us to investigate the characteristics of the passage of lipoproteins through endothelial monolayers. These endothelial cell monolayers displayed: (a) tight junctions, (b) an electrical resistance of 17 ± 4 Ohm.cm^2, (c) molecular sieving characteristics (17), and (d) a selectivity on electrical charge of the molecule. These characteristics resemble in vivo features of animal endothelial cells (47-49). However, it should be noted that in vivo data suggest a marked variation between various micro- and macrovascular endothelia, including the relative involvement of vesicles and inter/intracellular pores in the passage of macromolecules (9,10,47,50). Limited information is available about the arterial endothelium. Therefore, it is not yet possible to ascertain whether our endothelial cell monolayers reflect all aspects of the barrier function of the arterial endothelium or that they differ in certain aspects. It has been suggested that in vitro monolayers of endothelial cells contain local gaps (51). We did not find such gaps between the cells of our monolayers by electron microscopic examination, but it is difficult to exclude an incidental gap. In favor of the intactness of our monolayers, we observed no passage of intact ^{125}I-very low density lipoproteins (VLDL) through them

in preliminary experiments (only passage of radiolabel with a molecular weight of less than 50 kD was observed, probably apoC).

In studies on microvascular permeability in vivo (49,52), it was reported that negatively charged proteins pass at a lower rate than their neutral counterparts. Consistent with this phenomenon, we found a lower passage rate of the more negatively-charged acetylated LDL than that of nonmodified LDL particles.

In agreement with in vivo observations in the rabbit (16), no difference was found between the passage rate of LDL and that of Me-LDL. This confirms that the B,E-receptor is not involved in the LDL passage process across the endothelium. The passage of LDL through monolayers of human umbilical artery endothelial cells appeared to be a nonsaturable process for LDL concentrations up to 800 μg protein/ml (1.6 nmol/ml). Territo et al. (12) and Navab et al. (15) described that the passage of LDL through bovine and rabbit aortic endothelial cells is saturable above 100 μg/ml (0.2 nmol/ml) and 200 μg/ml (0.4 nmol/ml), respectively. These cells were cultured in the absence of lipoproteins, while our cells have to be cultured in serum to behave optimally. In pig aorta endothelial cell monolayers, which were also kept in serum-containing medium, a saturation of LDL passage was observed above 400 μg/ml LDL (0.8 nmol/ml) (14). The discrepancies between our data and those obtained with animal cells could be due to species differences, but more likely they reflect a difference in incubation period. We also observed a relative decrease in LDL passage at high LDL concentrations during the first hour after the addition of the LDL. This decrease, however, was accompanied by a similar decrease in the passage of peroxidase, suggesting that high concentrations of LDL induce a temporary decrease in the permeability of the monolayer. The mechanism behind this effect is still under investigation. When measured over longer time intervals, the passage rates of peroxidase were independent of the LDL concentration, and the apparent saturation of LDL passage disappeared completely. Therefore we conclude that the passage of LDL across cultured human endothelial cells is a nonsaturable process at physiological LDL concentrations.

Histamine induced a rapid increase in the passage rate of LDL and peroxidase, concomitant with a decrease in electrical resistance of the endothelial monolayer. The effect of histamine was transient, and several hours after addition of the histamine, the passage rate of LDL was even lower than that across nonstimulated cells.

Haddock et al. (53) have shown that desensitization of the histamine response in umbilical vein endothelial cells occurs and that it is due to competitive inhibition of the interaction of histamine with its receptor by an inactive histamine metabolite. This mechanism may explain the transience of the increase in LDL passage rate, but does not explain the subsequent decrease in LDL passage after prolonged exposure to histamine. The effect of histamine on the permeability of the endothelial cell monolayer proceeds via interaction with an H1 receptor. H1 receptors were also detected on human carotid artery (24), guinea pig aorta (25), and human umbilical vein (54) endothelial cells. Whether such receptors may play a role in the local regulation of macromolecular permeability of the arterial endothelium remains uncertain. The unexpected finding that histamine increases the passage of macromolecules through monolayers of cultured endothelial cells of human aorta and carotid artery may suggest this, but it may also reflect an in vitro activation of these cells. From in vivo experiments, Majno et al. (20) postulated that histamine acted on a contractile mechanism of rat and rabbit venule endothelial cells. In favor of this, Rotrosen et al. (53) demonstrated an increase in cytoplasmatic calcium concentration after stimulation of suspended human umbilical vein endothelial cells, which coincided with a change in F-actin content. An increase in cytoplasmatic calcium concentration was also observed after stimulation of human endothelial cells with thrombin (41,44). Here we have demonstrated that in endothelial cell monolayers also the cytoplasmatic calcium level was increased upon stimulation by vasoactive substances (histamine via H1 receptors, thrombin and calcium ionophore).

Under our experimental conditions, the addition of bradykinin resulted in only a small increase in cytoplasmatic calcium concentration in the cells, while it had no effect on the permeability of LDL and the electrical resistance across the monolayers. The lack of effect of bradykinin on human endothelial cells has also been found by other authors (23), but contrasts with observations on animal cells (20,55,56). Possibly human endothelial cells become desensitized for bradykinin in culture.

Because only the large increase in cytoplasmatic calcium elicited by histamine, thrombin, or calcium ionophore was associated with a change in LDL passage through endothelial cells monolayers, while the small increase elicited by bradykinin was not, our data fit in a model, in which a marked increase in cytoplasmatic calcium concentration results in contraction of endothelial cells and in an increase of the permea-

bility of the endothelial cell monolayer.

Despite of a general resemblance of the passage of lipoproteins in the in vitro model and in vivo, the actual passage rate of LDL was high as compared to in vivo observations (4,5,57). In experiments with monolayers of adult human aorta or carotid artery endothelial cells, we observed passage rates of LDL, which are threefold lower than those of umbilical artery endothelial cells. These values are in the same order of magnitude as those reported for rabbit and bovine aortic endothelial cell monolayers in vitro (12,15). Still the clearance rate of LDL in these models is at the higher limit of LDL influx into atherosclerotic human aortic intima, while this influx is 10-fold higher than that found with unaffected human arteries in vivo (4). After addition of the cAMP adenylate cyclase activating agent, forskolin, the passage rate of LDL in vitro becomes 5- to 10- fold lower and approximates the physiological clearance rate of LDL in vivo. Under these conditions, the lack of saturation of LDL passage and the relative increase in permeability by histamine are essentially the same. These data suggest that histamine not only has large effects on the permeability of "leaky" endothelia, but also may induce relative minute, but significant, increases in the permeability of tight arterial endothelial cell monolayers.

The model presented here can be used to evaluate the effects of platelet products, fibrin degradation products and vasoactive peptides on the permeability characteristics of human endothelial cells. Such studies will be helpful in clarifying the difference in permeability characteristics of normal and atherosclerotic arteries.

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CHAPTER 4

NOR-EPINEPHRINE AND ILOPROST IMPROVE THE BARRIER FUNCTION OF HUMAN ARTERY ENDOTHELIAL CELL MONOLAYERS

EVIDENCE FOR A CYCLIC AMP-DEPENDENT AND INDEPENDENT PROCESS

Erna G. Langeler and Victor W.M. van Hinsbergh

Gaubius Institute TNO, Leiden, The Netherlands

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ABSTRACT

The endothelium actively controls the influx of macromolecules into the arterial wall. The barrier function of human artery endothelial cell monolayers can be improved by addition of the adenylate cyclase activator, forskolin or the phosphodiesterase inhibitor, isobutyl methyl xanthine (IBMX), agents that increase the cellular cyclic adenosine 5'-monophosphate (cAMP) concentration. This is established by determining the passage rate of peroxidase through, and the transendothelial electrical resistance of, the monolayers. A continuous elevation of cAMP is necessary to maintain the improved barrier function of the endothelial cells.

The β-adrenergic agonist isoproterenol and the stable analogue for prostacyclin, iloprost increase the cellular cAMP concentration of endothelial cells 2.5- to 3- fold, and decrease the passage rate of peroxidase through the monolayers. The effect of isoproterenol is prevented by addition of the β-adrenergic antagonist propranolol. A direct correlation is found between the relative increases of the cellular cAMP concentration and the relative decreases in peroxidase passage after incubation of the cells with 0.25 μM and 2.5 μM forskolin, 10 μM isoproterenol and 10 μM iloprost. Nor-epinephrine increases the cAMP concentration of endothelial cells by only 44%. This is the result of interactions with β-adrenergic receptors, which increase cAMP and are blocked by propranolol, and with α-adrenergic receptors, which result in a decrease in cAMP, an effect that is prevented by the α-adrenergic antagonist phentolamine. The reduction of peroxidase passage is much larger than might be expected on the basis of the change in cAMP, (both in the presence and the absence of phentolamine), indicating that an additional cAMP-independent mechanism underlies the barrier improving effect of nor-epinephrine.

In addition, the changes in the permeability of endothelial cell monolayers are accompanied by changes in the configuration of microfilaments, as can be shown by double immunofluoresecence microscopy of the f-actin and myosin structures in the cells.

INTRODUCTION

Vascular endothelial cells separate the circulating blood from the surrounding vessel wall and tissues. One of the functions of these cells is the active control of the influx of macromolecules into the tissues. The extravasation of macromolecules can be

increased by pathophysiological conditions (1,2). Various vasoactive substances, like histamine and thrombin, induce an increase of endothelial permeability in vivo (3,4) and in vitro (5-7). In the early sixties, Majno and Palade (3,8) described the appearance of gaps between endothelial cells in postcapillary venules after exposure to histamine and they concluded that endothelial cell contraction occurred. At present the molecules involved in the regulation of this contraction are becoming recognized. They include intracellular calcium, calmodulin, actin, myosin and myosin-light-chain-kinase (9-14). An increased vascular permeability has also been indicated in ischemia (15), in tumors (16), and at focal sites in normal and arteriosclerotic arteries (17,18). Vascular leakage may result in edema, and subsequently in an increased cardiac work load and shock. Possibly, it contributes locally to undesirable accumulation of lipoproteins and fibrin (16-18).

Reduction of the extravasation of macromolecules may prevent these complications. In animal in vivo studies, phosphodiesterase inhibitors which increase cAMP gave a reduction of vascular leakage after toxic or inflammatory injury (19-23). In addition, in vitro studies with cultured endothelial cells indicated an improvement of the barrier function by elevation of the cellular cAMP concentration (7,24-27).

Activation of the adenylate cyclase can be induced by activation of the β-adrenergic receptor. This receptor type has been demonstrated on endothelial cells (28). Nor-epinephrine and the β-adrenergic agonist isoproterenol increase the cAMP concentration in bovine aorta and human umbilical vein endothelial cells (29,30). In other studies (25,31), a reduction of the permeability induced by these agents has been reported. On the other hand, infusion of nor-epinephrine, in the absence of any plasma protein, into animal arteries resulted in an increased extravasation of lipoproteins (32). Comparison of these data, however, is difficult, because different systems and different assays have been used. Here, we have evaluated the effects of forskolin, several adrenergic agents and the prostacyclin analogue iloprost on both the cAMP concentration and the permeability of human umbilical artery endothelial cells. Furthermore, the effects of these agents on the cellular actin and myosin configurations have been studied.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Corning (Corning, NY) or Costar (Cambridge, MA); Transwells (diameter 0.65 cm; pore size 3 μm) were from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al (33). Human serum was obtained from a local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY) and heat-inactivated before use (30 min, 56°C). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands). Horse-radish peroxidase (HRP), nor-epinephrine and isoproterenol were obtained from Sigma Chemical Company (St. Louis, MO), forskolin was from Hoechst (La Jolla, CA) and isobutyl methyl xanthine (IBMX) from Janssen Chimica (Beerse, Belgium). Rhodamine-phalloidin was purchased from Molecular Probes (Junction City, OR). Iloprost was a gift from Schering A.G. (West-Berlin, FRG). Rabbit anti-myosin immunoglobulins (non-muscle) were obtained from Sanbio B.V. (Uden, the Netherlands) and fluoresceine-conjugated swine anti-rabbit immunoglobulins from Dako-immunoglobulins (Glostrup, Denmark). Propranolol and phentolamine were obtained from Ciba-Geigy (Basel, Switzerland).

Culture of endothelial cells

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al (34). Human aorta and vena cava endothelial cells were isolated and characterized as described previously (35). Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/streptomycin. Cells were kept at 37°C under 5% CO_2 / 95% air.

For passage studies endothelial cells from umbilical artery (primary), aorta (fourth passage) or vena cava (sixth passage) were released with trypsin-EDTA (ethylene-diamine-tetraacedic) acid and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell system (36) and cultured as described above. Medium was renewed every other day. For the evaluation of the cytoskeleton configuration by

immunofluorescence, cells were cultured on coverslips coated with cross-linked gelatin (37).

Evaluation of the barrier function

a. Passage-experiments

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Passage of horse-radish peroxidase through human endothelial cell monolayers was performed as earlier described (36). Briefly, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) for 4-6 days to form a tight monolayer. Before the experiment, cells were preincubated for one hour in Medium 199 supplemented with 20% human serum. Experiments, in which the effects of catecholamines were evaluated, were performed in the presence of vitamin C. At the start of the experiment, 5 μg/ml horse-radish peroxidase in Medium 199 with 20% human serum was added to the upper compartment of the Transwell-system. At various time intervals samples were taken from the lower compartment (at the other side of the endothelial monolayer), and an equal amount of Medium 199-20% human serum was re-added to this compartment. Cells were kept at 37°C under 5% CO_2 / 95% air. All passage experiments are performed in triplicate. The peroxidase-concentration was determined in each sample as previously described. Passage rates are expressed as ng/h/cm^2 or ng/cm^2.

b. Transendothelial electrical resistance

The transendothelial electrical resistance (TEER) was measured as earlier described (36).

Immunofluorescence

Confluent endothelial cell monolayers cultured on coverslips were stimulated with or without forskolin (25 μM), nor-epinephrine (10 μM) or iloprost (10 μM) in complete culture medium for the indicated time-intervals. Medium was aspirated and cells were directly fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes, followed by two minutes in 0.1% Triton-X-100 in PBS at 4°C. The cells were gently washed with PBS. To visualize f-actin the cells were incubated with 3.3 10^{-8}M rhodamine-phalloidin in PBS for 30 minutes. To visualize myosin, cells were incubated with rabbit-anti-myosin (non muscle) immunoglobulin (37°C, 45 min),

washed and subsequently stained with fluoresceine-conjugated swine-anti-rabbit-IgG. After staining the cells, they were gently washed, p-phenylenediamine was added to each coverslip and cells were viewed in a fluorescence microscope (Leitz, Laborlux D). Photomicrographs were taken with Ilford HP5-film. To determine the amount of bound rhodamine-phalloidin, cells were stained with $5x10^{-8}$ M rhodamine-phalloidin, mixed with 10^{-7} or 10^{-6} M unlabelled phalloidin. After staining and washing the cells, rhodamine-phalloidin was extracted from the cells in methanol for 1 hour at 37°C, in the dark. Fluorescence in extracts were measured at emission wavelength 570 nm and excitation wavelength 540 nm.

Extraction and assay of cyclic AMP

Extraction and assay of cyclic AMP was performed according the method of Adams-Brotherton et al (38) with some modifications. Medium of confluent endothelial cell monolayers, cultured in 5 cm^2 wells, was renewed with Medium 199 supplemented with 20% human serum one hour before the incubation-period. Unless otherwise mentioned, 15 minutes before the start of the experiment IBMX (1 mM) was added to the medium. At the start of the experiment, cells were incubated with medium containing 20% human serum with or without stimulatory agents for an indicated time-interval. Hereafter, the medium was aspirated and 0.6 ml of ice-cold 5% tri-chloroacetic acid was directly added to each well. A small amount of [^{3}H]-cyclic AMP (Amersham, UK) was added to each well to monitor the recovery of cyclic AMP during the purification period. After ten minutes on ice, the extracts were collected and brought on a small DOWEX-50 WX4 column. Cyclic nucleotides were eluted with 1 N HCl, and air-dried. The concentration of cyclic AMP was determined using a radio-immunoassay (Amersham), and corrected for the recoveries (50-70%) of cyclic AMP in the various samples.

RESULTS

Effect of Forskolin on the cAMP Concentration in the Endothelial Cell

Confluent monolayers of human umbilical artery endothelial cells are stimulated for various time-intervals with forskolin, a direct activator of the adenylate cyclase. In the presence of isobutyl methyl xanthine (IBMX), a phosphodiesterase inhibitor, forskolin induces a marked, sustained and concentration-dependent increase of the

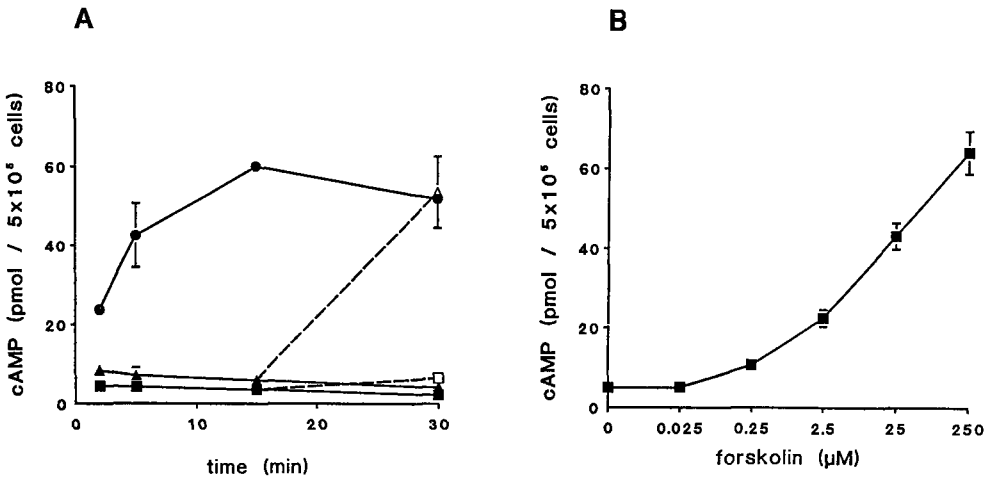

figure 1 Effect of forskolin on the cAMP concentration of human umbilical artery endothelial cells. Cells were incubated in Medium 199 with 20% human serum and with the indicated substances. Cyclic AMP was isolated and assayed by radioimmunoassay. Details are described in Materials and Methods. A: Time course of the cAMP concentration of cells after addition of 25 μM forskolin in the presence of 1 mM IBMX (●) and in the absence of IBMX (▲). Control incubations were performed without forskolin and IBMX (■). When IBMX was added at t=15 min to the forskolin-treated cells (△), a large increase in cAMP was found, comparable to the cells treated with forskolin and IBMX from the beginning. Addition of IBMX to untreated cells resulted in a two-fold increase of cAMP (□). B. Concentration dependent increase of cAMP in endothelial cells incubated for 5 min with forskolin in the presence of 1 mM IBMX. Values represent the mean ± range of a representative experiment, performed in duplicate.

cellular cAMP concentration (figure 1 A,B). In the absence of IBMX, incubation of the cells with forskolin for 15 minutes induces only a small increase of the cAMP concentration (1.3-fold vs. control; mean of 4 experiments). When IBMX (1 mM) is added to forskolin treated cells (for 15 minutes) a marked increase is observed (fig 1A), reaching a similar concentration as when IBMX and forskolin are continuously present. This indicates that forskolin continuously activates the adenylate cyclase. It may locally increase the cAMP level, but the elevation in cAMP generation is compensated by an elevated cAMP degradation, so that no increase in the total cAMP concentration is detected in cellular extracts.

Effect of Forskolin on the Transendothelial Electrical Resistance across Monolayers of Human Endothelial Cells

Measurements of the transendothelial electrical resistance (TEER) indicate that the continuous presence of forskolin is necessary to improve the barrier function of human endothelial cell monolayers. Addition of 25 μM forskolin to the medium results in a 1.5- to 2- fold increase of the TEER, while after removal of forskolin the TEER directly decreases. Upon re-addition of forskolin, the TEER immediately rises again (figure 2). When the cells are continuously incubated with forskolin, the resistance remain elevated for at least four hours (not shown).

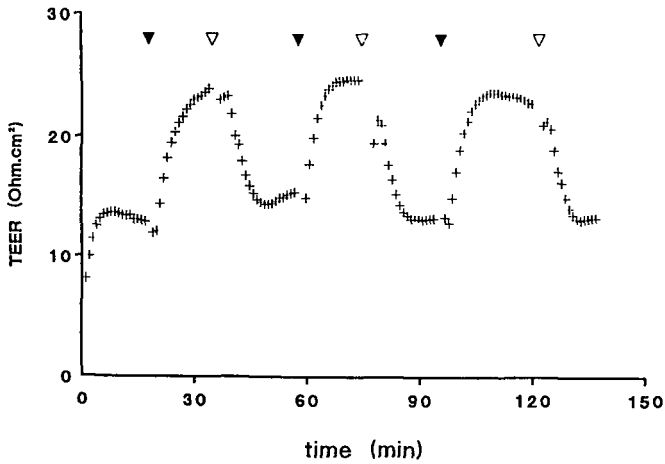

figure 2 The transendothelial electrical resistance (TEER) increases immediately after addition of 25 μM forskolin (▼), but returns to control-level after removal of forskolin (▽). The initial rise of resistance is due to mechanical stimulation of the endothelial monolayers (36).

Effect of Forskolin and IBMX on the Passage of Macromolecules through Monolayers of Human Endothelial Cells

Incubation of endothelial cell monolayers with forskolin induces a concentration-dependent reduction of the passage rate of macromolecules as is shown in figure 3A for peroxidase. This effect can directly be observed after addition of forskolin, and sustains for at least 12 hours (fig 3B). The mean reduction of the passage of LDL, HRP or sucrose induced by 25 μM forskolin is 82, 74 and 55% respectively (not shown). In the presence of IBMX alone, a similar reduction of the passage rate is observed as with forskolin, while in most experiments, in the presence of both substances the passage rate is slightly but not significant further reduced (fig 3B).

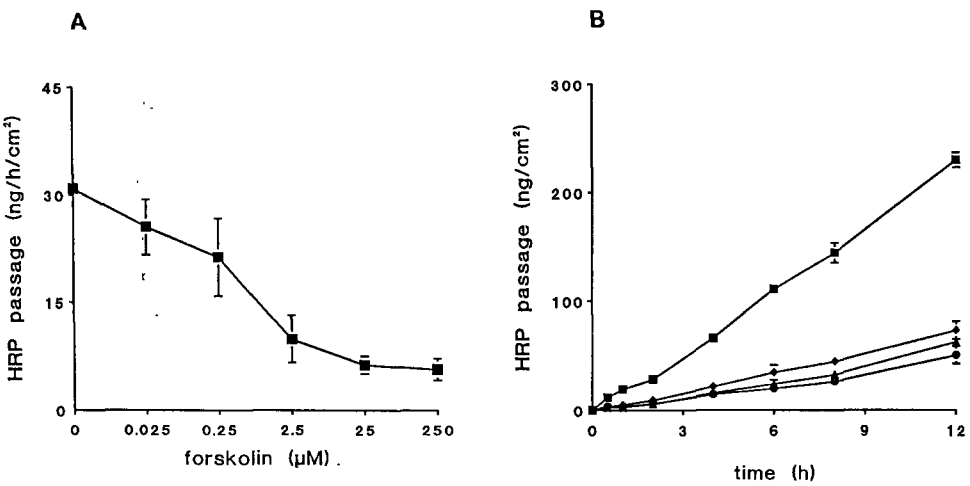

figure 3 Effect of forskolin on the passage rate of horse-radish peroxidase (HRP) through human umbilical artery endothelial cell monolayers. A. A concentration-dependent reduction of the HRP passage was observed over a two hour period after addition of forskolin. B. Time course of the effects of 25 μM forskolin (◆), 1 mM IBMX (▲) and their combination (●) on the passage of HRP. Forskolin and IBMX were added at the start of the experiment. (■): control incubations. Values are the mean $\pm$ S.D. of triplicate filters of a representative experiment.

Effect of Adrenergic Substances on Human Endothelial Cell Monolayers

To investigate whether endothelial barrier enhancement can be evoked by physiological stimuli of the cellular cAMP concentration, we tested the effects of the stable analogue for prostacyclin, iloprost, and the β-adrenergic agonist, isoproterenol, on the cAMP-concentration of and the passage of peroxidase through endothelial cell monolayers. In the presence of IBMX, 10 μM iloprost as well as 10 μM isoproterenol induces a two to four -fold increase of the cAMP-concentration (table 1). Furthermore, the passage rate of peroxidase decreases upon addition of one of these substances (table 2). The effects of isoproterenol on cAMP and peroxidase passage can be blocked by the addition of the β-adrenergic antagonist, propranolol (10 μM). Nor-epinephrine acts on endothelial cells not only through β-adrenergic receptors (28), but also via α-adrenergic receptors (39). Therefore we evaluated the effects of α-adrenergic agonists on permeability. Activation of the α-receptors may result in calcium-influx (α1/α2), IP_3-formation (α1) and inactivation of the adenylate cyclase via a G_i-protein (α2). Since activation of the calcium/calmodulin complex and a decrease in cAMP level results in an increased endothelial permeability, we expected

that activation of α-receptors would result in a decrease of the barrier function. As can be seen in table 2 activation of endothelial cells with the α-adrenergic agonists clonidine, methoxamine or phenylephrine results in an increase of the passage rate. In response to nor-epinephrine (10 μM) only a small increase of the cellular cAMP concentration is observed. This increase is always observed 5 minutes after addi-tion (table 1). This increase can be blocked by propranolol to the same extent as in case of isoproterenol (table 1). In addition, in the presence of the α-blocker phentol-amine (10 μM) the cAMP concentration is slightly elevated above that observed with nor-epinephrine (table 1). In the presence of nor-epinephrine, the passage rate of HRP is significantly reduced (63 ± 16% of control values; mean ± s.d, 8 experiments). Upon addition of either phentolamine or propranolol the effect of nor-epinephrine is only partly blocked (table 2).

table 1 Effect of isoproterenol, nor-epinephrine and iloprost on the cyclic AMP concentration in human umbilical arterial endothelial cells.

Addition	cyclic AMP-concentration (pmol/ $5x10^5$ cells)		
	exp 1	exp 2	exp 3
None	6.2 ± 0.8	5.6 ± 0.8	3.5 ± 0.2
Isoproterenol	13.0 ± 1.6	10.1 ± 0.8	13.0 ± 1.3
+ phentol-amine	17.8 ± 1.4	10.4 ± 1.5	11.4 ± 1.0
+propranolol	8.8 ± 2.0	4.8 ± 0.4	5.6 ± 0.3
Nor-epinephrine	9.1 ± 1.1	6.6 ± 0.0	5.8 ± 0.6
+phentol-amine	10.5 ± 1.4	7.2 ± 0.2	7.1 ± 0.1
+propranolol	7.8 ± 0.4	4.7 ± 0.8	4.5 ± 0.5
Phentol-amine	8.6	4.3 ± 0.1	3.4 ± 0.1
Propranolol	6.2 ± 1.1	3.1 ± 0.4	4.8 ± 0.2
None	5.7 ± 0.7	4.7 ± 0.0	2.3 ± 0.3
Iloprost	18.5 ± 0.1	18.1 ± 0.7	4.7 ± 0.7

Confluent human arterial endothelial cells (first passage) were preincubated for one hour in Medium 199 with 20 % human serum. During the last 15 minutes 1 mM IBMX was present. At the start of the experiment at each well 0.5 ml 20% human serum in Medium 199 without supplement or supplemented with either 10 μM isoproterenol (± 10 μM phentolamine or 10 μM propranolol) or 10 μM nor-epinephrine (± 10 μM phentolamine or 10 μM propranolol) or 10 μM phentolamine or 10 μM propranolol or 10 μM iloprost was added. After a 5 minute-incubation, medium was aspirated from each well and cAMP was extracted and determined as described in Material and Methods. Values represent mean ± range of duplicate wells and are given for three independent experiments (exp).

table 2 Effects of iloprost and various adrenergic substances on the passage of peroxidase through monolayers of human umbilical artery endothelial cells.

Addition	HRP passage (% of control)	
None	100	
Iloprost	63 ± 16	(3)
Isoproterenol	77 ± 17	(3)
+phentolamine	79 ± 20	(3)
+propranolol	98 ± 6	(3)
Nor-epinephrine	62 ± 13	(8)
+phentolamine	74 ± 16	(8)
+propranolol	80 ± 23	(8)
Phentolamine	101 ± 21	(8)
Propranolol	94 ± 23	(8)
Clonidine	145 ± 6	(2)
Methoxamine	128 ± 13	(2)
Phenylephrine	130 ± 6	(2)

Values (% of control) represent the means ± S.D. (or range in case of two experiments) of independent experiments (numbers in parentheses) performed with human umbilical artery endothelial cells from different donors. Passage of HRP (5 μg/ml) was determined in the absence and presence of the indicated substances (all concentrations: 10 μM), as described in Materials and Methods. Passage of peroxidase was determined in three separate filters after a two-hour incubation and is expressed as percentage of the value observed in control monolayers (also determined in triplicate).

To investigate whether the effects of nor-epinephrine on the permeability of umbilical artery endothelial cell monolayers also extends to monolayers of endothelial cells from adult arteries and veins, we tested the effects of nor-epinephrine and forskolin on monolayers of human aorta and vena cava endothelial cells. In agreement with our earlier findings (7), human aorta endothelial cells display a lower basal permeability for peroxidase than umbilical artery endothelial cells. The peroxidase passage was further reduced by 36% by nor-epinephrine and by 53% by forskolin. The permeability of vena cava endothelial cell monolayers was reduced by these agents by 55% and 71%, respectively (figure 4).

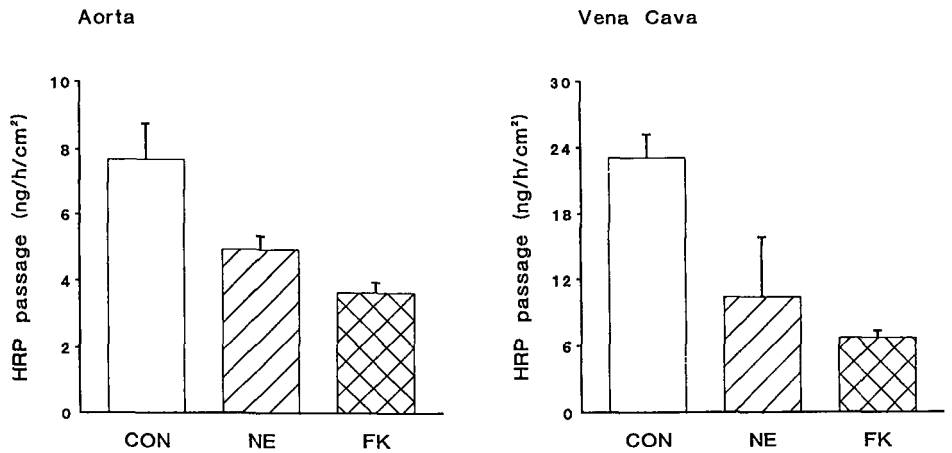

figure 4 Effect of nor-epinephrine (NE, 10 μM) and forskolin (FK, 25 μM) on the passage rate of peroxidase (HRP) through human aorta (left) and vena cava (right) endothelial cell monolayers. Peroxidase (5 μg/ml) is added to upper compartment and two hours later the passage rate is determined as described in Materials and Methods. CON: control cells. Values represent the mean ± S.D. of three monolayers of the same endothelial cell culture.

Effect on Actin/Myosin Configuration

Cultured human arterial endothelial cells in a confluent monolayer contain prominent stress fibers. F-actin becomes visible by staining with rhodamine-phalloidin, while the non-muscle myosin can be stained by a specific antibody. Both actin and myosin are present in stress fibers of the untreated endothelial cells (figure 5 a,b). Incubation of human umbilical artery monolayers with forskolin (25 μM, 1 hour) results in a loss of integrity of the stress fibers: f-actin is no longer visible in stress fibers but appears in a more diffuse pattern, while myosin is also present in a more diffuse pattern (figure 5 c,d). Effects of nor-epinephrine, iloprost (fig 5 e-h) and isoproterenol (not shown) are less pronounced than those of forskolin.

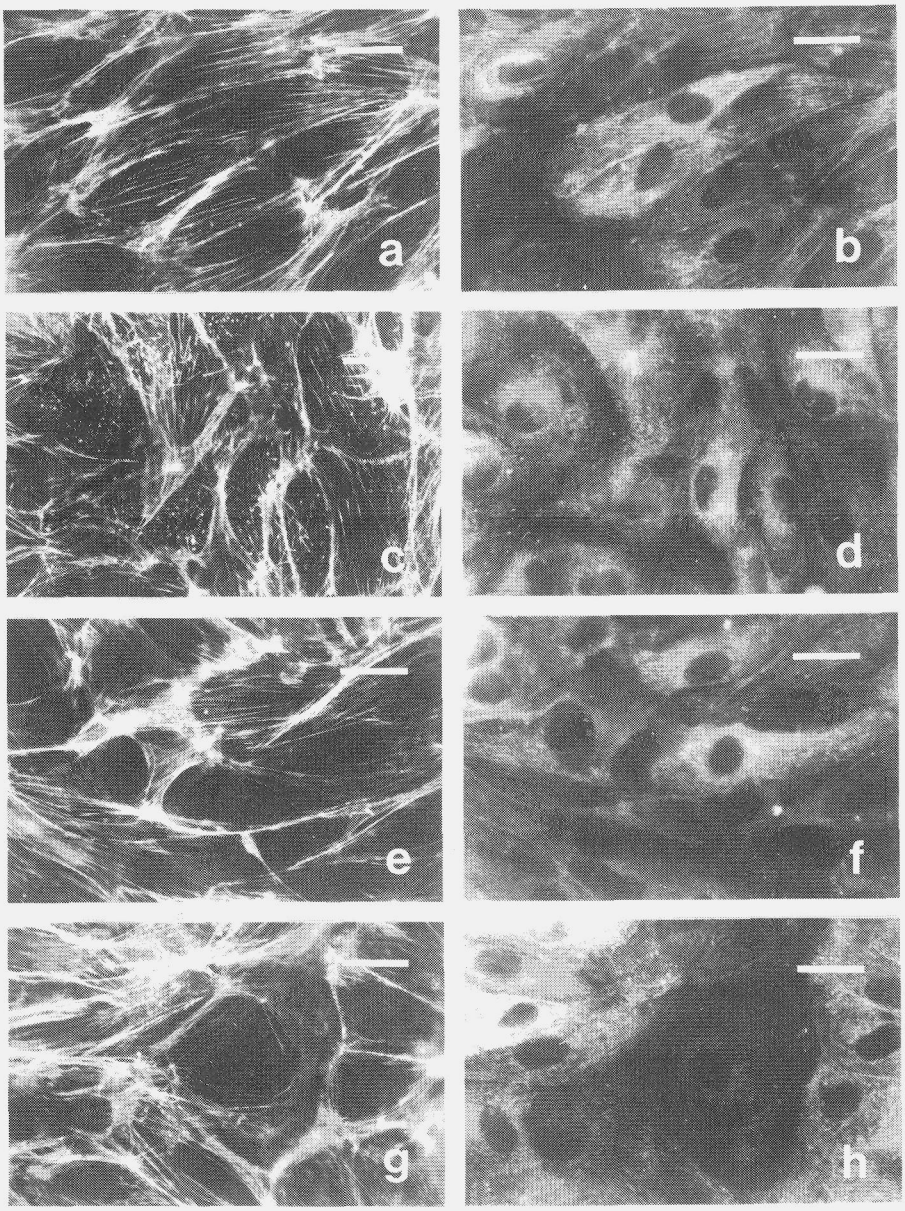

figure 5 Fluorescence photomicrographs of double stained human umbilical artery endothelial cells. Cells are labelled for f-actin with rhodamine-phalloidin (a,c,e,g) and for myosin with a specific antibody (b,d,f,h). Control cells contain prominent stress fibers, containing both f-actin (a) and myosin (b). After incubation with forskolin (25 μM) for one hour stress fibers are disrupted in many cells and f-actin (c) and myosin (d) are visible in a more diffuse pattern. After incubation with nor-epinephrine (e,f) or iloprost (g,h) changes are similar but less pronounced. Bars represent 10 μm.

The total amount of bound rhodamine-phalloidin can be measured after extraction of the cells in methanol. Low doses of rhodamine-phalloidin resulted in a non-saturable staining of the f-actin, while addition of high doses of rhodamine-phalloidin will introduce too high concentrations methanol for staining. To obtain adequate measurements of the f-actin content we incubated cells with a fixed amount of rhodamine-phalloidin and two concentrations of unlabelled phalloidin. The total f-actin content of endothelial cells exposed to 25 μM forskolin is 91 ± 8% of the amount extracted from untreated cells (four experiments, each in duplicate, not significant).
Antonov et al (40) suggested that effects of forskolin are mediated by the interaction of intermediate filaments. They described that colchicine (by disruption of microtubules), prevents the effect of forskolin on cell shape. In our model, monolayers do not remain intact upon addition of colchicine (0.1 μM) alone. One hour after addition, the passage is 1.5 ± 0.2- fold higher than through control monolayers. In the presence of forskolin, monolayers remain intact longer upon addition of colchicine. But about 4 hours after this addition these monolayers loose their tight barrier function and subsequently the passage rate increases even above control values (data not shown).

DISCUSSION

The integrity of the endothelium has an essential role in controlling vascular permeability. Factors that influence endothelial permeability directly affect the influx of macromolecules and solutes into the vessel wall. Furthermore, endothelial cells are the first cells in the vasculature to be exposed to circulating hormones, such as the catecholamines. In the present study we have demonstrated that (a) agents that increase cellular cAMP, including catecholamines and iloprost, decrease endothelial permeability, (b) nor-epinephrine decreases endothelial permeability to a larger extent than that might be expected on the basis of its effects on cAMP, and (c) these changes in endothelial permeability are paralleled by changes in the cellular configuration of actin and myosin.
Forskolin induces a concentration-dependent decrease of endothelial permeability and a parallel concentration-dependent increase of intracellular cAMP. The effects of forskolin and other agents which activate adenylate cyclase on the cellular cAMP-concentration are much more pronounced in the presence than in the absence of the

phosphodiesterase inhibitor IBMX. Without IBMX, only marginal changes can be established, probably due to the ability of the cells to compensate for the increased adenylate cyclase activity by increasing their phosphodiesterase activity. A similar phenomenon has recently been described for fat cells that were stimulated by insulin or isoprenaline (41). It is likely, however, that a continuously enhanced generation of cAMP, which occurs locally but does not influence the total cellular cAMP-concentration, underlies the improved barrier function of the endothelial cell monolayers. Firstly, because forskolin has to be continuously present for an improved barrier function, as becomes clear from the determinations of the transendothelial electrical resistance (figure 2), and secondly, because forskolin activates the adenylate cyclase during the whole incubation period, as has been shown by the later addition of IBMX (figure 1A, open triangle).

Other investigators have shown an increase of cAMP or a decrease of the permeability after treatment with isoproterenol (25,42-44), nor-epinephrine (5,31,45), prostaglandins (30,46,47) and in vivo the stable prostacyclin analogue iloprost (48,49). However, a comparison between these results is difficult because various cell types and various conditions have been used. In the present study we have shown that the β-adrenergic agonist isoproterenol increases the cAMP concentration 2.5-fold, whereas nor-epinephrine induces a lesser increase at the same concentration. Two causes may underlie the difference between isoproterenol and nor-epinephrine. Firstly, Steinberg et al. (28) reported that isoproterenol binds with a 100-fold higher affinity to the β2-receptors of human endothelial cells than nor-epinephrine, which may result in a difference in receptor occupancy even at the high concentration that we have used. Secondly, nor-epinephrine may interact not only with β-adrenergic receptors (resulting in an activation of adenylate cyclase), but also with α-adrenergic receptors (leading to inactivation of adenylate cyclase). The addition of the α-receptor antagonist, phentolamine, together with nor-epinephrine elevates the increase in the cellular cAMP concentration in our cells, while addition of the β-receptor antagonist propranolol reduces the increase. Notwithstanding the small increase in cAMP, nor-epinephrine causes a marked reduction of endothelial permeability. In figure 6, the relative decrease of the peroxidase passage induced by the various mediators in our study are expressed as a function of the relative increase in cellular cAMP. A linear relationship between the reduction of the peroxidase passage and the elevation

of the cAMP concentration is observed for iloprost, isoproterenol and 0.25 and 2.5 μM forskolin (F1 and F2). Nor-epinephrine, on the other hand, decreases the permeability of endothelial cell monolayers much more than might be expected on the basis of the small increase in cAMP. This indicates that the large reduction of permeability, induced by nor-epinephrine, can only for a minor part be explained by involvement of cAMP and that there is an additional mechanism involved in the reduction of endothelial permeability, induced by nor-epinephrine.

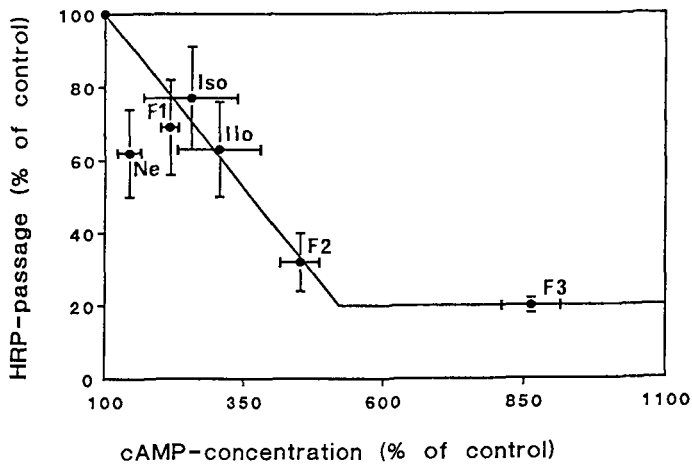

figure 6 Relative changes in cAMP are plotted against relative changes in endothelial permeability for peroxidase (HRP). Data for cAMP concentration and endothelial permeability are taken from tables 1 and 2 and figures 1 and 3. A correlation between cAMP and endothelial permeability is found for isoproterenol (Iso), iloprost (Ilo) and forskolin (F1: 0.25 μM; F2: 2.5 μM). The large reduction of the permeability, induced by nor-epinephrine (Ne) is paralleled by a relatively small increase of cAMP. No further reduction of the permeability has been found, if the cAMP is elevated above five times control level (F3: 25 μM forskolin). Values represent the mean ± S.E.M. for the amount of experiments as mentioned in the tables and the figures, respectively.

Changes in the cytoskeletal organization may underlie the improved barrier function, induced by cAMP. Antonov et al. (20) have reported closer and more complicated cell to cell contacts after stimulation of human endothelial cells with forskolin, and Bensch et al. (27) have shown that bovine endothelial cells tend to spread upon elevation of their cAMP concentration. Also in epithelial cells a change in micro-filaments has been observed after elevation of their cellular cAMP concentration (50) and peri-junctional actin-myosin interaction has been implicated in the regulation of the permeability of these cells (51). Also in endothelial cells the interaction between

actin and non-muscle myosin is involved in the regulation of permeability (13,14). The involvement of f-actin was demonstrated both in vitro (52-54) and in vivo (55). Here, we have shown by double fluorescence microscopy that the stress fibers structures, which consist of f-actin and non-muscle myosin molecules, disintegrate upon incubation of the cells with forskolin. The total amount of f-actin was hardly influenced under this condition. Whether the dissociation of stress fibers plays a direct role in endothelial permeability is uncertain. It may reflect a general dissociation of actin and myosin in the cell. Possibly the peri-junctional actin-myosin interaction is a more important regulator of the permeability of endothelial cell monolayers.

Although in vitro monolayers can only be used as an approximation of physiological or pathological processes, the in vivo relevance of our data is supported by several reports. In various in vivo conditions, in which the vascular permeability is increased by toxic, mechanical or other injury, prostacyclin or iloprost (48,49,56), β-adrenergic agonists (22,49,57,58) and di-buturyl cAMP (21) reduce this increased permeability. In summary, forskolin, nor-epinephrine, the β-adrenergic agonist isoproterenol and the prostacyclin analogue iloprost decrease endothelial monolayer permeability and increase the cAMP concentration in an in vitro system. Although there is a relationship between cellular cAMP concentration and endothelial permeability, the effect of nor-epinephrine on endothelial permeability is much larger as would be expected on the basis of the small elevation on cAMP. This indicates that another mechanism exists by which nor-epinephrine induces an improved barrier function. Further studies have to clarify which mechanism is involved in this process.

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CHAPTER 5

EFFECTS OF TUMOUR NECROSIS FACTOR ON PROSTACYCLIN PRODUCTION AND THE BARRIER FUNCTION OF HUMAN ENDOTHELIAL CELL MONOLAYERS

Erna G. Langeler[1], Walter Fiers[2] and Victor W.M. van Hinsbergh[1]

[1]Gaubius Institute TNO, Leiden, The Netherlands
[2]Laboratory of Molecular Biology, The State University of Gent, Gent, Belgium

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ABSTRACT

The endothelium controls the influx of macromolecules into the tissues, a process that may be disturbed at sites of inflammation and in the atherosclerotic plaque. In this report we have evaluated the effects of the inflammatory mediator, tumour necrosis factor (TNF) on the production of prostacylin and the barrier function of human endothelial cell monolayers in an in vitro model. TNF (500 U/ml) induced within 10 minutes a 10- fold stimulation of the production of 6-keto-prostaglandin $F1_{\alpha}$ by primary human umbilical artery and vein endothelial cells, suggesting an immediate release of prostacyclin. Incubation for two to four hours with 500 U/ml TNF resulted in a decreased passage rate of both peroxidase and LDL through monolayers of human umbilical artery endothelial cells. Incubation for 24 hours with TNF induced an increased permeability in these cells, whereas no change in permeability of human aorta endothelial cells was found. The reduction of the passage rate during short time-incubation of umbilical artery endothelial cells induced by TNF is paralleled by a change in actin-configuration. The adenylate cyclase activator, forskolin, induced a similar, but more pronounced effect on the permeability and the cytoskeleton. However, the effect of TNF on the permeability of endothelial monolayers appeared not to be dependent on cyclic adenosine 5'-monophosphate (cAMP) or prostacyclin production by the endothelial cells, because (a) no effect on cAMP concentration in the cell by addition of TNF could be observed, (b) in the presence of forskolin, TNF was able to reduce the passage rate and (c) the effect of TNF on permeability was not influenced by aspirin.

INTRODUCTION

The vascular endothelium actively regulates the influx of solutes, macromolecules and leucocytes into the tissues and the arterial intima. At sites of inflammation (1,2) and in the human arteriosclerotic plaque (3) the passage of macromolecules through the endothelium is increased. In inflammation, direct interactions of leucocytes and of histamine and other inflammatory mediators with the endothelium play an important role in the vascular leakage phenomenon (4). The underlying cause of the impaired barrier function of the endothelium of arteriosclerotic lesions is less clear. Since the human atheromata contain numerous macrophages, in which, in the cytoplasm tumor necrosis factor (TNF) can be detected by immunohistochemical techniques (5), and

endothelial cells in vitro show structural changes after incubation with TNF (6-8), we set up a series of experiments to evaluate the effect of TNF on endothelial permeability. We have used an in vitro model which we have previously developed and characterized (9,10), to study the permeability of human endothelial cells.
Two groups of investigators have recently reported that TNF increased the permeability of sheep and bovine endothelial cell monolayers on porous filters (11,12). On the other hand, the passage of albumin through human umbilical vein monolayers did not increase for up to four hours after addition of TNF or interleukin-1 (IL-1), while the diapedesis of neutrophils did increase during the same time period (13). In the present report, we describe that high concentrations of TNF have a dual, time-dependent effect on the permeability of monolayers human umbilical artery endothelial cells. In addition, we have evaluated whether TNF affects the immediate release of prostacyclin and the cAMP concentration in the cells, and whether prostacyclin and cAMP may be involved in the effects of TNF on endothelial cell permeability.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Corning (Corning, NY) or Costar (Cambridge, MA); Transwells (diameter 0.65 cm; pore size 3 μm) were from Costar. Human recombinant Tumour Necrosis Factor (TNF) was a gift from Dr. Jan Tavernier (Biogent, Gent). The preparation contained 2.45 x 10^7 U/mg protein and less than 40 ng LPS per mg protein. Human recombinant IL-1α (pI 5) was purchased from Genzyme (Haverhill, U.K.). Interleukin-2 and penicillin/streptomycin were purchased from Boehringer (Mannheim, FRG). Iloprost was a gift from Schering AG (Weesp, the Netherlands). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al (14). Human serum was obtained from a local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY) and heat-inactivated before use (30 min, 56°C). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands); pyrogen-free human serum albumin was from the Red Cross Central Blood Transfusion Laboratory (Amsterdam, the Netherlands). Horse-

radish peroxidase was obtained from Sigma Chemical Company (St. Louis, MO), forskolin from Hoechst (La Jolla, CA). Rhodamine-phalloidin was purchased from Molecular Probes (Junction City, OR).

Culture of Endothelial Cells

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al (15). Endothelial cells from vena cava and aorta were isolated and characterized as described previously (16). Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/streptomycin. Cells were kept at 37°C under 5% CO_2-95% air.

For passage studies, confluent monolayers of endothelial cells from umbilical artery (primary), aorta (after one passage) or vena cava (fifth till seventh passage) were released with trypsin-EDTA (ethylenediaminetetraacedic) acid and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell system and cultured as described above. Medium was renewed every other day. For the evaluation of the actin configuration by immunofluorescence, cells were cultured on coverslips coated with cross-linked gelatin (17).

Passage-experiments

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Passage of horse-radish peroxidase through human endothelial cell monolayers was measured as earlier described (9). In short, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) for 4-6 days to form a tight monolayer. Before the experiment cells were incubated for one hour in Medium 199 supplemented with 20% human serum. At the start of the experiment 5 μg/ml horse-radish peroxidase in Medium 199 with 20% human serum was added to the upper compartment of the Transwell-system. At various time intervals samples were taken from the lower compartment (at the other side of the endothelial monolayer), and an equal amount of Medium 199-20% human serum was re-added to this compartment. All passage experiments are performed in triplicate. The peroxidase-concentration was determined in each sample as previously described. Passage rates are expressed as ng/h/cm^2 or ng/cm^2.

Immunofluorescence

Confluent endothelial cell monolayers cultured on coverslips were stimulated with or without 500 U/ml TNF in complete culture medium for the indicated time-intervals. Medium was aspirated and cells were directly fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes, followed by two minutes in 0.1% Triton-X100 in PBS at 4°C. Cells were gently washed with PBS. To visualize f-actin the cells were incubated with 3.3 x 10^{-8} M rhodamine-phalloidin in PBS for 30 minutes. After staining the cells, they were gently washed, p-phenylenediamine was added to each coverslip and cells were viewed in a fluorescence microscope (Leitz, Laborlux D). Photomicrographs were taken with Ilford HP5-film.

Assay of 6-Keto-Prostaglandin-F1$_\alpha$

The prostacyclin metabolite 6-keto-PGF1$_\alpha$ was assayed by radioimmunoassay (Amersham). Human umbilical artery endothelial cells (confluent, primary or once subcultured) in 2 cm^2 wells were washed with Medium 199 and incubated for 30 minutes in Medium 199 supplemented with 0.03% human serum albumin. Hereafter, a sample was taken from the medium to determine basal 6-keto-PGF1$_\alpha$-production in each well. Medium with or without TNF (final concentration: 500 U/ml) or histamine (final concentration 10^{-5} M) was re-added; after both 10 and 60 minutes incubation samples were taken from the medium. Samples were stored at -80°C until the assay of 6-keto-PGF1$_\alpha$ was performed. Total 6-keto-PGF1$_\alpha$-concentration was determined in each sample and corrected for the basal production in each well before stimulation.

Extraction and Assay of cyclic AMP

Extraction and assay of cAMP was performed according to Adams-Brotherton et al (18) with some modifications. Confluent endothelial cell monolayers, cultured in 5 cm^2 wells, were incubated in Medium 199 supplemented with 20% human serum one hour before the incubation-period. fifteen minutes before the incubation period isobutyl methyl xanthine (IBMX, 1 mM final concentration) was added to the medium. At the start of the incubation period, stimulatory agents were added to the medium and incubations were continued for another 15 minutes at 37°C. Subsequently 0.6 ml of ice-cold 5% trichloroacetic acid was added to each well. A small

amount of [^{3}H]-cAMP (Amersham, UK) was added to each well to monitor the recovery of cAMP during the purification. After ten minutes on ice, the extracts were collected and brought on a small DOWEX-50 WX4 column. Columns were washed with 0.05 N HCl and cyclic nucleotides were eluted with 1N HCl, and air-dried. Concentration of cAMP was determined using a radioimmunoassay (Amersham), and corrected for the recoveries of cAMP in the various samples.

RESULTS

Effect of Inflammatory Mediators on Endothelial Permeability

Human umbilical artery, aorta and vena cava endothelial cells, cultured on porous filters, form a tight monolayer, with an average transendothelial electrical resistance of 17 Ohm.cm^2 (umbilical artery), 10 Ohm.cm^2 (aorta) and 8 Ohm.cm^2 (vena cava), respectively. The passage rate of peroxidase through human umbilical artery endothelial cell monolayers was comparable with the passage rate through human vena cava and aorta endothelial monolayers and 60-fold lower than through filters without an endothelial monolayer. Pretreatment of human umbilical artery endothelial monolayers with 500 U/ml tumour necrosis factor (TNF) for 24 hours resulted in a

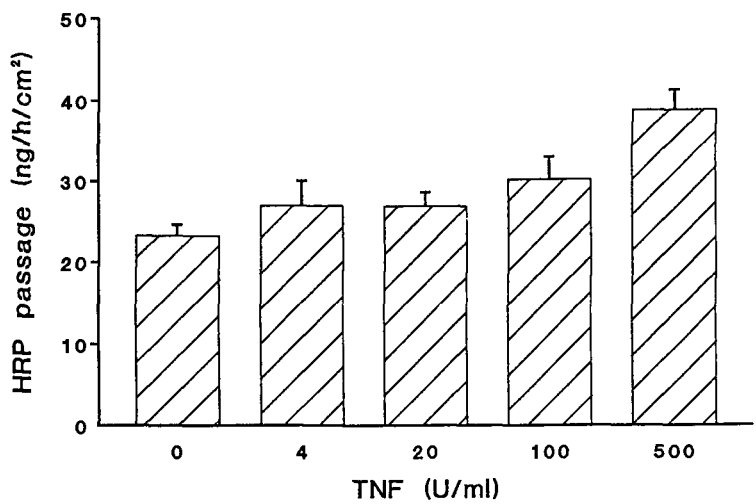

figure 1 Human umbilical artery endothelial cell monolayers on porous filters were incubated for 24 hours with various concentrations TNF. Subsequently, 5 μg/ml peroxidase was added to the upper compartment without replacing total medium. After 2 hours the amount of peroxidase was determined in the lower compartment. Values represent the mean ± SD of two independent experiments, each performed in triplicate.

1.9 ± 0.3- fold increase of the passage rate (mean ± SD; four independent experiments). This effect was independent of the presence of endothelial cell growth factor (not shown). When the TNF preparation was inactivated by heating for 15 minutes at 90°C before addition, no change in permeability was observed. Figure 1 shows the concentration dependency of the effect of TNF. Although there was a tendency to an increased peroxidase passage at lower TNF-concentrations, this passage was only significantly increased at 500 U/ml TNF. Incubation of the cells for 24 hours with 50 U/ml interleukin-2 did not change the peroxidase passage, whereas exposure of the cells for the same period to 5 U/ml interleukin-1 induced a small increase in peroxidase passage in only one out of three experiments.

When subcultured vena cava endothelial cells were treated with 500 U/ml TNF for 24 hours the passage rate increased by 25% (table 1). Similar results were obtained when the passage of LDL was studied (not shown). No significant increase of the permeability was observed after treatment of human aortic endothelial cell monolayers with 500 U/ml for 24 hours (table 1).

table 1 Effect of TNF on peroxidase passage through monolayers of human umbilical artery, vena cava and aorta endothelial cells.

Endothelial Cells	Peroxidase passage (ng/cm^2)	
	Without TNF	With TNF
Umbilical artery (two hours)	51 ± 18 (7)	36 ± 14 (7) **
Umbilical artery (24 hours)	856 ± 102 (6)	1274 ± 185 (6) *
Vena cava (24 hours)	907 ± 169 (4)	1131 ± 160 (4) *
Aorta (24 hours)	746 ± 10 (2)	742 ± 148 (2)

Passage of peroxidase (added in upper compartment 5 $\mu g/ml$) through human endothelial cell monolayers was determined in the absence and presence of 500 U/ml TNF. Passage of peroxidase was determined in triplicate after 2 or 24 hours. Data of aorta and umbilical artery endothelial cells represent the mean ± SD of 2 and 7 experiments, respectively, performed with cultures of two and seven different donors. The data on human vena cava endothelial cells are the mean ± SD of 4 independent experiments with cells from one donor.

* ($p < 0.05$); ** ($p = 0.02$): values statistically different from control values.

When we evaluated the effect of TNF on the permeability through human umbilical artery endothelial monolayers over shorter time-intervals, a concentration-dependent decrease in peroxidase passage was observed (figure 2a,b). Table 1 shows the data of seven different cultures of umbilical artery endothelial cells. In five of these cultures the peroxidase passage was reduced 2 hours after addition of TNF by 33% on the average, whereas in two of these cultures no change in permeability was observed. Taken over the seven experiments together the decrease of the passage rate was statistically significant ($p = 0.02$) (table 1). In two additional experiments with human aortic endothelial cells, no significant reduction of the permeability was observed two hours after addition of 500 U/ml TNF (85 ± 12% and 84 ± 10% of mean control values).

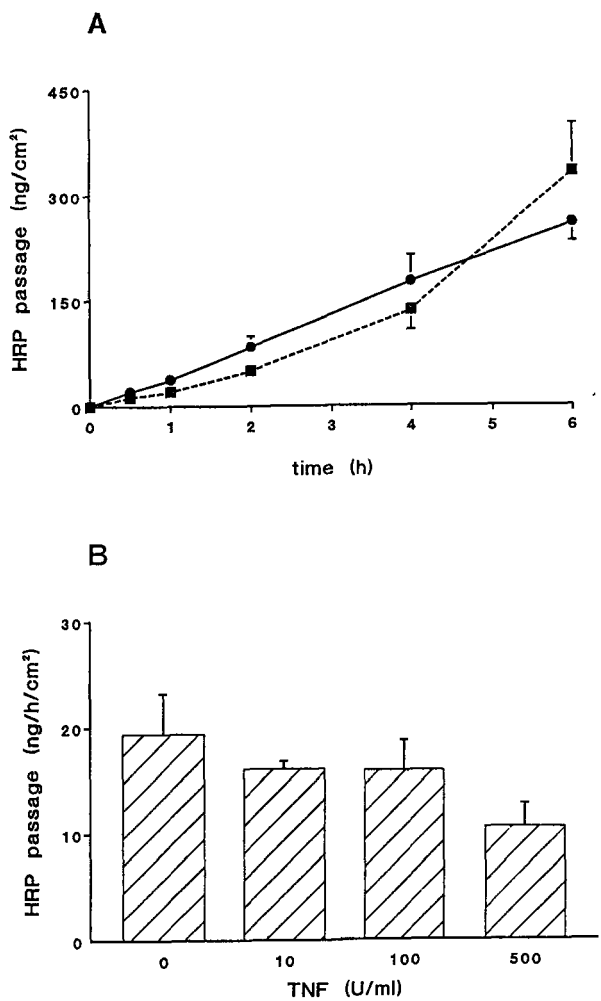

figure 2 Effect of TNF on the passage of peroxidase through monolayers of human umbilical artery endothelial cells. A: passage of peroxidase was followed over a 6 hours-period in the absence (●) or presence (■) of 500 U/ml TNF. B: various monolayers were incubated for four hours with various concentrations TNF. TNF was added to both upper and lower compartment, while peroxidase was only added to the upper compartment at the start of the experiment.
Values are mean ± SD of one representative experiment, performed in triplicate.

Effect of TNF on Actin-configuration in the Cell

Human umbilical artery endothelial cells in a confluent monolayer are flat closely apposing cells with prominent stress fibers, which contain f-actin, as visualized by staining with rhodamine-phalloidin (figure 3A). F-actin was also present in the peripheral zone of the cells. Treatment of these cells with TNF (500 U/ml) resulted in a time-dependent alteration of the configuration of the cytoskeleton. After 30-60 minutes exposure to TNF the f-actin staining of the stress fibers was less prominent (two of four tested cultures) or even disappeared largely (two of four experiments) as is depicted in figure 3C. The cells remained closely apposed. After 30-60 minutes

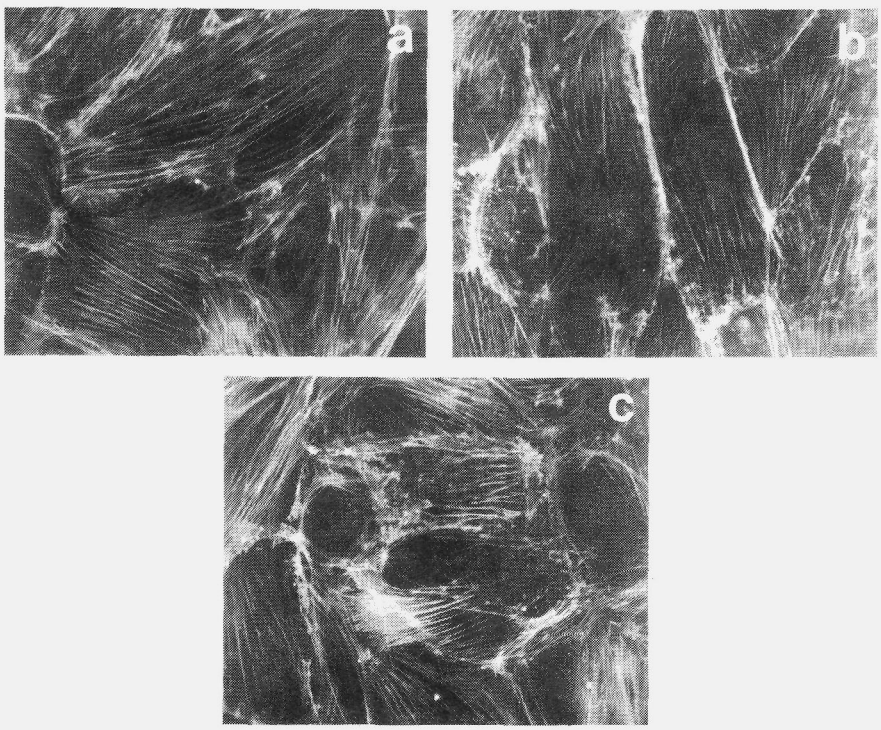

figure 3 Human umbilical artery endothelial cells were cultured on gelatin-coated coverslips and incubated for 60 minutes without (A), with 25 μM forskolin (B), or with 500 U/ml TNF (C). Cells were stained with rhodamine-phalloidin as described in Materials and Methods. Magnification 7000 x.

treatment with TNF the peripheral f-actin staining remained similar or occasionally became more pronounced. After 24 hours exposure to 500 U/ml TNF small gaps between the cells became visible (figure 4), while the stress fibers have become clearly visible again.

We have recently shown that agents that increase the cellular cAMP-level also induce a decrease in the permeability of human endothelial cell monolayers (10). Treatment of human endothelial cells with the adenylate cyclase activator forskolin (25 μM) resulted within 30-60 minutes in a loss of stress fibers in many cells, while the f-actin staining in the cell periphery became more prominent.

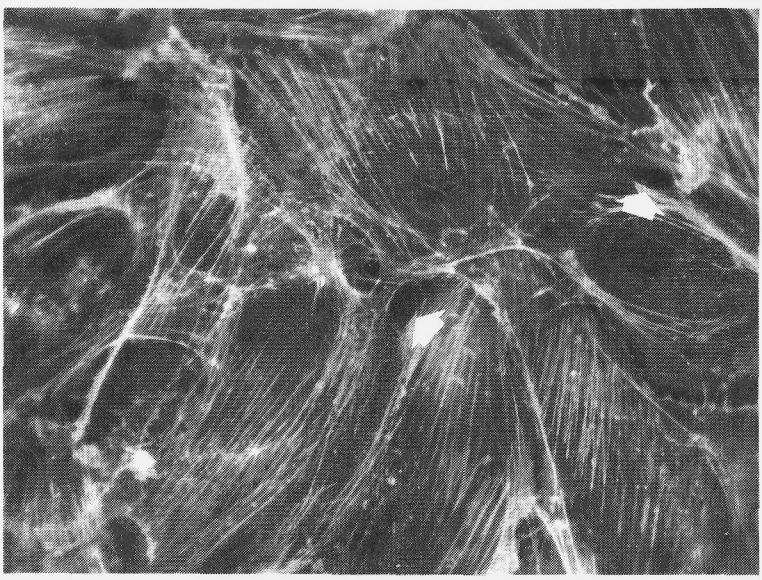

figure 4 Cells were cultured on coverslips and stained with rhodamine-phalloidin after a 24 h incubation with 500 U/ml TNF. Note the gaps between the adjacent cells (arrows). Magnification 7000 x.

Involvement of Prostacyclin and cAMP in the Effects of TNF

TNF induced a temporary decrease of the passage of macromolecules and the changes in the cellular distribution of f-actin gave a weak reflection of the changes obtained with forskolin. Therefore, we investigated whether TNF may induce prostacyclin production, which may then elevate the cAMP level in endothelial cells

(8,19,20), and whether TNF may elevate the cAMP level of these cells directly. Addition of 10^{-5} M iloprost, a stable prostacyclin analogue, induces 30% reduction of the passage rate of peroxidase and increases the cAMP level in the cells 3-fold (not shown). Since TNF can stimulate prostacyclin production in various cell types by direct activation of phospholipase A2 (21), we evaluated whether TNF increases the production of prostacyclin. As is shown in table 2, TNF, as well as histamine, stimulates the prostacyclin production by primary cultures of endothelial cells, as measured by the accumulation of 6-keto-PGF1$_\alpha$. This increase was found both in umbilical artery and vein endothelial cells (7 different cultures). However, after one passage of the cells, the prostacyclin production in unstimulated cells was below detection level, and remained so after stimulation with TNF (four independent cultures). The lack of response to TNF was not due to a general desensitization of the cells for TNF, since TNF still induced urokinase and plasminogen activator inhibitor type-1 in our subcultured cells (22). It is unlikely that TNF acts via prostacyclin production because the effect of TNF on the permeability of endothelial cell monolayers was evaluated with cells that had been passed once. This conclusion is further strengthened by the observation that pretreatment of the cells with the cyclooxygenase inhibitor aspirin (25 μM) could not prevent the effect of TNF on

table 2 Effect of TNF and histamine on the production of 6-keto-prostaglandin F1$_\alpha$ by primary human umbilical artery endothelial cells.

	6-keto-PGF1$_\alpha$ (pg/well)	
Addition	10 minutes	60 minutes
None	0.4 ± 0.4	1.0 ± 1.1
500 U/ml TNF	13.1 ± 5.2	14.7 ± 6.0
10^{-5} M histamine	20.2 ± 6.8	21.4 ± 8.9

Confluent cultures, grown in 2 cm^2 wells, were used for the assay of 6-keto-prostaglandin F1$_\alpha$. Cells were incubated and prostacylin-production was calculated as described in Materials and Methods. Values represent the mean ± SD of six determinations, performed with three different cultures.

table 3 Effect of TNF on the passage of peroxidase through monolayers of human umbilical artery endothelial cells, in the absence or presence of aspirin or forskolin.

	Passage rate of peroxidase ($ng/h/cm^2$)	
Addition	Without TNF	With TNF
None	33 ± 2	23 ± 2
Aspirin	31 ± 1	17 ± 2
None	30 ± 7	22 ± 1
Forskolin	8 ± 3	4 ± 0

Effects of 25 μM aspirin and 25 μM forskolin on the passage of peroxidase through monolayers of human umbilical artery endothelial cells. In the presence of these substances TNF (500 U/ml) has an effect on the permeability of the monolayers similar to that on control monolayers. Peroxidase concentration in the lower compartment was determined two hours after addition of the substance(s) to the medium. Passage rates are calculated and expressed as $ng/h/cm^2$. Values represent the mean ± SD of determinations performed with two different cell cultures.

table 4 Effect of TNF on cAMP concentration in human endothelial cells under various conditions.

	Cyclic AMP (pmol /10^5 cells)	
Addition	Control	500 U/ml TNF
None	1.6 ± 0.3	2.2 ± 1.2
IBMX	3.1 ± 0.9	3.6 ± 1.2
Forskolin	1.7 ± 0.2	1.9 ± 0.3
IBMX and forskolin	9.0 ± 1.6	8.9 ± 1.0

Human umbilical artery endothelial cells (after one passage) were grown to confluence in 5 cm^2 wells. Isobutyl methyl xanthine (IBMX 1mM) was added 15 minutes before the incubation period. Forskolin (25 μM) and TNF (500 U/ml) were added during 15 minutes to the culture medium. Extraction and determination of cAMP were performed as described in Materials and Methods. Values represent the mean ± SD of six determinations, performed in three independent experiments.

endothelial cell permeability (table 3). This observation also excludes the involvement of other prostaglandins.

Three experiments have been performed to investigate whether the effect of TNF on endothelial cell permeability depends directly on an elevation of the cAMP level in

the cells. When our cells were stimulated for 15 minutes with forskolin alone, virtually no increase of the cAMP concentration could be observed, while incubation with IBMX resulted in a sustained two-fold increase of the cAMP-concentration. The presence of both stimulators appeared to be necessary for a sustained increase of the cAMP concentration. However, substitution of either forskolin or IBMX for TNF did not result in an increase of cAMP (table 4).
Furthermore, addition of TNF to endothelial cells in the presence of forskolin resulted in an additional decrease of the passage rate of peroxidase (table 3). We conclude that it is likely that the decrease in endothelial cell permeability occurs by a mechanism that is independent of the cAMP concentration.

DISCUSSION

In this study we have evaluated the effects of TNF on the barrier function of human umbilical artery endothelial cell monolayers. Endothelial cells were cultured on porous filters. They form a tight monolayer, characterized by the presence of tight junctions, an adequate electrical resistance, molecular sieving characteristics and a response to vasoactive substances (9,10).
Incubation of human endothelial cells for two to four hours with TNF resulted in a decreased passage rate of macromolecules. Several investigators (11,12) have shown that TNF induces a large increase of the passage through monolayers of endothelial cells from animal origin, whereas Moser et al (13) reported that incubation of human endothelial cell monolayers with low concentrations of TNF did not result in an increased albumin passage. The discrepancy between the results with animal endothelial cells and our results are probably due to species differences, but an in vitro adaptation can not be excluded, since we maintained our cells in a very high density and subcultured them once, while other investigators have used cell-lines or subcultured cells. When endothelial monolayers of umbilical artery and vena cava cells were incubated for prolonged periods (24 hours) with high concentrations of TNF, we found a slight, but significant increase of the passage of peroxidase. No change in permeability of human aorta endothelial monolayers was observed under these conditions.
Incubation of primary cells for short periods of time (10 minutes) with 500 U/ml TNF resulted in an increased release of prostacyclin. This effect is rapid and repre-

sents another effect of TNF on prostacyclin production, independent of the long-term increase of prostacyclin production probably by induction of cyclo-oxygenase, which has been reported by other investigators (24-26). The effect of TNF on immediate prostacyclin release could only be demonstrated in primary cultures of endothelial cells. Although cells, which have been subcultured once, could be stimulated by histamine or thrombin, no response on prostacyclin production was observed after 10 minutes incubation with TNF. We have investigated whether prostacyclin is involved in the mechanism of the passage process, because it can induce cAMP in endothelial cells (18-20) and an increase of cAMP in endothelial cells is paralleled by a decrease of the protein passage rate (10,23). However, the reduction of the passage rate after 2 hours incubation with TNF can not be explained by the involvement of prostacyclin because 1) we always used cells, which produced very little prostacyclin as they had been passaged once and 2) the reduction of the passage rate was also observed in the presence of the cyclo-oxygenase inhibitor aspirin.

The adenylate cyclase activator, forskolin induces a large increase of cAMP in endothelial cells (27,28). Many of these experiments are performed in the presence of the phosphodiesterase inhibitor IBMX. When we evaluated the effect of forskolin on human endothelial cells in the absence of IBMX, no or only a small increase of cAMP was observed 15 minutes after addition of forskolin. Further studies have shown that forskolin in the absence of IBMX induced a small, rapid increase of cAMP, but this increase is rapidly downregulated by endogenous phosphodiesterase activity (Langeler and van Hinsbergh, unpublished results). Upon addition of IBMX, a sustained increase in cAMP concentration was found (table 4). Apparently, only in the presence of both an activator of the synthesis of cAMP and a phosphodiesterase inhibitor a marked increase of cAMP was found. Therefore, we have evaluated the effects of TNF on the cAMP concentration in endothelial cells in the presence of forskolin as well as in the presence of IBMX. Because under both conditions no further increase of cAMP was induced by TNF, it is not likely that TNF decreases endothelial cell permeability via a cAMP dependent pathway.

In the presence of forskolin, TNF was still able to induce a transient reduction of the passage rate of peroxidase. This finding supports the idea that the reduction is cAMP-independent. In addition, it suggests that the results are not due to a relative

high basal level of the passage rate through cultured human endothelial cell monolayers on artificial surfaces, because the passage rates in the presence of forskolin approximate physiological values (10).

Gap formation between endothelial cells is induced by cellular contraction (29-32) and is caused by the interaction between actin and myosin, which are structural proteins present in the stress fibers and in the cell periphery. This cellular contraction is regulated via the phosphorylation of the myosin light chain by the calcium/calmodulin complex (33). Stress fibers are found in arterial cells, both in vivo under various conditions (34,35) and in vitro (36). They have been postulated to play a role in cellular contraction (37,38). We have shown here that treatment of human endothelial cells with forskolin results in a considerable loss of stress fibers in many of the cells. Treatment of the endothelial monolayers with TNF (500 U/ml) for one hour results in a less prominent but visible alteration of the cytoskeleton. However, it remains to establish whether the disappearance of stress fibers is only the result of a general dissociation of actin and myosin interaction, or that it plays a direct, active role in the regulation of endothelial cell permeability.

In summary, in this report we have shown that high concentrations TNF induce an increase of the prostacyclin production in primary human endothelial cells. During short time incubations TNF induces a reduction of the passage rate of macromolecules through monolayers of human umbilical artery endothelial cells. Although changes in actin configuration induced by TNF are comparable with changes upon an increasement of the cAMP-concentration in the cell, it is not likely that TNF reduces the permeability via a cAMP dependent pathway.

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CHAPTER 6

REGULATION OF THE PERMEABILITY AND PRODUCTION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY HUMAN UMBILICAL ARTERY AND AORTA ENDOTHELIAL CELLS

EFFECT OF FIBROBLAST GROWTH FACTORS AND PHORBOL ESTER

Marcel M. Verbeek, Erna G. Langeler, Teake Kooistra
and Victor W.M. van Hinsbergh

Gaubius Institute TNO, Leiden, the Netherlands.

ABSTRACT

Extravasation of (macro)molecules and fibrinolysis play an important role in the deposition and removal of fibrin within and around the vessel wall. To understand the regulation of the permeability at a biochemical level, we have studied the effects of fibroblast growth factors (FGFs) and the phorbol ester, phorbol 12-myristate 13-acetate (PMA) on the passage of peroxidase through tight monolayers of human umbilical artery and aorta endothelial cells. In addition, we have evaluated the production of tissue-type plasminogen activator (t-PA), a key regulator of fibrinolysis, by these cells. Our results demonstrate the following:

1. Basic-FGF (b-FGF) or acidic-FGF (a-FGF) are necessary to maintain the barrier function of human umbilical artery endothelial cell monolayers.
2. The adenylate cyclase activator forskolin decreases endothelial permeability. b-FGF does not activate adenylate cyclase activity. It acts additive to forskolin in decreasing permeability.
3. Low concentrations of PMA also decrease the permeability of umbilical artery endothelial monolayers. PMA and b-FGF act via different mechanisms, because (a) the decrease by PMA is significantly inhibited by H-7, whereas that by b-FGF is not, and (b) in the presence of forskolin, b-FGF further reduces the permeability of these cells, while PMA does not.
4. PMA and b-FGF increase the production of t-PA by umbilical artery endothelial cells. The increase induced by PMA, but not that by b-FGF is inhibited by H-7. The marked stimulation of t-PA production after the addition of PMA is synergistically enhanced by forskolin, whereas the effect of b-FGF is not further stimulated by forskolin.
5. PMA and b-FGF have no effect on the permeability of human aorta endothelial cells, while PMA several-fold increases the production of t-PA by these cells. This suggests that either different types of protein kinase C are involved in the induction of the decrease of permeability and the stimulation of t-PA production, or that the pathway distal to protein kinase C activation does not transduce the signal into a change in permeability in human aorta endothelial cells.
6. Forskolin induces a decrease in permeability of human aorta endothelial cells, further strengthening the conclusion that PMA and b-FGF act by a different mechanism than forskolin does.

INTRODUCTION

Endothelial cells play a crucial role in the homeostasis of blood vessels. Two of their most important functions are the active control of the extravasation of (macro) molecules and the regulation of the fibrinolytic system. These processes play an important role in the deposition and removal of fibrin within and around the vascular wall. Such fibrin formation occurs in many diseases, such as tumors [1,2], arteriosclerosis [3], and rheumatoid arthritis [4]. Particularly in the case of rheumatoid arthritis and certain tumors, diseases with vascular proliferation, fibroblast growth factors (FGFs) are expressed and affect endothelial cell metabolism [5-7].

Cell division and activation of phospholipase C result in an increase in the permeability of endothelial cell monolayers [8,9]. An increase in endothelial cell permeability might be expected after exposure to FGFs, because acidic-FGF (a-FGF) and basic-FGF (b-FGF) stimulate endothelial cell proliferation and activate phospholipase C-gamma by tyrosine phosphorylation [10-12]. In addition to their effect on endothelial cell proliferation, FGFs are necessary to maintain human umbilical vein and artery endothelial cells in a stable confluent monolayer over prolonged periods [13]. Mioh and Chen [14] reported that a-FGF increases adenylate cyclase activity in smooth muscle cells. Because elevation of the cellular cAMP concentration results in a decrease of endothelial permeability [15-17], and putative elevation of cAMP concentration of endothelial cell monolayers by FGFs may improve their barrier. In the present report, we describe the effects of human b-FGF and a-FGF on the permeability of human artery endothelial cell monolayers in vitro. These monolayers have previously been characterized [16,18] by the presence of tight junctions, an adequate electrical resistance, molecular sieving characteristics, repulsion of negatively-charged proteins, dependence of the barrier function on albumin and calcium ions, and a responsiveness to vasoactive substances.

Furthermore, we have evaluated the effect of stimulating protein kinase C (PKC) on the permeability of these monolayers. Presta et al. [19] have recently reported that b-FGF induces u-PA in bovine endothelial cells via a PKC pathway, which was mimicked by PMA, while the effect of b-FGF on endothelial cell proliferation is independent on PKC activation. The phorbol ester PMA causes endothelial cell damage and vascular leakage upon superfusion of animal arteries [20,21] and pig aorta endothelial cells in vitro [22,23]. Here, we demonstrate that the permeabilities

of human umbilical artery and aorta endothelial cell monolayers respond differently to the addition of PMA. Furthermore, we compare the response of these cells to PMA and b-FGF. Because tissue-type plasminogen activator (t-PA) is induced in human endothelial cells by PMA [24,25], we have also evaluated whether the effects of PMA and b-FGF on the permeability of monolayers of human umbilical artery and aorta endothelial cells and the production of plasminogen activators by these cells are regulated by similar or different mechanisms.

MATERIALS AND METHODS

Materials

Medium 199, supplemented with 20 mM Hepes was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA, USA) or Corning (Corning, NY, USA); Transwells (exposed area 0.33 cm^2, pore size 3μm) were from Costar. A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain as described by Maciag et al [26]. Human recombinant a-FGF was a gift from T. Maciag (American Red Cross, Biotechnology Research Centre, Rockville, MD, USA). Human recombinant b-FGF and penicillin-streptomycin were obtained from Boehringer Mannheim (Mannheim, FRG). Collagenase type 1 CLS was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA). Heparin was obtained from Leo Pharmaceutical Products (Weesp, The Netherlands). Human fibronectin was a gift of J.A. van Mourik (Red Cross Central Blood Transfusion Laboratory, Amsterdam, The Netherlands). Human serum was prepared in our laboratory from fresh blood of healthy donors, pooled and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY, USA) and was heat-inactivated at 56°C for 30 minutes before use. H-7 (1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine, phorbol 12-myristate 13-acetate (PMA), 4α-12,13-didecanoate (4α-PDD), o-sphingosine, o-dianisidine and horse-radish peroxidase (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); HA1004 (N-(2-guanidinoethyl)-5-isoquinolinylsulfonamide hydrochloride) from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Forskolin was from Hoechst (La Jolla, CA, USA); isobutyl methyl xanthine (IBMX) from Janssen Chimica (Beerse, Belgium); suramin from Bayer (Leverkusen, FRG).

H-7 and HA1004 stocks were made at 10 mM in distilled water and stored at 4°C. PMA and 4α-PDD stocks were prepared at 100 μM in ethanol and stored at -20°C. The o-sphingosine stock was prepared at 25 mM in DMSO and stored at -20°C. IBMX stock was prepared at 100 mM in 1 N NaOH and stored at -20°C.

Isolation and culture of endothelial cells

Endothelial cells from human umbilical arteries, umbilical veins and human aortas, were isolated by 0.075% (w/v) collagenase treatment according to the method of Jaffe et al [27]. Cells were characterized as previously described [13,28]. Cells were cultured on fibronectin-coated dishes in Medium 199, supplemented with 10% human serum, 10% newborn calf serum, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/streptomycin and kept at 37°C under 5% CO_2/95% air. At confluence cells were released from the culture dishes with trypsin/EDTA and transferred to new dishes with a split ratio of 1:3 for further propagation.
For permeability studies endothelial cells from umbilical artery (primary culture) or aorta (first passage) were released as described above, but were seeded on a filter-surface that was two times smaller than the original dish surface, in order to obtain high density cultures. Four to six hours after seeding, non-attached cells were removed. Filters were incubated at 37°C under 5% CO_2/95% air in the same medium as given above, and the culture medium was replaced every other day. Experiments were done 4 to 7 days after seeding the cells [18].

Passage studies

Passage of horse-radish peroxidase (HRP) through human endothelial cell monolayers was studied with the Transwell system as earlier described [18]. Twenty-four hours before the start of an experiment, the medium was replaced by medium 199, supplemented with 20% human serum, 5 U/ml heparin, penicillin/streptomycin and human recombinant b-FGF (usually 5 ng/ml). At the start of each experiment, a known amount of HRP in Medium 199 supplemented with 20% human serum and heparin, containing different concentrations of human recombinant b-FGF was added at the top of the endothelial monolayer (upper compartment, 150 μl), and at the other side (lower compartment, 700 μl) the same medium was added, but without HRP.

During the experiment the filters were kept at 37°C under 5% CO_2/ 95% air atmosphere. At several time points samples (100 μl) were taken from the lower compartment, and the same volume of the appropriate medium was replaced in this compartment to prevent fluid permeation due to hydrostatic pressure. The HRP concentration in the samples was determined spectrophotometrically (408 nm) after incubation with o-dianisidine and hydrogen peroxide. Passage rate is expressed in ng/h/cm^2 or ng/cm^2.
When indicated, human recombinant a-FGF was used instead of b-FGF. Stimulatory or inhibitory agents were always added to both the upper and lower compartment, when investigated for their influence on the passage of HRP. In experiments where the involvement of protein kinase C (PKC) activity in the passage of HRP was investigated, inhibitors of PKC-activity were added 1 hour before the start of the experiment in the absence of b-FGF. PMA, 4α-PDD and forskolin were always added at the start of an experiment. To evaluate the competition of suramin with b-FGF for receptor-interaction, suramin was always added at the start of an experiment.

Transendothelial electrical resistance (TEER)
The transendothelial electrical resistance (TEER) was measured as previously described [18]. In short, an alternating current (50 μA) was passed across the monolayer (one pulse, every minute). The measured electrical potential difference was used to calculate the electrical resistance by Ohm's law.

Extraction and assay of cyclic AMP
Extraction and assay of cyclic AMP was performed according the method of Adams-Brotherton et al [29] with some modifications. Confluent monolayers of human umbilical artery endothelial cells (first passage) were incubated 24 hours before the start of an experiment with Medium 199, supplemented with 10% human serum, 10% newborn calf serum, 5 U/ml heparin and different concentrations of human recombinant b-FGF. At the start of the experiment the medium was replaced by the same medium supplemented with 1 mM IBMX, unless otherwise stated. Stimulatory agents were added 15 minutes after the start of the experiment. After an indicated time period, the medium was aspirated and 0.6 ml of ice-cold 5% trichloroacetic acid was directly added to each well. A tracer amount of [^{3}H]-cyclic AMP (Amersham, UK)

was added to each well to monitor the recovery of cyclic AMP during the purification procedure. After ten minutes on ice, the extracts were collected and brought on a small Dowex 50 W X-4 column. Cyclic nucleotides were eluted with 1 N HCl, and air-dried. Concentration of cyclic AMP was determined using a radio immuno assay (Amersham, UK), and corrected for the recoveries of cyclic AMP in the various samples.

Assay of tissue-type plasminogen activator antigen

Confluent monolayers of endothelial cells from human umbilical artery and vein and human aorta were washed once with serum-free medium and incubated with Medium 199, supplemented with 10% human serum and different concentrations of human recombinant b-FGF. After twenty-four hours preincubation, the medium was replaced by the same medium supplemented with the same amount of b-FGF and, if indicated, additional agents. The inhibitors H-7 and HA1004 were added 1 hour prior to the start of the incubation and were present throughout the incubation period. After 24 hours incubation the supernatants were collected, centrifuged and stored at -20°C. t-PA antigen was determined by an enzyme immuno assay (Thrombonostika t-PA, Organon-Teknika, Turnhout, Belgium) with human two-chain t-PA as a standard.

RESULTS

b-FGF and a-FGF improve the barrier function of human umbilical artery endothelial cell monolayers.

The monolayers of human endothelial cells are maintained in the presence of endothelial cell growth factor (ECGF). Omission of the growth factor preparation from the medium for 24 hours results in a marked increase in the permeability of the monolayers (figure 1). Basic and acidic fibroblast growth factor (b-FGF and a-FGF) can totally substitute for the crude ECGF-preparation (figure 1; table 1), suggesting that FGF is indeed the active component in the ECGF-preparation. The effects of b-FGF and a-FGF are concentration dependent, and are shown in table 1 by decreased rates of peroxidase passage and increased transendothelial electrical resistances. They probably act via FGF-receptors, since in the presence of 1 mM suramin, which is known to displace b-FGF from its receptor [30], b-FGF is no longer able to improve the barrier function (without suramin a 40 ± 6% reduction of the peroxidase passage by 5 and 10 ng/ml b-FGF is observed, while no reduction is evident with suramin).

table 1 Effect of ECGF, a-FGF and b-FGF on the permeability and electrical resistance of human endothelial cell monolayers.

Addition		Peroxidase passage (% of control)	Transendothelial electrical resistance (% of control)
None		100	100
ECGF (150 μg/ml)		43 ± 13 (8)*	256 ± 63 (7)*
b-FGF	0.5 ng/ml	77 ± 25 (4)	125 ± 7 (3)
	2 ng/ml	62 ± 17 (6)#	232 ± 18 (3)**
	10 ng/ml	54 ± 33 (8)##	364 ± 3 (3)**
	50 ng/ml	32 ± 8 (2)	N.D.
a-FGF	2 ng/ml	66 ± 7 (2)	123 ± 19 (3)
	10 ng/ml	48 ± 14 (3)	185 ± 15 (3)*

Human umbilical artery endothelial cell monolayers were preincubated for 24 hours in and subsequently incubated in Medium 199 with 20% human serum and supplemented with crude endothelial cell growth factor (ECGF), hr-b-FGF and hr-a-FGF in the presence of 5 U/ml heparin or with heparin alone (control).
Passage of horse-radish peroxidase and the transendothelial electrical resistance were determined as described in Materials and Methods. The data represent the mean ± SD of the number of experiments indicated between parentheses. Control values of peroxidase passage were assayed after 6 hours of incubation and were 59 ± 30 ng/h/cm^2 (mean ± SD, 8 experiments). Values of the transendothelial electrical resistance were measured after a 15 min equilibrium period and were for control cells (24 hours without ECGF) 7.1 ± 1.1 Ohm.cm^2. *: P<0.01; **: P<0.001; #: P< 0.02; ##: P< 0.05.

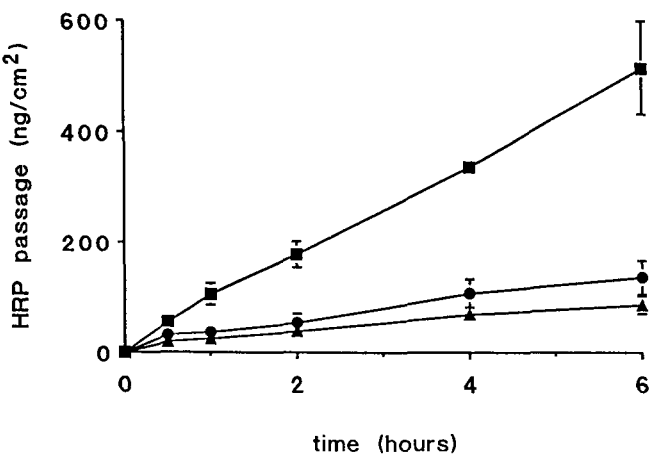

figure 1 Effect of b-FGF and ECGF on the permeability of monolayers of human umbilical artery endothelial cells. The cells were preincubated for 24 hours and subsequently incubated in Medium 199 supplemented with 20% human serum and 5 U/ml heparin. During the preincubation and incubation period 10 ng/ml b-FGF (▲) or 150 μg/ml ECGF (●) was added, or no addition (■) was made. The incubation medium was renewed one hour before the addition of horse-radish peroxidase (HRP) to the luminal site of the cells. HRP passage was measured as indicated in Materials and Methods. The data represent the mean ± SD of triplicate filters.

Effect of PMA on the permeability of monolayers of human umbilical artery endothelial cells

When human umbilical artery endothelial cells are incubated with low concentrations of the phorbol ester PMA, a concentration-dependent decrease in endothelial cell permeability is observed (figure 2a). At 50 nM PMA the permeability of the monolayer increases again, probably due to some cell toxicity. The decrease in endothelial cell permeability occurs immediately upon addition of PMA (figure 2b). The non-tumor promoting analogue 4α-PDD does not effect the endothelial cell permeability (figure 2a, open symbols), which suggests that PMA acts via activation of protein kinase C (PKC). The involvement of PKC has been further established by the use of inhibitors. The isoquinolinylsulfonamide inhibitor H-7 effectively blocks the decrease in peroxidase passage induced by PMA, while its structural analogue HA1004, which inhibits PKC less efficiently, is relatively inactive at the same concentration (figure 3). H-7 does not prevent the decrease in endothelial cell permeability induced by b-FGF (figure 3).

Addition of another PKC inhibitor sphingosine (5-25 μM) results in a rapid and large increase in endothelial cell permeability, probably due to activation or damage of the endothelial cells (not shown).

Effect of b-FGF on the cellular cAMP concentration

The permeability of human endothelial cell monolayers can be decreased by elevation of the cellular cAMP concentration [15-17]. The adenylate cyclase activator forskolin increases the cellular cAMP concentration (table 2) and markedly decreases endothelial cell permeability (table 3). Addition of b-FGF alone does not increase the cAMP concentration in cells that are incubated in the presence or absence of the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX). Only a small increase in cAMP has been observed when the cells have been simultaneously incubated with forskolin and b-FGF in the absence of IBMX. Therefore a small inhibitory effect of b-FGF on phosphodiesterase activity can not be excluded. In favour of this suggestion we observed that the decrease in peroxidase passage is enhanced by the simultaneous addition of forskolin and b-FGF (table 3). In contrast to b-FGF, addition of PMA together with forskolin failed to further reduce the endothelial cell permeability as compared with the addition of forskolin alone (table 3). Similar results are obtained when ECGF has been used instead of b-FGF (not shown).

table 2 Effect of forskolin and b-FGF on the cAMP concentration of human umbilical artery endothelial cells.

Addition	Cyclic AMP (pmol/well)	
	Without IBMX	With IBMX
None	5.5 ± 0.4	7.9 ± 0.3
b-FGF (10 ng/nl)	5.2 ± 0.8	7.0 ± 0.4
Forskolin (25 μM)	5.6 ± 0.4	24.4 ± 0.9
b-FGF (10 ng/ml) + Forskolin (25 μM)	7.2 ± 0.2	23.4 ± 0.4

Cyclic AMP was assayed 15 minutes after addition of the various mediators and after 24 hours preincubation with or without b-FGF. Data are the mean ± range of duplicate wells (5 cm^2) of a representative culture.

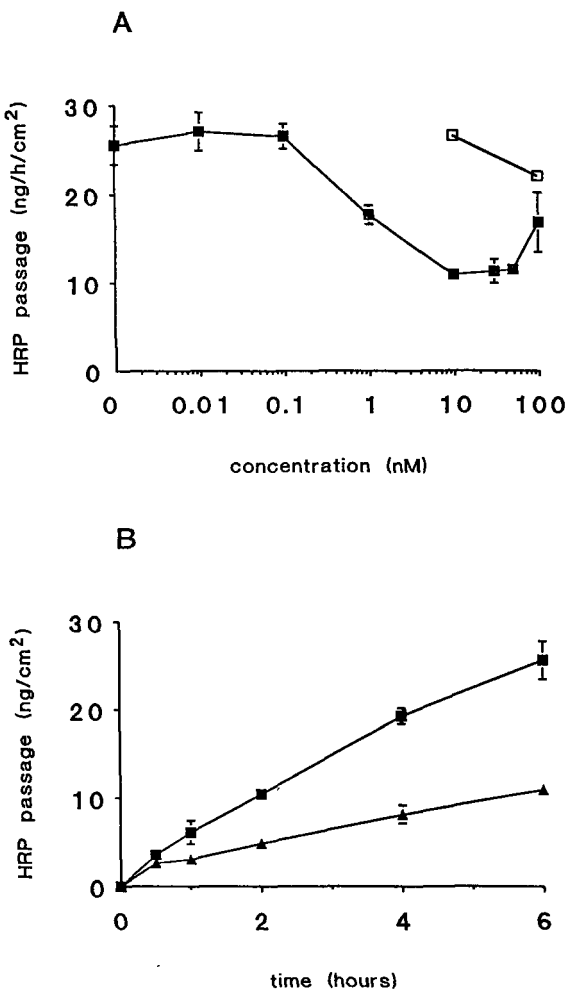

figure 2 Effect of phorbol ester on the permeability of monolayers of human umbilical artery endothelial cells. A. Concentration dependent effect of phorbol myristate acetate (■, PMA) and 4α-PDD (□). B. Time course of the passage of horse-radish peroxidase (HRP) upon addition of 10 nM PMA (▲) or without addition (■). The data are the mean ± SD of triplicate filters. Similar results were obtained with two other cultures.

table 3 Effect of forskolin on the permeability of b-FGF and PMA-stimulated human endothelial cell monolayers.

Addition	Peroxidase passage (ng/h/cm^2)	
	Culture 1	Culture 2
None	43 ± 6	27 ± 2
Forskolin (25 μM)	11 ± 1	6 ± 1
b-FGF (10 ng/ml)	26 ± 4	22 ± 2
b-FGF (10 ng/ml) + Forskolin (25 μM)	7 ± 1	4 ± 1
PMA (10 nM)	17 ± 1	14 ± 2
PMA (10 nM) + Forskolin (25 μM)	11 ± 1	6 ± 1

Passage of peroxidase was measured over a 6 hours period as described in Materials and Methods. The data are the mean ± SD of triplicate filters of two different cultures of human umbilical artery endothelial cells.

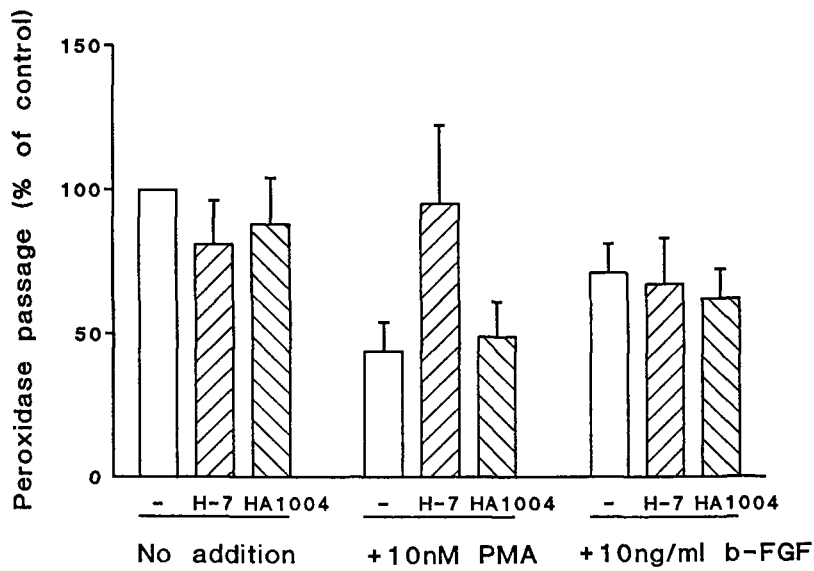

figure 3 Inhibition of the effect of PMA on endothelial permeability by the inhibitors H-7 and HA1004. Human umbilical artery endothelial cells were preincubated for 24 hours in Medium 199 with 20% human serum and 5 U/ml heparin supplemented with 10 ng/ml b-FGF or without b-FGF (Control, PMA). One hour before addition of peroxidase and PMA the medium was replaced by the same medium supplemented, if indicated, with 25 μM H-7, 25 μM HA1004 and 10 ng/ml b-FGF. At the start of the incubation PMA was added to both the upper and lower compartments, and peroxidase was added to the upper compartment. The data represent the mean ± SD of 4 independent cultures.

Effect of b-FGF and PMA on the production of t-PA by umbilical artery endothelial cells

Activation of protein kinase C by PMA induces the production of t-PA in human umbilical artery endothelial cells (figure 4). The induction increases with the concentration of PMA till 100 nM. At 100 nM PMA, the increase in the production of t-PA levels off and, in several experiments, even decreases again, probably because the PMA becomes toxic for the cells.

While PMA increases the t-PA production by endothelial cells, its non-active analogue 4α-PDD is ineffective (table 4). Forskolin did not affect t-PA production itself, but, in agreement with previous reports [24,25], it acts synergistically with PMA in enhancing the t-PA production. b-FGF increases the basal t-PA production by 67%. It had no additional effect when it was added together with PMA. However, the effects of PMA and b-FGF appear to be different, since forskolin did not enhance the t-PA production by b-FGF. Furthermore, as shown in figure 5 for a representative experiment, both the PMA-stimulated and the basal production of t-PA antigen are inhibited by H-7, whereas the increase in t-PA production induced by b-FGF appears less sensitive for H-7.

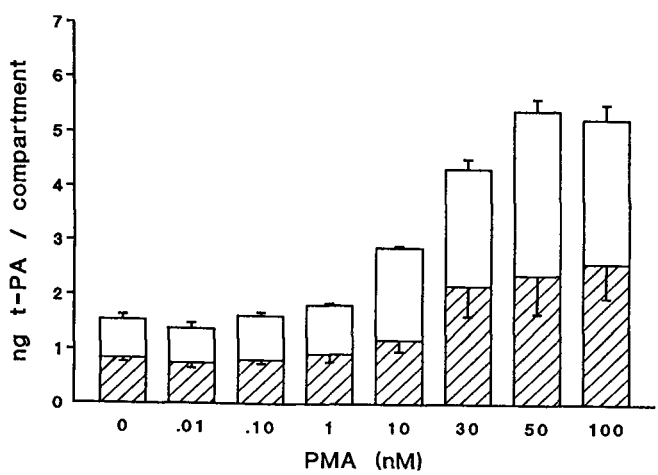

figure 4 Effect of phorbol myristate acetate (PMA) on the production of t-PA antigen by human umbilical artery endothelial cells. After 24 hours incubation the medium above and below the endothelial cell monolayers were collected and assayed for t-PA antigen. The data represent the mean ± range of two independent cultures; the permeability data of one of these cultures is given in figure 2a. open bars: upper compartment; shaded bars: lower compartment.

table 4 Effect of b-FGF and PMA on the production of tissue-type plasminogen activator (t-PA) by human umbilical artery endothelial cells.

Addition	t-PA Antigen (% of control)	
	Umbilical artery EC	Aorta EC
None	100 *	100 #
Forskolin (25 μM)	89 ± 18 (5)	86 ± 14 (5)
b-FGF (10 ng/ml)	167 ± 25 (5)	134 ± 27 (4)
b-FGF (10 ng/ml) + Forskolin (25 μM)	144 ± 11 (5)	102 ± 27 (4)
PMA (10 nM)	387 ± 162 (4)	294 ± 106 (5)
PMA (10 nM) + Forskolin (25 μM)	597 ± 262 (4)	345 ± 184 (5)
PMA (10 nM) + b-FGF (10 ng/ml)	331 ± 180 (4)	275 ± 59 (2)
4α-PDD (10 nM)	111 ± 15 (3)	82 ± 27 (2)
4α-PDD (100 nM)	133 ± 42 (4)	149 ± 40 (2)

Endothelial cells were incubated for 24 hours in Medium 199 supplemented with 20% human serum and t-PA antigen was assayed as indicated in Materials and Methods. Data represent the mean ± SD of the number of different cultures of human umbilical artery endothelial cells (each from a different donor) given in parentheses. The data from human aorta endothelial cells represent independent experiments with cells from 2 or 3 different donors. Control values were 5 ± 4 ng/10^5 cells (*) and 19 ± 6 ng/10^5 cells (#), respectively.

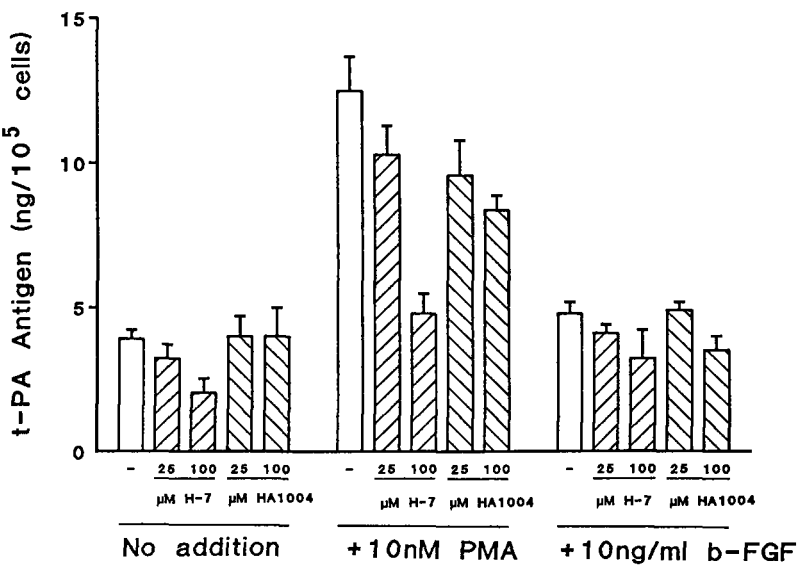

figure 5 Effect of the inhibitors H-7 and HA1004 on the basal and PMA- and b-FGF-stimulated production of t-PA by human umbilical artery endothelial cells. Cells on 5 cm^2 wells were incubated for 24 hours, after which t-PA antigen was assayed in the supernatant medium.

Effect of b-FGF and PMA on the permeability and t-PA production of human aorta endothelial cell monolayers

To investigate whether umbilical artery endothelial cells respond differently towards b-FGF and PMA than do endothelial cells from adult human arteries, we studied the effect of these agents on the permeability of human aorta endothelial cell monolayers. Figure 6a shows a representative experiment, from which it is clear that neither b-FGF nor PMA change the permeability of human aorta endothelial cell monolayers. These findings have been confirmed in another experiment with aorta endothelial cells from a second donor. To evaluate whether the aorta cells still respond to PMA, we have assayed the production of the t-PA by the same culture. From the results in figure 6b it is clear that the aorta cells continue to respond to PMA. Similar results were obtained with endothelial cells obtained from different donors (table 4). Hence, aorta and umbilical artery endothelial cells respond differently to PMA with respect to the regulation of their barrier function (cf. figures 2 and 6), whereas the regulation of t-PA production by both cell types proceeds in a similar way (cf. table 4).

DISCUSSION

The availability of a well characterized model of human artery endothelial cell monolayers on porous filters [16,18] enables us to obtain information about the regulation of human endothelia at the biochemical level. In previous reports we have described the involvement of cytoplasmatic calcium [16] and cyclic AMP [31] in the regulation of endothelial permeability. Their involvement in human endothelial cells is comparable to that in animal endothelial cells [15,17,32-34]. Here, we report the effects of FGFs and stimulation of protein kinase C (PKC) on the permeability of human arterial endothelial cells and give evidence that the involvement of PKC in this process varies in different types of endothelial cells. We further demonstrate that the regulation of endothelial permeability and the production of t-PA by phorbol ester is differently regulated in these cells.

Effect of fibroblast growth factors

Our experiments demonstrate that b-FGF or a-FGF are necessary to maintain the barrier function of human umbilical artery endothelial cell monolayers. FGFs are

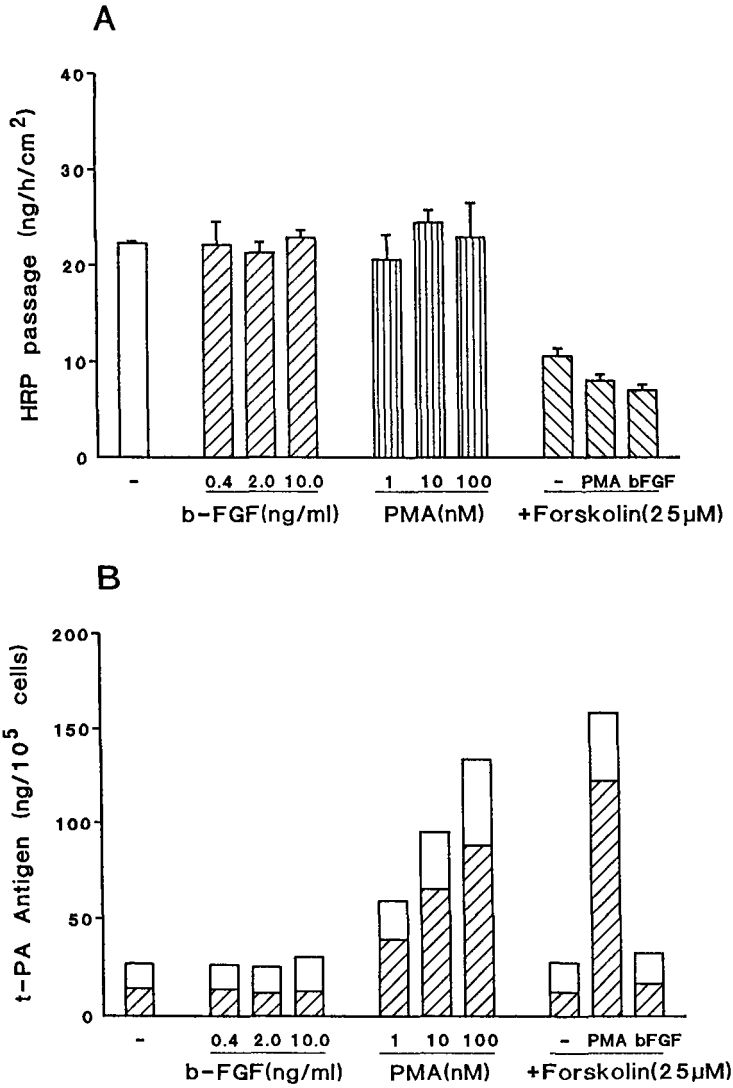

figure 6 Effects of b-FGF, PMA and forskolin on (A) the permeability of and (B) the production of t-PA by human aorta endothelial cell monolayers. Passage of horse-radish peroxidase (HRP) was determined over a 2 hours period. The production of t-PA antigen was assayed in the upper (open bars) and lower (shaded bars) compartments around the same monolayers after a 24 hours incubation period. The permeability data represent the mean ± SD of triplicate filters; the triplicate conditioned media were pooled before assay of t-PA antigen. In the presence of forskolin, 10 nM PMA and 10 ng/ml b-FGF has been used, respectively.

strong mitogens for endothelial cells in vitro [11,35] and can induce angiogenesis in vivo [36]. The effect of FGFs on endothelial cell permeability may seem unexpected, because Chien et al [8] recently demonstrated that focal leakage of macromolecules in arteries and veins occurs around dividing endothelial cells. However, although both a-FGF and b-FGF are potent growth factors in subconfluent cells, their effectiveness in inducing mitosis may be far less in very dense populations of endothelial cells. As pointed out by Ingber et al [37,38] the degree of cell spreading is an important determinant for the onset of cell division. Therefore, in our confluent monolayers, the limited cell spreading may not allow a-FGF and b-FGF to induce mitosis. In favor of this suggestion is our unpublished observation that b-FGF (2-10 ng/ml) did not increase [^{3}H]-thymidine incorporation in these highly confluent monolayers.

The barrier function of the endothelial monolayer can be improved by increasing the cAMP concentration in the endothelial cells [15-17], as demonstrated here with forskolin. Mioh and Chen [14] have reported that FGF activates adenylate cyclase in smooth muscle cells. However, under our experimental conditions, b-FGF (and ECGF) does not activate the adenylate cyclase but it has a small effect on cAMP in the presence of forskolin; in addition it acts additive to forskolin in decreasing permeability. Therefore, a small inhibitory effect of b-FGF on phosphodiesterase activity can not be excluded. It remains uncertain whether this inhibition underlies the improvement of the endothelial barrier function induced by b-FGF.

Role of protein kinase C

Furthermore we have demonstrated that stimulation of protein kinase C (PKC) by low concentrations of PMA improved the barrier function of human umbilical artery endothelial cells. After completion of our study, Yamada et al [39] reported similar findings on human umbilical vein endothelial cells. These observations contrast with observations on animal aorta endothelial cells, where an increase in permeability with increasing PMA concentration was found [22,23]. Interestingly, in human aorta endothelial cell monolayers, concentrations of PMA up to 100 nM have no effect on the permeability. Apparently, both species differences and regional differences between different types of blood vessels occur with respect to the PKC induced changes in endothelial permeability.

PMA and b-FGF act on the permeability of umbilical artery endothelial cells via different mechanisms, because (a) the decrease by PMA was significantly inhibited by

H-7, whereas that by b-FGF was not, and (b) in the presence of forskolin, b-FGF further reduced the permeability of these cells, while PMA did not further decrease the permeability.

Stimulation of t-PA production by PMA and b-FGF

Our data confirm and extend previous findings that PMA enhances t-PA production by human umbilical vein endothelial cells, and that this enhancement is enlarged by elevation of the cellular cAMP concentration [24,25]. Induction of t-PA mRNA transcription underlies this stimulation [24]. We also show that b-FGF moderately increases the production of t-PA by umbilical artery endothelial cells. This occurs by a mechanism that is different from that by PMA, because (a) the increase induced by b-FGF is not inhibited by the protein kinase C inhibitor H-7, in contrast to the increase induced by PMA, and (b) the effect of b-FGF is not further stimulated by forskolin.

Differences in protein kinase C mediated processes

The difference in the effects of PMA on the permeability of umbilical artery and aorta endothelial cells was not found for the increase of t-PA production by PMA in these cells. An additional effect of cAMP elevation by forskolin on the induction of t-PA was also found in both cell types. Both permeability changes and t-PA induction by PMA could be inhibited by H-7, albeit that some difference in effectiveness was observed. It is probable that different types of PKC are involved in the regulation of permeability and that of t-PA induction. Human endothelial cells contain different types of PKC [40]. On the other hand, we cannot yet exclude the possibility that a step in the regulation of the permeability of endothelial cell monolayers distal to PKC underlies the variation between different types of endothelial cells.

ACKNOWLEDGEMENTS

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CHAPTER 7

EFFECTS OF LIPOPROTEINS AND FATTY ACIDS ON THE PERMEABILITY OF HUMAN ARTERIAL ENDOTHELIAL CELL MONOLAYERS

Erna G. Langeler[1], Renée C.R.M. Vossen[2], Maria C.E. van Dam-Mieras[2] and Victor W.M. van Hinsbergh[1]

[1]Gaubius Institute TNO, Leiden, the Netherlands
[2]Department of Biochemistry, University of Limburg, Maastricht, the Netherlands

ABSTRACT

The endothelium controls the influx of macromolecules into the arterial wall, a process that can be influenced by e.g. oxidized lipoproteins and (metabolites of) fatty acids. We have used an in vitro model, in which human umbilical artery or aorta endothelial cells are cultured on porous filters, to study the effects of lipoproteins and free fatty acids on the endothelial permeability. The addition of 800 μg/ml low, high or very low density lipoproteins (LDL, HDL or VLDL) and, to a lesser extent, 100 μg/ml β-migrating VLDL (β-VLDL), results in a reduction of the passage rate of peroxidase through monolayers of human umbilical artery endothelial cells, during the first two hours after addition. The effect is transient: six hours after addition the effect on permeability has disappeared. The effect of LDL on endothelial permeability is likely not mediated via a prostacyclin, cyclic adenosine 5'-monophosphate (cAMP) or protein kinase C dependent pathway. In the presence of either aspirin or the protein kinase C inhibitor H-7, LDL was still able to reduce the permeability. Furthermore, no effect of LDL has been observed on either the cellular cAMP concentration or on the production of prostacyclin.

In addition, the effects of palmitic (16:0), oleic (18:1), arachidonic (20:4) and eicosapentaenoic (20:5) acid on endothelial permeability have been studied. At short-time intervals (up to 4 hours) these fatty acids (except for palmitic) slightly reduce the passage rate of peroxidase through the endothelial monolayers. At longer time intervals, and after a culture period of 10 days in the presence of the fatty acids, all fatty acids, except for arachidonic acid increase the passage rate. This effect is not due to a general growth inhibition induced by the fatty acids.

INTRODUCTION

The endothelium, a single cell layer lining the blood vessels, acts as a selective barrier for plasma components. It actively controls the influx of macromolecules into the arterial wall. Damage to the endothelium may result in a reduced effectiveness of the barrier-function. High concentrations of plasma triglyceride and low density lipoproteins (LDL) have been implicated in the injury process of the endothelium and are indicated as important risk factors for heart and vessel diseases (1,2).

It has been reported that oxidized lipids, several fatty acids (oleic and linoleic acid) and β-VLDL increase the permeability of monolayers of porcine pulmonary (3-8),

bovine pulmonary (9) and rabbit aorta (10) endothelial cells. In addition, arachidonic acid increases the diapedesis of polymorphonuclear leukocytes through monolayers of bovine aortic endothelial cells (11). Furthermore, agents as tumour necrosis factor (TNF) (12,13) and the protein kinase C activator, phorbol myristate acetate (PMA), (14-16) increase the permeability of animal endothelial monolayers.

Human endothelial cells, exposed for a prolonged period to high concentrations LDL in the absence of whole serum or any other anti-oxidizing agent, undergo distinct morphological changes, resulting in detachment of cells (17-19). On the other hand, we have recently shown that the addition of high concentrations LDL in the presence of serum or other oxidation preventing conditions, induces a temporary reduction of the permeability of human umbilical artery and aorta endothelial cell monolayers (20). Up till now, no information is available about the effects of other lipoproteins and fatty acids on the permeability of human endothelial cell monolayers.

Several mediators have been described which induce a decrease of the permeability of macromolecules across human endothelial cell monolayers. They include agents, that decrease the permeability for a prolonged period, as agents that raise the cellular cAMP concentration (20-22) and agents which stimulate the protein kinase C activity (22,23). Nor-epinephrine (24) and TNF (25) induce a temporary reduction of the permeability, in human endothelial cells.

On the contrary, PMA and TNF induce an increase of the permeability through animal endothelial monolayers. Because of this discrepancy between human and animal endothelial cells, we wondered whether β-VLDL and free fatty acids may influence the permeability of human endothelial cell monolayers.

In the present study, we have evaluated whether the LDL-induced reduction was specific for this class of lipoproteins or could also be induced by other types of lipoproteins, HDL, VLDL and β-VLDL. Subsequently, we have investigated whether this effect could be attributed to an increase in cAMP or to activation of protein kinase C. Furthermore, we studied the effects of palmitic (16:0), oleic (18:1), arachidonic (20:4) and eicosapentaenoic (20:5) acid on human endothelial cell permeability.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Corning (Corning, NY) or Costar (Cambridge, MA); Transwells (diameter 0.65 cm; pore size 3 μm) were from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al (26). Human serum was obtained from a local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY) and heat-inactivated before use (30 min, 56°C). Human serum albumin was from the Red Cross Central Blood Transfusion Laboratory (Amsterdam, the Netherlands). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands). Horse-radish peroxidase, phorbol 12-myristate 13-acetate (PMA), H-7, fatty acids and vitamin C were obtained from Sigma Chemical Company (St. Louis, MO), HA1004 was from Seikagaku Kogyo Co.Ltd. (Tokyo, Japan), forskolin from Hoechst (La Jolla, CA) and isobutyl methyl xanthine (IBMX) was from Janssen Chimica (Beerse, Belgium). Aspirin was obtained from Merck (Darmstadt, FRG).

Culture of Endothelial Cells

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al (27). Endothelial cells from human aortas were isolated (28) and characterized as described previously (28,29). Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/streptomycin. Cells were cultured at 37°C under 5% CO_2 / 95% air.

For passage studies, endothelial cells from umbilical artery (primary or first passage) or aorta (fourth passage) were released with trypsin-EDTA and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell system (30) and cultured as described above. Medium was renewed every other day.

In the experiments in which the long term effects of fatty acids were evaluated, cells were cultured until confluence (umbilical artery: primary; aorta: third passage) in the same medium as described above. Hereafter, cells were trypsinized (split ratio 1:3)

and cultured during five days in Medium 199 supplemented with 20% human serum, endothelial cell growth factor, heparin, penicillin/streptomycin and 300 μM of the indicated fatty acid. Subsequently, cells were again trypsinized and cultured on the fibronectin-coated filters in the same medium. As a control, endothelial cells from the same culture were cultured in parallel wells to monitor morphology and growth rate during the subculture in the presence of the various fatty acids. After 5 days (similar to passage studies), these cells were trypsinized, stained with trypan blue and counted using a hemocytometer.

Preparation of Lipoproteins

Very low, low and high density lipoproteins (VLDL, LDL and HDL) were isolated from fresh serum prepared from the blood of healthy volunteers by gradient ultracentrifugation according to the method of Redgrave et al (31). β-VLDL from Watanabe rabbits and a type III-patient was kindly provided by Drs. Atsma and Gevers Leuven (Gaubius Institute TNO, Leiden, the Netherlands) respectively. Lipoproteins were stabilized by the addition of 1% human serum albumin and dialysed against phosphate-buffered saline and subsequently against Medium 199. Lipoproteins were sterilized, stored at 4°C and used within 7 days after isolation. Lipoproteins concentrations are expressed in μg protein/ml. Protein concentration was determined by the method of Lowry et al (32) with albumin as a standard.

Passage Experiments

a. Lipoproteins experiments

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Passage of horse-radish peroxidase through human endothelial cell monolayers was performed as earlier described (30). In short, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) for 4-6 days to form a tight monolayer. Before the experiment, cells were preincubated for one hour in Medium 199 supplemented with 20% human serum or 1% human serum albumin (as indicated). At the start of the experiment 5 μg/ml horse radish peroxidase in Medium 199 with either 20% human serum or 1% human serum albumin supplemented with the indicated amount of lipoprotein was added to the upper compartment of the Transwell system, while in the lower compartment Medium 199 and 20% human

serum or 1% human serum albumin was present. At various time intervals samples were taken from the lower compartment, and an equal amount of Medium 199 with either 20% human serum or 1% human serum albumin was re-added to this compartment. All passage experiments are performed in triplicate. The peroxidase-concentration was determined in each sample as previously described. Passage rates are expressed as ng/h/cm^2 or ng/cm^2.

b. Fatty acids experiments

Experimental set up for the diffusion assay was comparable as described above. Fatty acids were present in both compartments, during the culture (as indicated) and the experiment. The mix of fatty acids consists of 34% palmitic acid (16:0), 12% stearic acid (18:0), 34% oleic acid (18:1) and 20% linoleic acid (18:2), which is comparable to the free fatty acid composition of human serum. Experiments, in which the effects of fatty acids were evaluated, were performed in the presence of vitamin C.

Extraction and Assay of cyclic AMP.

Extraction and assay of cyclic AMP was performed according the method of Adams-Brotherton et al (33) with some modifications. Medium of confluent endothelial cell monolayers, cultured in 5 cm^2 wells, was renewed with Medium 199 supplemented with 1% human serum albumin one hour before the incubation-period. Unless otherwise mentioned, 15 minutes before the start of the experiment IBMX (1 mM) was added to the medium. At the start of the experiment, cells were incubated with medium containing 1% human serum albumin with or without lipoproteins for an indicated time-interval. Hereafter medium was aspirated and 0.6 ml of ice-cold 5% trichloroacetic acid was directly added to each well. A small amount of [^{3}H]-cyclic AMP (Amersham, UK) was added to each well to monitor the recovery of cyclic AMP during the purification period. After ten minutes on ice, the extracts were collected and brought on a small DOWEX-50 WX4 column. Cyclic nucleotides were eluted with 1 N HCl, and air-dried. Concentration of cyclic AMP was determined using a radioimmunoassay (Amersham), and corrected for the recoveries of cyclic AMP in the various samples (50-70%). Concentration is expressed as pmol/amount of cells, consuming an equal amount of cells in each well.

RESULTS

Effects of Lipoproteins on the Permeability of Human Arterial Endothelial Monolayers

The effects of β-migrating very low density lipoproteins (β-VLDL) and low density lipoproteins (LDL) on endothelial permeability have been evaluated in Medium 199 supplemented with either 1% human serum albumin or 20% human serum. Table 1 shows that, under both conditions, two hours after addition, both β-VLDL and LDL slightly reduce the permeability of the endothelial cell monolayers for peroxidase. After 24 hours incubation, no significant difference has been observed between the passage rate of peroxidase through control monolayers and those incubated with either β-VLDL or LDL. Similar results have been obtained with β-VLDL preparations prepared from either the blood of Watanabe rabbits or the blood of a type III hyperlipidemic patient (table 1).

table 1 Effect of β-VLDL and LDL (100 μg/ml) on the passage of peroxidase through monolayers of human umbilical artery endothelial cells.

	PASSAGE of HRP (% of control)			
	in 1% HUMAN SERUM ALBUMIN incubation time		in 20% HUMAN SERUM incubation time	
addition	2hr	24hr	2hr	24hr
β-VLDL (wat)	74 ± 7%	130 ± 20%	64 ± 6%	115 ± 3%
LDL (human)	68 ± 3%	99 ± 5%	70 ± 12%	108 ± 6%
β-VLDL (pat)	77 ± 8%	123 ± 28%	88 ± 11%	105 ± 3%
LDL (human)	85 ± 28%	148 ± 5%	90 ± 6%	103 ± 3%

Human umbilical artery endothelial cells are cultured on porous filters. At the start of the experiment (as described in Materials and Methods) medium supplemented with or without either β-VLDL (from Watanabe rabbits (wat) or from a type III patient (pat)) or LDL from a healthy donor has been added to both the upper and the lower compartment. Experiments are performed in the presence of either 1% human serum albumin or 20% human serum. At 2 and 24 hours, respectively after addition, the passage of peroxidase has been determined. Values are means ± SD of triplicate filters of two independent experiments as compared with control values (100%).

Recently, we have shown that LDL reduces the permeability in a time and concentration dependent way (20). After 2 hour incubation of umbilical artery endothelial cells with 800 μg/ml LDL a 61 ± 13% reduction of the passage of peroxidase has been found as compared to cultures without additional lipoproteins (mean ± SD, seven experiments). Because β-VLDL also induced a transient decrease in permeability (table 1), we evaluated whether other lipoproteins have an effect on peroxidase passage through endothelial cell monolayers. Figure 1 shows that HDL and VLDL at 800 μg/ml concentration reduced the passage rate of peroxidase over a 2-hour period similar to LDL. The reduction is transient. After six hours incubation with these lipoproteins the passage rate of peroxidase was not significantly different from control values (82 ± 7%, 90 ± 1%, 84 ± 5% for HDL, VLDL and LDL, respectively, means ± SD of triplicate filters).

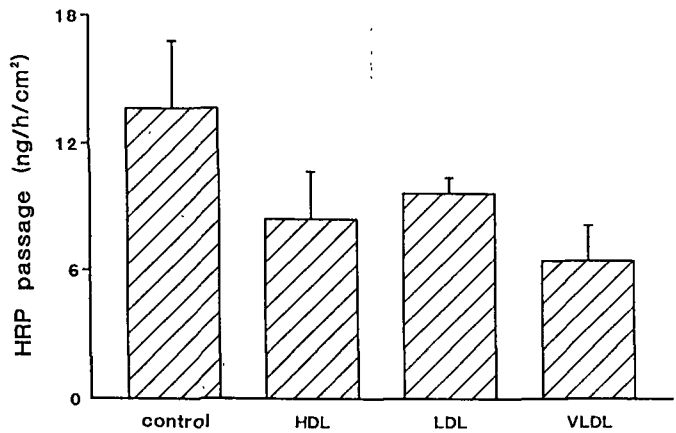

figure 1 Effect of various lipoproteins on the passage of peroxidase (HRP) through human umbilical artery endothelial cell monolayers. At the start of the experiment either HDL, LDL or VLDL (800 μg/ml) in Medium 199 supplemented with 1% human serum albumin and 5 μg/ml peroxidase are added to the upper compartment of the assay system. In the lower compartment Medium 199 with 1% human serum albumin is present. Passage of peroxidase is determined after 2 hours incubation. Values represent the means ± SD of a representative experiment, performed in triplicate.

Involvement of Prostaglandins and cAMP in the Reduction of the Permeability Induced by LDL

The prostacyclin analogue iloprost increases the cellular cAMP concentration and reduces the permeability of human endothelial cell monolayers (21). To evaluate whether prostaglandins may be involved in the reduction of the endothelial permeability induced by high concentrations of LDL, aspirin has been added to the

monolayers to inhibit cyclo-oxygenase activity. Incubation of endothelial cells in the presence of 25 μM aspirin prevented the formation of prostacyclin, as estimated by its degradation product 6-keto-prostaglandin F1α, but did not prevent the LDL-induced reduction of the passage of peroxidase (three independent experiments, not shown). Other agents that increase the cellular cAMP concentration, such as the adenylate cyclase activator, forskolin, and the phosphodiesterase inhibitor, isobutyl methyl xanthine (IBMX), also reduce the permeability of human endothelial cell monolayers (20,21). Therefore, we evaluated the effects of LDL and HDL on the cAMP concentration of our cells. The cAMP concentration of the endothelial cell monolayers was not changed either at various time intervals after addition of LDL and HDL as compared to control cells (figure 2A), or after addition of various concentrations of LDL to the endothelial cells, both in the presence and absence of the phosphodiesterase inhibitor, IBMX (figure 2B).

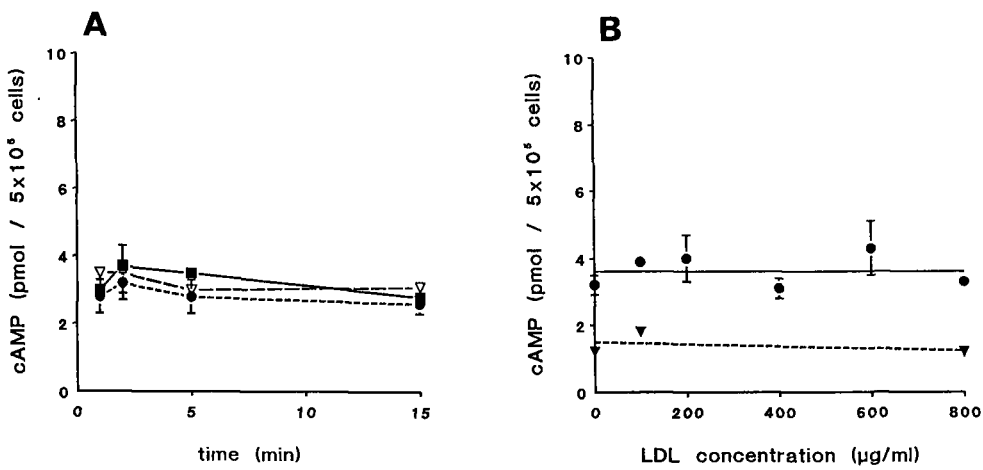

figure 2 Effect of LDL and HDL on the cAMP concentration of human umbilical artery endothelial cells. Cells are incubated in Medium 199 supplemented with 1% human serum albumin and the indicated amount of LDL or HDL. Cyclic AMP has been isolated and assayed by radioimmunoassay as described in Materials and Methods. **A:** Time course of the cellular cAMP concentration without lipoproteins (■) and after addition of 800 μg/ml LDL (●) or 800 μg/ml HDL (▽), all in the presence of 1 mM isobutyl methyl xanthine, IBMX. **B:** Various concentrations LDL are added to the medium at the start of the experiment; effects are evaluated both in the presence of 1 mM IBMX (●) and in the absence (▼).

Involvement of Protein Kinase C in the Reduction of the Permeability Induced by LDL

In human umbilical vein and artery endothelial cells, low concentrations of phorbol myristate acetate (PMA) improve the barrier function of the endothelial cell monolayer (22,23). Addition of 10 nM PMA reduced the passage rate of peroxidase to 52 ± 16% of the control incubations (mean ± SD, 6 experiments). This effect sustained for at least 6 hours. The decrease induced by 10 nM PMA is effectively prevented by the addition of 25 μM of the isoquinolinylsulfonamide inhibitor H-7 (82 ± 29% of control values) whereas the structural analogue HA1004 had no significant effect on the stimulation by PMA (56 ± 23% of control). This suggests that PMA acts via activation of protein kinase C. Table 2 shows the effect of H-7 on the LDL-induced reduction of peroxidase passage through umbilical artery endothelial cells. In the presence of albumin, 25 μM H-7 influenced the basal passage rate of peroxidase. However, the relative reduction of LDL is in the presence of H-7 not significantly different from the relative reduction in the absence of any supplement or in the presence of vitamin C. In addition, the reduction of the passage through human aorta endothelial cells, induced by the addition of 800 μg/ml LDL, in the presence of 20% human serum can also not be prevented by the addition of H-7 (table 2).

table 2 Effect of low density lipoproteins (800 μg/ml) on the permeability of human umbilical artery (HUAEC) and human aorta endothelial cells in the absence or the presence of either H-7 or vitamin C.

addition	HUAEC-I	HUAEC-II	HUAEC-III	Aorta EC
None	7.2 ± 0.3	13.7 ± 3.2	9.0 ± 0.9	5.3 ± 0.3
LDL	3.8 ± 0.6	9.6 ± 0.7	6.6 ± 0.6	1.3 ± 0.1
H-7	13.2 ± 2.0	15.8 ± 0.4	11.4 ± 2.7	3.4 ± 0.8
H-7 + LDL	10.2 ± 0.7	13.4 ± 2.9	3.9 ± 1.2	1.0 ± 0.3
vitamin C	7.0 ± 0.4	13.0 ± 1.5	6.3 ± 0.9	N.D.
vitamin C + LDL	5.1 ± 1.4	8.3 ± 1.8	3.0 ± 1.2	N.D.

The effect of high concentrations low density lipoproteins (800 μg/ml) on the passage rate of peroxidase is determined in the absence and the presence of 25 μM H-7 or 100 μM vitamin C. Both the lipoproteins and peroxidase are present in the upper compartment, while H-7 or vitamin C are present in both compartments. Experiments are performed in either 1% human serum albumin (umbilical artery, UA) or 20% human serum (aorta). Passage of peroxidase is determined after 2 hours incubation and is expressed as ng/h/cm^2 and is given for three experiments (umbilical artery) and one experiment (aorta), respectively. Values respresent means ± SD of triplicate filters. N.D.: not determined.

Effects of Fatty Acids on Human Endothelial Cell Permeability

The effects of various free fatty acids on human endothelial permeability are studied during a 24 hour incubation after initial addition of free fatty acids and after a culture period of 10 days in media supplemented with 20% pooled human serum and various free fatty acids.

Short-time effects of fatty acids (up to 24 hours)

Short-time effects of fatty acids are studied in the presence of either 1% human serum albumin or 20% human serum. In the presence of human serum albumin, some of the fatty acids, in particular oleate (18:1), linoleic (18:2) and arachidonate (20:4), reduce transiently the permeability of the endothelial cell monolayers (figure 3A, open bar; B). This effect disappeared after 4 hours of incubation. After 24 hours incubation a slight increase in permeability was found with palmitate, stearate, oleate, lineate and linoleate, whereas in the presence of arachidonate similar values for the passage rate were obtained as through monolayers incubated without supplemented fatty acids (figure 3A, hatched bar). The initial effects of the fatty acids were much less when the cells were incubated in the presence of human serum (figure 4A, open bar). Oleate, arachidonate and eicosapentaonate reduced the permeability by only 8.5%, 16% and 15.5% respectively. Twenty four hours after addition of the fatty acid-enriched media an increase in permeability was found when palmitate, oleate, eicosapentaonate or a mix of fatty acids was used, whereas a relatively small increase was found with arachidonate (figure 4A, striped bar).

Long term effects of fatty acids

To evaluate the long-term effects of the presence of fatty acids on endothelial permeability, endothelial cells were cultured during two subcultures (10 days) in the presence of 300 μM of the indicated fatty acids. The presence of the fatty acids did not induce visible morphological changes of the cells. After 10 days of culture, equal amounts of cells were present in the various wells. Through monolayers of the same culture, the passage rate of peroxidase has been determined in the presence of these fatty acids. Although all monolayers formed a relatively tight barrier for peroxidase, passage rates under the various conditions were different (figure 4B). Most of the tested fatty acids, except for arachidonate, increased the passage rate (figure 4A, hatched bar and 4B). A similar tendency was observed when peroxidase passage through monolayers of human aorta endothelial cells was determined after 10 days incubation in medium enriched with various fatty acids (figure 5).

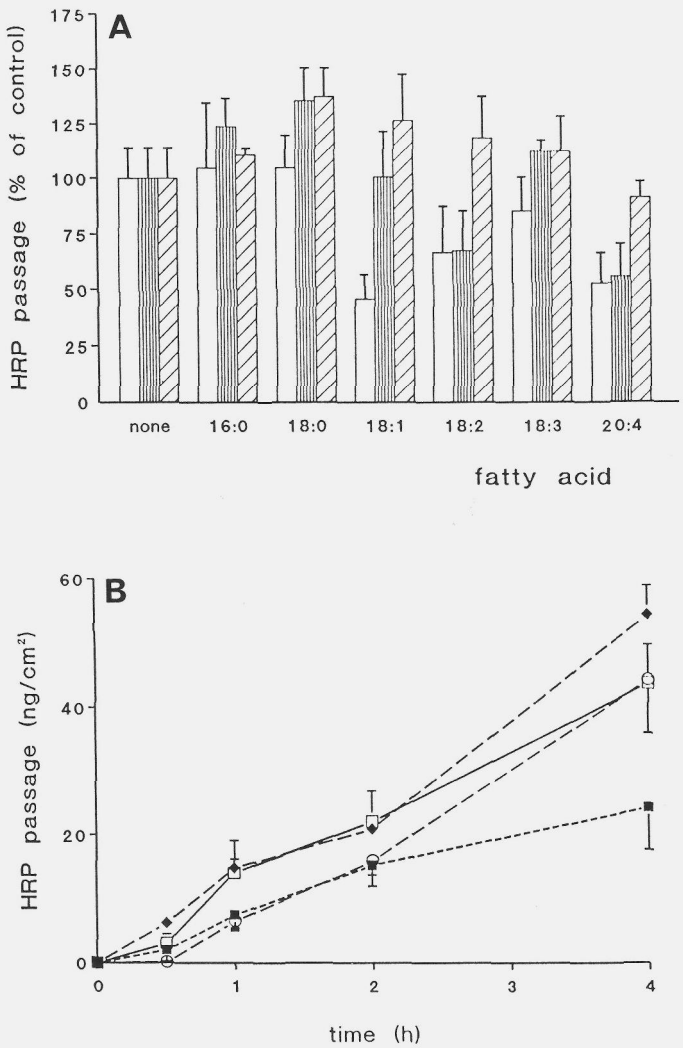

figure 3 Effects of various fatty acids on different time intervals on the passage of peroxidase (HRP) through human umbilical artery endothelial cells. Cells are cultured in the presence of 20% serum. At the start of the experiment the indicated fatty acid (300 μM) is added to both compartments in the presence of 1% human serum albumin, while peroxidase is only added to the upper compartment. **A:** Passage of peroxidase is determined after one hour (open bar), 4 hours (striped bar), and 24 hours (hatched bar) incubation, respectively, and is expressed as percentage of control values (100%). **B:** Time curves of the effects of palmitate (◆), oleate (○) and arachidonate (■) on the passage of peroxidase. (□): control incubations.
Values represent the means ± SD of three different filters of a repesentative experiment.

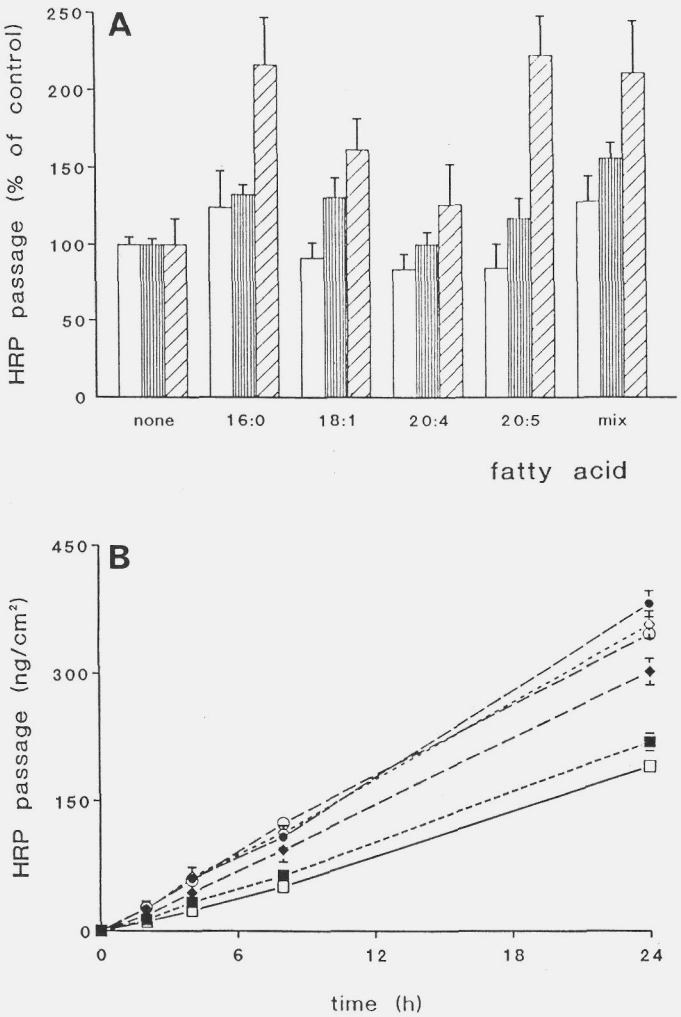

figure 4 Effects of various fatty acids on different time intervals on the passage of peroxidase (HRP) through human umbilical artery endothelial cells. The cells are cultured during two subcultures in the presence of 20% human serum without additional fatty acids (A: open and striped bar) or the indicated fatty acid (hatched bar and B). At the start of the experiment (as described in Materials and Methods) the indicated fatty acid (300 μM) is added to both compartments in the presence of 20% human serum. Peroxidase (5 μg/ml) was only added to the upper compartment.
A, open and striped bar: (no fatty acids during culture) passage of peroxidase is determined after 2 hours and 24 hours incubation, respectively.
A, hatched bar and B: (cells were cultured in the presence of the fatty acids) at two hours and different time intervals, respectively after addition of the peroxidase the passage rate of peroxidase has been determined. (□: control incubations; ◆: palmitate; ○: oleate; ■: arachidonate; ●: eicosapentaenate; ◇: a mix of various fatty acids in the same concentration as in human serum).
Values represent: (A) the means ± SD of six different filters (two different experiments, each performed in triplicate) as compared with control values (100%) or (B) the means ± SD of a representative experiment, performed in triplicate.

To evaluate whether the responsiveness of the endothelial cell monolayers to vasoactive substances may change by this prolonged incubation in fatty acids-enriched medium, we measured the effect of the addition of 10 μM histamine on the permeability of human umbilical artery endothelial cell monolayers that have been incubated for 10 days in medium enriched with 300 μM of the above indicated fatty acids. Both the increase in permeability seen after one hour and the subsequent reduction of the permeability determined after 24 hours -as described previously (20)- was similar in histamine-treated monolayers as compared to control monolayers (not shown).

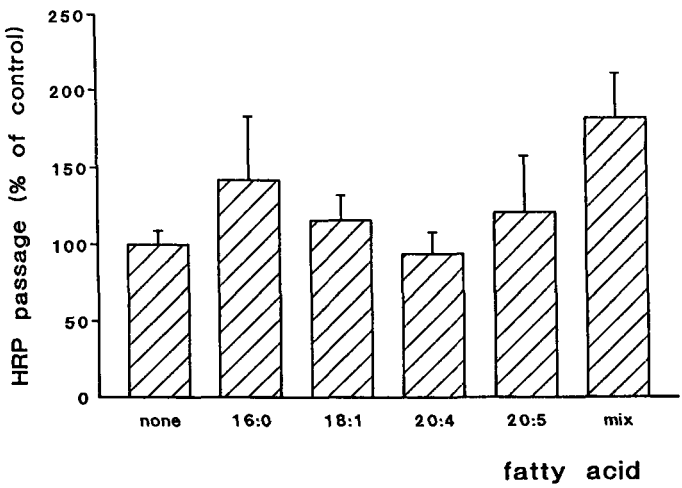

figure 5 Effects of various fatty acids on the passage of peroxidase through human aorta endothelial cells. Cells are cultured during two passages (10 days) in the presence of 20% human serum supplemented with the indicated fatty acids. At the start of the experiment the indicated fatty acid (300 μM) is added to both compartments in the presence of 20% human serum. Passage of peroxidase is determined after 2 hours incubation, and are expressed as percentage of control values (100%). Values represent the means ± SD of three different filters.

DISCUSSION

Several studies suggest that damage to the endothelium and the resulting disturbance in endothelial integrity may be the initiating event in atherosclerotic lesion formation. Any disturbance in endothelial integrity may thus allow an increased penetration of plasma components into the arterial wall. In this series of experiments, we have studied the effects of lipoproteins and fatty acids on human endothelial integrity. We have used an in vitro model, in which human endothelial cells obtained from the umbilical artery or the aorta are cultured on porous filters. This model has been

described and characterized previously (20,30). As marker protein for the evaluation of the permeability, horse-radish peroxidase, an independent molecule (no interactions with the endothelium, lipoproteins or fatty acids), has been used.
High concentrations LDL induce a temporary reduction of the permeability of both LDL and peroxidase (20). Here, we have shown that this effect is not specific for LDL, but that also other lipoproteins, HDL and VLDL induce this decrease. This suggests that a specific agent associated to or generated from all lipoproteins is responsible for the reduction. The addition of 100 μg/ml β-VLDL to human umbilical artery endothelial cell monolayers did not result in an increase of the permeability, as described by Navab et al. (10) for rabbit aorta endothelial cells. Their experiments were done in the presence of lipoprotein depleted serum (LPDS) and were continued for 5 days. Human endothelial cells do not remain intact in the presence of LPDS for such a long period. These differences may influence the observed results.
A mechanistic explanation for the observed effects of LDL can not be given on base of the current data. Although it has previously been shown that both cAMP increasing agents, as forskolin, IBMX (20,21) and iloprost (stable prostacyclin analogue) (21) and protein kinase C activators (22,23) are able to reduce the permeability of monolayers of human umbilical artery endothelial cells, these processes are likely not involved in the underlying reduction, induced by LDL. In the presence of 25 μM aspirin (cyclo-oxygenase inhibitor) LDL was able to reduce the permeability. Furthermore, no effect of LDL was observed on both the cAMP concentration (figure 2) and the production of prostacyclin, as measured by the accumulation of 6-keto-prostaglandin F1α in the culture medium (not shown). This last phenomenon is in agreement with previous observations of Nordoy et al (34).
The involvement of protein kinase C also is not likely, because the protein kinase C inhibitor, H-7, which prevented the effects of phorbol esters, did not prevent the effect of LDL. However, caution should be taken, because only low concentrations of H-7 could be used as H-7 also may affect the organization of actin filaments in endothelial cells (35) and, furthermore, the presence of high concentrations of LDL may affect the effective concentration of the lipophylic inhibitor H-7.
Previous studies on animal endothelial cells have suggested an important negative effect of free fatty acids, particularly oleic acid, on the endothelial barrier function (3-11). Effects of fatty acids on the permeability of human endothelial cell

monolayers are studied on different time-intervals. On short-time intervals, the effects of fatty acids are, especially in the presence of human serum, rather marginal. While several unsaturated fatty acids slightly reduced the permeability, palmitic acid did not. Direct activation of protein kinase C by the fatty acids as demonstrated in membrane preparations (36) can not be excluded. The facts that activation of protein kinase C results in animal endothelial cells in an increased permeability, while it does not in human ones and that both oleic and arachidonic acid directly increase the permeability in bovine pulmonary (9) and aorta (11) endothelial cells, respectively, are in favour of this suggestion.

Hennig et al (4,5,8) described for porcine pulmonary artery endothelial cells, that 24 hours after addition, 100 to 300 μM oleic and linoleic acid increased 1.5- to 3- fold the passage of both albumin and LDL, whereas palmitic, linolenic, arachidonic and eicosapentaenoic acid had no effect. In these experiments, the animal endothelial cells were pre-incubated with the indicated fatty acid in medium supplemented with 5% fetal bovine serum and 100 μM fatty acid free bovine albumin, while during the diffusion assay (for one hour) no fatty acid, serum and albumin were present in the medium. We have used human endothelial cells in a slighlty different experimental set-up (see Materials and Methods), but we observed only a slight (less than 1.5-fold) increase of the permeability, after 24 hours incubation with palmitic acid, oleic acid, eicosapentaenoic acid or a mix of fatty acids, and no increase in the presence of arachidonic acid.

After a culture period of 10 days in the presence of the various fatty acids, human endothelial cells still form a relative tight monolayer. Although no effect was observed on both the morphology and the growth rate of the endothelial cells, the passage rates for peroxidase are higher than those of control monolayers. The effect of arachidonic acid is smaller than of the other tested fatty acids. The presence of a mix of fatty acids, in the same concentrations as in human serum induces a relative large increase of the permeability. A similar phenomenon was observed in case of human aorta endothelial cells. This may indicate that an elevated fatty acid concentration in plasma may contribute to an increased influx of macromolecules into the arterial wall. It has been described by Vossen et al (37) that culturing of human endothelial cells in the presence of a mix of fatty acids (in the same concentration as in control serum) does not result in an altered cell-membrane composition of fatty

acids. Therefore, this can not be the explanation for our results.
The continuous presence of the fatty acids not only during preincubations but also during the assay of the peroxidase passage is necessary for the observed effects. After preincubation for 24 hours with oleic acid, the passage rate of peroxidase was higher if oleic acid remained present during the experiment, than if oleic acid was removed (139 ± 2%, two experiments).
Although the fatty acid concentration in our experiments may seem rather high, they are still comparable to values that can occur in humans in (patho-) physiological conditions (38). High plasma free fatty acid concentrations occur during severe stress (39), uncontrolled diabetes (40), starvation (41) and after prolonged exercise (42). Local concentrations of fatty acids during hydrolysis of lipoprotein triglycerides may even exceed the high levels, used in our studies.
In summary, in this study we have shown that high concentrations of various lipoproteins reduce the permeability of human umbilical artery endothelial cell monolayers during short time incubations. This effect was likely not mediated via an elevation of the cellular cAMP concentration and could not be attributed to the activation of protein kinase C. Furthermore, we have shown that during short time incubations some fatty acids (oleic and arachidonic acid) may reduce the permeability, but on longer time intervals, high concentrations of various fatty acids, but not moderately, increase the permeability of endothelial monolayers.

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CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

At the start of this study, in 1986, limited information was available about the passage process of macromolecules through the endothelial monolayer. On the basis of experiments with capillary endothelium, it was generally accepted that the passage of molecules, as low and high density lipoproteins, occurred via micropinocytotic vesicles (Renkin et al 1982). Morphological observations also suggested a vesicle-associated transport (Stein et al 1973, Vasile et al 1983). Intercellular passage had also been described under pathological conditions (hypertension, stimulation by vasoactive substances, high plasma-cholesterol level) (Hüttner et al 1973, Stemerman 1981). However, the biochemical background of the passage process was poorly understood. The use of cultured endothelial cells made it possible to study the endothelial permeability in vitro, without interactions of other cells or other body functions. In 1986, a few models had been described to study animal endothelial permeability, but no model was available to study human endothelial cell permeability. It was generally assumed, that human umbilical cord endothelial cells could not be cultured on porous filters to form a tight monolayer. In 1986, two articles were published about the permeability of monolayers of human umbilical vein endothelial cells (Killackey et al, Rotrosen and Gallin). The models used in these reports were not characterized nor were they compared with other models or in vivo data in any way. In this thesis, two in vitro models for the study of human arterial endothelial permeability are described. In the first model, fluid passage under hydrostatic pressure can be studied. In the second one, a diffusion model, the passage of (macro-) molecules through an endothelial monolayer can be studied, while in parallel wells the transendothelial electrical resistance can be measured. After our initial demonstration that is was possible to culture tight human endothelial monolayers on polycarbonate filters, other investigators also have used in vitro models to study human endothelial permeability (Huang et al 1988, Carson et al 1989, Casnocha et al 1989, Furie and McHugh 1989, Moser et al 1989).

8.2 IN VITRO MODELS TO STUDY ENDOTHELIAL PERMEABILITY

8.2.1 Comparison of Various in vitro Models

Before using an in vitro model it is necessary to evaluate some basal characteristics of the monolayer in relation to known in vivo data. Minimal requirements for a cultured endothelial monolayer are: 1) a large difference between the passage of macromolecules through filters with an endothelial monolayer and filters without a monolayer has to exist, 2) large molecules should pass at a slower rate than small molecules, while very large molecules should not pass the monolayer, 3) monolayers have to respond to various biological agents such as vasoactive amines, 4) the presence of albumin and calcium ions is necessary to maintain the barrier function for prolonged periods and 5) the presence of an adequate transendothelial electrical resistance (TEER) across the monolayers. As described in chapters 2 and 3, our model meets these criteria well.

Transendothelial electrical resistance has been measured by various authors. Although, it is not exactly known which component in the monolayer is responsible for the measured value; two authors (Navab et al 1986; van Bree et al 1990) have found a relationship between the passage of sodium and the TEER, for rabbit aorta and bovine brain endothelium, respectively. This suggests that the integrity of the tight junctions is important for the appearance of an electrical resistance. The TEER is generally accepted as a value for the integrity of the endothelial monolayer. In table 1 some values are listed. For all in vitro monolayers the resistance is between 7 and 20 Ohm.cm^2, which is comparable with the observed value for comparable endothelia in vivo (chapter 1, table 1).

table 1 Electrical resistances across monolayers of endothelial cells in vitro

Reference	Endothelial cells from	Electrical resistance (Ohm.cm^2)
Territo et al (1984)	Bovine Aorta	15
Hashida et al (1986)	Porcine Aorta	10
Navab et al (1986)	Rabbit Aorta	14
Shasby and Shasby (1986)	Porcine Pulmonary artery	5-7
Huang et al (1988)	Human Umbilical Vein	8-9
Albelda et al (1988)	Bovine Aorta	20
this thesis	Human Umbilical Artery	17
this thesis	Human Aorta	10

8.2.2 Energy Dependency of the Passage Process

By several authors it has been suggested that the passage process is energy-dependent. This may suggest, that the passage occurs via vesicles. However, Hinshaw et al (1988) described, more recently, that a (slight) contraction of the endothelial cells occurs in vitro and is energy-dependent. By the addition of an inhibitor of the energy metabolism, the contraction may be decreased, by which cells flatten and make closer cell to cell contacts, through which fewer molecules can be passed. Therefore, the energy-dependency cannot be used as a criterium to demonstrate that the passage of macromolecules is mediated via the transcytosis of vesicles.

8.2.3 LDL Passage through Monolayers of Human Arterial Endothelial Cells

The specific characteristics of the passage of low density lipoproteins (LDL) are described in chapter 3. Although several investigators have claimed a saturation of the passage process of LDL, our results do not confirm this conclusion. On short time intervals the addition of high concentrations LDL results in a temporary reduction of the passage of both LDL and other molecules. This phenomenon results in an apparent saturation of the passage process. In chapter 7, this phenomenon has been described in greater detail. Not only LDL, but also HDL and VLDL induce a temporary reduction of the passage. At present, this effect can not be attributed to an effect on cAMP or protein kinase C.

On endothelial cells a LDL-(B,E)-receptor has been described and characterized by various authors. Methylation of the LDL-particles inhibits their ability to bind to this receptor. However, the passage rates of both LDL and Me-LDL are not significantly different, indicating that the receptor is not involved in the passage process. By acetylation of the LDL-particle, the charge on the molecules has been decreased. Acetylated LDL pass in a slower rate than native LDL, indicating that more negative particles pass at a slower rate than its neutral analogues. This phenomenon is probably caused by the presence of negative charges on the surface of the cells (Simionescu et al 1981). These two characteristics (no receptor-involvement and no saturation) indicate that the passage of LDL is a rather a-selective process. Therefore, in further studies on endothelial permeability an independent marker protein (peroxidase) has been used.

8.3 MECHANISMS, UNDERLYING REGULATION OF ENDOTHELIAL PERMEABILITY

8.3.1 Introduction

The mechanisms by which substances traverse the vascular endothelium is of fundamental physiological and pharmacological importance. Classically, the micro-vascular endothelium has been considered as a static heteroporous barrier analogous to that of an artificial semi-permeable membrane. It has been accepted since the time of Starling that the transvascular flux of water and hydrophillic solutes are passive events governed by physical determinants. The classical view of the microvascular membrane emerged because the vascular endothelium was considered functionally inert, and the majority of the findings from transport studies under basal conditions could be satisfactorily explained by diffusion/filtration events, as postulated in the "pore theory". A static heteroporous model of the microvascular endothelium, however, cannot explain the formation of for example inflammatory edema in the absence of injury to the vascular endothelium. The opening of the large pores disturbs the equilibrium of the Starling forces, virtually eliminating the transmural colloid osmotic pressure gradient by permitting plasma proteins to penetrate into the subendothelial space at virtually any microvascular pressure.

Majno (1961,1967) provided the first evidence that inflammatory mediators produced transient, spontaneously-reversible increases in vascular permeability to macromolecules subsequent to the formation of specific leakage sites in the postcapillary venules. This hypothesis was based on the idea that endothelial cells contain receptors and contractile proteins. Leakage occurred after contraction of the cells, resulting in gap-formation between the cells.

In the mid 1970's, agents were discovered, which inhibited mediator stimulated formation of venular leakage sites and edema formation. These inhibitors were called "endothelial cell stabilizers". The inhibition is receptor-operated, but uses a receptor other than that stimulated by the inflammation mediator. Nor-epinephrine and other drugs which stimulate β-adrenergic stimulants are among the substances which may function as physiological antagonists of mediator-stimulated increases in vascular permeability.

8.3.2 Mechanisms Involved in the Regulation of Contraction of Mesenchymal Cells

If cellular contraction is involved in endothelial cell permeability, more about the regulation of these processes may be learned from the regulation of cellular contraction of other mesenchymal cells such as smooth muscle cells and mesengial cells. In these cell types contraction forces are basically generated by Ca^{++} and ATP dependent interaction of actin and myosin. This process is regulated by the myosin light chain (MLC), which is a regularly protein that becomes activated upon phosphorylation by the MLC-kinase. At least three pathways regulate this process (figure 1). Firstly, activation of phospholipase C, by e.g. histamine or thrombin, results in an increase of the intracellular calcium concentration. Subsequently, calcium is able to bind to calmodulin which activates the myosin light chain kinase, which transfers the gamma phosphate from ATP to myosin. The light chain phosphorylation permits activation of myosin ATP-ase by actin and is thereby believed to

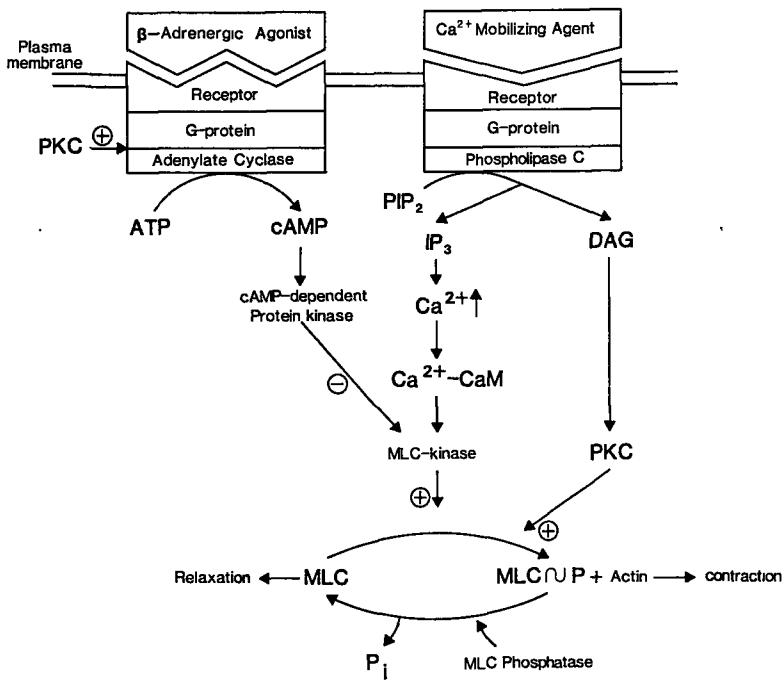

figure 1 Schematic view of various pathways to influence contraction of smooth muscle cells. + and - indicate stimulation and inhibition, respectively. Abbreviations used: CaM, calmodulin; PIP_2, phosphatidylinositol 4,5-biphosphate; IP_3, inositol 1,4,5-triphosphate; DAG 1,2-diacylglycerol; PKC, protein kinase C; G-protein, GTP-binding protein; MLC, myosin light chain.

initiate the contractile events resulting in the shortening and tension development of smooth muscle cells. Secondly, an elevation of the cAMP concentration in the cell have the opposite effect. Cyclic AMP activates the cAMP-dependent protein kinase, which inactivates the myosin light chain kinase.
The role of protein kinase C is less clear. It may activate adenylate cyclase, by which it increases the cAMP concentration in the cell, and subsequently decrease the permeability. On the other hand, it can also catalyze the phosphorylation of the light chains of myosins, resulting in a contraction of the cell.
Furthermore, for mesengial cells it has also been shown that disruption of stress fibers, induced by myosin light chain phosphorylation has been associated with shape change of the cells (Kreisberg et al 1985).

8.3.3 Mechanisms Underlying the Regulation of Endothelial Permeability

It is likely that similar mechanisms are involved in the regulation of the barrier function of endothelial cells, as has been described above for smooth muscle cells. Contractile proteins, including actin, myosin, α-actinin and vinculin have been identified in endothelial cells (Becker and Nachman 1973, Drenckhahn 1983). Endothelial cells in regeneration areas, overlapping atherosclerotic lesions or under shear stress possess prominent stress fibers, which contain actin and myosin (Gabbiani et al 1983, Gotlieb et al 1984, White et al 1988, Guyton et al 1989, Kim et al 1989a,b). The presence of contractile proteins is, of course, not an evidence of a contractile function. In 1982, Shasby et al already suggested that endothelial cell cytoskeleton may be important determinants of endothelial permeability. More recently, Wysolsmerki and Lagunoff (1990) described the involvement of the myosin light chain in endothelial cell contraction, while recent experiments from Schnittler et al have shown that gap formation between endothelial cells requires ATP, and occurs only when free myosin binding sites are available on endothelial actin. This suggests that an interaction between actin and myosin is necessary for the contraction of the endothelial cell.
In figure 2 the effects of various substances, as described in this thesis, on the permeability of human umbilical artery endothelial cells (on short time intervals) are summarized, while in table 2 an overview of effects on various cell types and two time-intervals has been given.

table 2 Comparison of the effects of various studied mediators on the permeability of human and animal endothelial cell monolayers in vitro.

	HUAEC		HAEC			
addition	2h	6h	2h	6h	animal EC	
histamine	++	0	++	0	++	(a)
thrombin	++	+	ND	ND	++	(b)
forskolin/IBMX	---	---	---	---	---	(c)
isoproterenol	-	0	--	0	--	(d)
iloprost	-	0	ND	ND	--	(e)
nor-epinephrine	--	0/-	--	0	--	(f)
TNF	-	0	0	0	+	(g)
LDL (high conc)	-	0	-	0		
oleic acid	-	0	-	0	+	(h)
PMA	--	--	0	0	+	(i)

references: (a) Bottaro et al (1986); Doukas et al (1987); (b) Garcia et al (1986); Siflinger-Birnboim et al (1988); Lum et al (1989); (c) Mizuno-Yagyu et al (1987); Stelzner et al (1989); Minnear et al (1989); (d) Haselton et al (1989); Minnear et al (1989); (e) in vivo: Müller et al (1987); Erlansson et al (1989); (f) Bottaro et al (1986); Doukas et al (1987); (g) Horvath et al (1988); Clark et al (1988); Brett et al (1989); Goldblum et al (1989); Royall et al (1989); (h) Hennig et al (1984, 1985, 1989); Cooper et al (1987); (i) Gudgeon and Martin (1989); Lynch et al (1990).
EC: Endothelial cells, HUAEC: Human Umbilical Artery EC, HAEC: Human Aorta EC, ND: not determined. +, 0 and - indicate increase, no effect and decrease of the permeability, respectively.

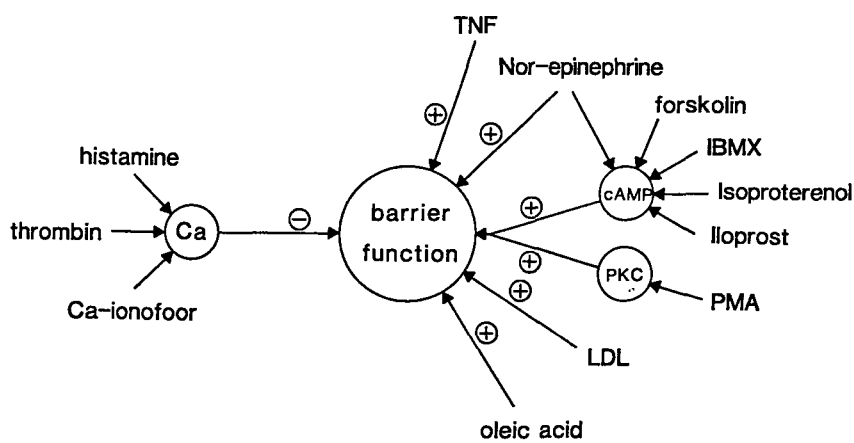

figure 2 Involvement of various substances in the regulation of the permeability of human umbilical artery endothelial cells. The passage rate of peroxidase has been determined two hours after addition of the indicating substance to the medium, and has been used as an indicator of the barrier function. + and - indicate an increase and a decrease of the barrier function respectively. Abbreviations used: TNF, Tumour necrosis factor; PMA, phorbol myristate acetate; LDL, low density lipoproteins; PKC, protein kinase C; IBMX, Isobutyl Methyl Xanthine.

In chapter 3, several vasoactive substances have been described, which increase the endothelial permeability. The effect on permeability was accompanied by an increase of the intracellular calcium concentration. The involvement of IP_3 in this process has nowadays been described (Carson et al 1989). The effect of thrombin on the intracellular calcium concentration is attributed to interaction of thrombin with its receptor(s) resulting in activation of phospholipase C for the initial rise (Magnuldo et al 1987, Jaffe et al 1987) and possibly also of phospholipase A2 for the sustained rise in calcium concentration (Goligorsky et al 1989).
Forskolin and IBMX, two substances which directly influence the actual and local cAMP concentration in the endothelial cells, decrease the basal passage rate of various molecules through the endothelial monolayer. In chapter 4, a direct correlation has been described between the passage of peroxidase through the endothelial monolayer and an increase in cAMP concentration for isoproterenol, iloprost and forskolin.
Low concentrations of the protein kinase C activator, phorbol myristate acetate (PMA), have also been shown to reduce the permeability of human umbilical artery endothelial cell monolayers. The observation that in the presence of forskolin, PMA does not further reduce the permeability, may suggest that one "step" in the pathways induced by cAMP and protein kinase C is essentially the same.
During the study, several substances have been found which increase the barrier function of human umbilical artery endothelial cells (figure 2 and table 2), but the mechanism underlying the change has yet to be identified. Nor-epinephrine reduces the permeability to a large extent, but its effect on cAMP is very limited and it cannot be entirely responsible for the reduction. Involvement of protein kinase C cannot be excluded, but is unlikely, because in human aorta cells (table 2), activation of protein kinase C does not result in an altered permeability, while nor-epinephrine does.
Furthermore, tumour necrosis factor also reduces the permeability on short time intervals; this effect is likely not mediated via a cAMP (chapter 5) or a protein kinase C dependent pathway (effect of TNF cannot be prevented by H-7, one preliminary experiment). It has recently been described that TNF activates the phosphorylation of various (unidentified) proteins (Robaye et al 1989, Krönke et al 1990), but it is not yet known whether (one of) these proteins is involved in the

regulation of contraction of the endothelial cytoskeleton.
The addition of high concentrations of lipoproteins to the endothelial cells, resulted in a temporary reduction of the permeability. This effect was observed for all tested lipoproteins, suggesting that the effective component resides in the lipid part and not in the apolipoprotein part of the lipoproteins. The effect of LDL and HDL could not be attributed to an effect on the cellular cAMP concentration. The involvement of protein kinase C also could not be demonstrated, since the protein kinase C inhibitor, H-7, did not prevent the effect of LDL.

8.3.4 Comparison of Endothelial Cells Obtained from Various Blood Vessels

8.3.4.1 Animal versus Human Endothelial Cells

From this study various remarkable differences between our results with human endothelial cells and published data obtained with animal endothelial cells became clear. Firstly, in several reports it has been described that stimulation of animal endothelial cells with TNF results in a direct and large increase of the permeability, while in human umbilical artery endothelial cells only a small effect can be observed after a lengthy incubation, and TNF does not effect the permeability of human aorta endothelial cells (within 24 hours) (chapter 5). Secondly, in our cells the activation of protein kinase C by PMA did not result in an increase of endothelial permeability and actually reduced the passage rate of peroxidase through human umbilical artery endothelial monolayers. Recently, similar results have been described for human umbilical vein endothelial cells (Yamada et al 1990). In contrast, it has been reported that PMA induces a pronounced increase of the permeability for animal endothelial cells (Gudgeon and Martin 1989, Lynch et al 1990).
Thirdly, the presence of an endothelial cell growth factor in the culture medium is essential for the growth and proliferation of human umbilical artery endothelial cells in vitro. Without this addition, it is very difficult to culture a tight monolayer (van Hinsbergh et al 1986). Furthermore, the used growth factor appeared not only to function prior to the confluence of the cells, but also acted as a kind of "stabilizing" factor after confluency was reached. Confluent human endothelial cell monolayers appeared to be very sensitive for removal of this "growth-stabilizing" factor. Twenty-four hours after removal of this factor, the monolayers become partially leaky (chapter 6). It has been described for animal endothelial cells that these monolayers

were kept for several days in the absence of this factor (Hennig et al 1984, Navab et al 1986).
Although the involvement of an in vitro adaptation of the cells in these differences cannot totally be excluded, it seems more likely that human endothelial permeability is partly regulated by another process than that of animal endothelial cell monolayers. The mechanism underlying these differences is not yet resolved. Most likely, an additional pathway exists that results in the reduction of the permeability of human endothelial cells. Since, in human endothelial cells, all substances which decrease the permeability also change the actin-myosin configuration, activation of this pathway may result either in the in-activation of the myosin light chain kinase or directly in the de-phosphorylation of the myosin light chain.

8.3.4.2 Human Aorta versus Human Umbilical Artery Endothelial Cells

The use of umbilical artery endothelial cells for standard laboratory techniques has different practical advantages. In this study, experiments are initially performed with umbilical artery endothelial cell and are later checked with human aorta endothelial cells. Most permeability characteristics are similar for both kind of cells, e.g. an apparent saturation of the LDL-passage has been found in both kind of cells; forskolin and nor-epinephrine decrease the passage rate of molecules through both kind of monolayers. However, several differences are observed. The basal passage rate of large molecules, in particular LDL, through monolayers of human aorta endothelial cells is lower than through monolayers of human umbilical artery endothelial cells. However, the transendothelial electrical resistance across human umbilical artery endothelial cells is higher than that of aorta endothelial monolayers. Since the electrical resistance has been described as a parameter of ion flux across the monolayer, this may suggest a different molecular selectivity for the two types of cells.
Two substances, TNF and PMA, temporarily reduce the passage through human umbilical artery endothelial cells, while neither substance influence the passage rate through human aorta endothelial cells. Although this difference may be due to differences between these cells, a difference due to subculturing cannot entirely be excluded. Human umbilical artery endothelial cells have been used after one passage, while human aorta endothelial cells have been used after at least two passages.

8.4 CONCLUSIONS

In this thesis, two models are described to study the permeability characteristics of human endothelial cell monolayers in vitro. The first is a model to study fluid transport under hydrostatic pressure, and secondly, a model to study (macro-) molecular transport in a diffusion assay. Basal characteristics of these models were in agreement with in vivo observations, according to electrical resistance, molecular sieve characteristics, morphology and dependency of plasma proteins and calcium ions.

Modulation of the permeability of the human arterial endothelial monolayer has been described in the following ways:

-an increase of the permeability, induced by vasoactive substances, Ca-ionophores
-a sustained decrease of the permeability, induced by forskolin and IBMX
-a temporary reduction of the permeability, induced by catecholamines, LDL and fatty acids.
-for umbilical artery endothelial cells: a reduction induced by TNF and PMA

Several mechanisms (may) underlie the described changes. An elevation of the intracellular calcium concentration is paralleled by an increase of the endothelial permeability. On the other hand, an increase of the cyclic AMP concentration and activation of protein kinase C are associated with a (sustained) reduction of the permeability. Hence, the regulation of human endothelial permeability may partly occur in another way than the regulation of animal endothelial permeability. Further studies are necessary to find out which mechanisms are involved in the reduction of endothelial permeability, induced by nor-epinephrine, TNF and fatty acids.

8.5 PERSPECTIVES

The in vitro system as described in this thesis provides a well established model to study endothelial permeability. Although an in vitro model remains an approximation of the in vivo situation, it may help firstly to understand the basic principles involved in the regulation of the passage of macromolecules through the endothelium and secondly to clarify, through which process endothelial permeability is increased in pathological situations. Furthermore, it may give suggestions to prevent an increase of endothelial permeability, induced by stimulation of the cells as occurs in various vascular diseases. The described in vitro model is also suitable for screening of agents on its effect on endothelial permeability.

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SUMMARY

In this thesis, an in vitro system of human endothelial cells, cultured on porous filters, suitable for the study of endothelial permeability, has been described.
In chapter 1 the theoretical background of the study has been described. Various diseases, as idiopathic edema, bronchial and allergic airway diseases, tumour development and arteriosclerosis are associated with increases in endothelial permeability. At the start of this study, it was generally accepted that active processes underlie both an increase and a decrease of the barrier function, but the biochemical knowledge about endothelial permeability was very limited.
In chapter 2, two in vitro models are described, to study human endothelial permeability. In the first one, fluid passage under hydrostatic pressure can be evaluated. The fluid passage appeared to be dependent on the presence of plasma proteins and calcium ions in the medium. In the second model, a diffusion-assay, the passage of (macro-) molecules through the endothelial monolayer, without hydrostatic pressure can be studied. The monolayers were characterized by: a) a size selectivity of the molecule on the passage rate, b) an adequate electrical resistance, c) the presence of tight junctions between adjacent cells (shown by electron microscopy) and d) a dependency of plasma proteins and calcium-ions on the integrity. Furthermore, the addition of 2-deoxy-d-glucose also inhibited the passage rate of peroxidase. This suggested that the passage is energy-dependent. However, at present, it is not known which process (vesicle transport or cellular contraction) is inhibited by this substance.
The passage of low density lipoproteins (LDL) has been described in chapter 3. Within about four hours after addition, high concentrations LDL passaged relatively slower than low concentrations LDL. This effect was not due to a saturation of the passage process, but to a general reduction of the permeability. This effect is not specific for LDL, but is also observed for other lipoproteins as (β-) very low and high density lipoproteins (chapter 7).
In addition, in chapter 3, a correlation has been described between an influx of calcium-ions into the cytoplasm and a contraction of the endothelial cell (shown by intercellular gaps between the cells by electron microscopy), resulting in an increase of the permeability. This effect has been identified for both histamine (H1 receptor mediated), thrombin and calcium ionophore.

On the other hand, an elevation of the cellular cyclic AMP concentration resulted in a decrease of the endothelial permeability, as shown for forskolin and isobutyl methyl xanthine (chapter 3 and 4) and for isoproterenol and iloprost (chapter 4). This effect was accompanied by a change in the actin-myosin configuration in the cell. Nor-epinephrine had a large effect on permeability, while it induced only a small increase of the cyclic AMP concentration. This suggests that nor-epinephrine activates another mechanism resulting in a reduction of the permeability.

During the study, two remarkable effects were observed in human endothelial cells. Both tumour necrosis factor (TNF) and phorbol myristate acetate (PMA), known as "destructors" of the integrity of animal endothelial monolayer, reduced temporary and sustained, respectively, the passage of peroxidase through human umbilical artery endothelial cells (chapter 5 and 6 respectively), while both substances did not influence the passage through monolayers of human aorta endothelial cells. Although TNF stimulated the production of prostacyclin in primary endothelial cells, this mechanism was likely not involved in the reduction of the permeability. Also in the presence of aspirin, TNF was able to reduce the permeability, and furthermore, TNF did not influence the cellular cAMP concentration.

The lack of effects of TNF and PMA on human aorta endothelial cells was not due to a general de-sensitization of these cells for the substances. The production of tissue-type plasminogen activator was influenced by both substances.

In chapter 7, the effects of lipoproteins and fatty acids (palmitic, oleic, arachidonic and eicosapentaenoic acid) are described. It seems unlikely that the reduction of the passage induced by all tested lipoproteins has been mediated by a cyclic AMP or a protein kinase C dependent pathway. The effect of fatty acids are studied on various time-intervals. While on short-time intervals most of the tested fatty acids induce a marginal reduction of the permeability, on longer time intervals all fatty acids (except for arachidonic acid) induce an increase.

In chapter 8, results are summarized and discussed. It is emphasized that it is very likely that certain aspects of the regulation of animal endothelial permeability is regulated in another way than that of human endothelial cell monolayers.

Nederlandse Samenvatting

Dit proefschrift beschrijft een in vitro systeem om de doorlaatbaarheid van een monolaag van humane endotheel cellen te kunnen bestuderen.
In hoofdstuk 1 wordt de theoretische achtergrond van het onderzoek beschreven. Daartoe worden zowel de functies van verschillende typen endotheel als verschillende passage routes voor macromoleculen door het endotheel besproken. In verschillende ziekte-beelden, zoals idiopathisch oedeem, bronchiale infecties, tumor ontwikkeling en arteriosclerosis is er sprake van toegenomen influx van macromoleculen in de vaatwand en het onderliggende weefsel. Bij aanvang van de studie werd algemeen aangenomen, dat het endotheel een actieve rol speelt bij de regulatie van deze influx, maar de biochemische kennis over het proces was zeer beperkt.
In hoofdstuk 2 worden twee modellen besproken om de doorlaatbaarheid van monolagen van humaan endotheel te kunnen bestuderen. Met behulp van het eerste model kan de doorlaatbaarheid van vloeistof door deze monolagen onder hydrostatische druk bestudeerd worden. De vloeistofpassage is afhankelijk van de aanwezigheid van plasma eiwitten en calcium-ionen in het medium. In het tweede model kan de passage van (macro-) moleculen door een monolaag van endotheelcellen vervolgd worden. In dit model worden humane endotheelcellen op een polycarbonaat filter gekweekt; na enkele dagen in kweek, vormen deze cellen een hechte monolaag. Als maat voor de doorlaatbaarheid van deze monolagen, wordt de passage-snelheid van peroxidase door de monolagen bepaald. De monolagen worden gekarakteriseerd door: a) een afhankelijkheid van de grootte van het te passeren molecuul op de passage-snelheid, b) een adequate transendotheliale electrische weerstand, c) de aanwezigheid van tight junctions tussen aaneenliggende cellen en d) een afhankelijkheid van de aanwezigheid van serum-eiwitten en calcium-ionen. Toevoeging van 2-deoxy-d-glucose resulteert in een afname van de passage. Hiermee wordt gesuggereerd dat het passage proces energie-afhankelijk is. Het is echter niet duidelijk welk proces hiermee geremd wordt: het transport van vesicles of een contractie van de cellen (hetgeen in in vitro cellen voor kan komen).
De passage van low density lipoproteins (LDL) wordt beschreven in hoofdstuk 3. Toevoeging van hoge concentraties LDL aan het medium, leidt tot een tijdelijke verlaging van de permeabiliteit. Hoewel dit lijkt op een verzadiging van het passage

proces, lijkt dit onwaarschijnlijk, omdat ook de passage van andere moleculen geremd wordt. Dit effect is niet specifiek voor LDL, maar wordt ook gevonden voor andere lipoproteinen, zoals high density lipoproteins en (β-) very low density lipoproteins (beschreven in hoofdstuk 7). Een betrokkenheid van de LDL-receptor in het passage proces kon niet worden aangetoond, terwijl wel de lading van het LDL-molecuul van belang bleek te zijn voor de passage-snelheid. Meer negatief geladen moleculen passeren langzamer, dit wordt waarschijnlijk veroorzaakt door het negatief geladen oppervlak van de endotheelcel.

Eveneens in hoofdstuk 3, wordt een relatie beschreven tussen de influx van calcium ionen in het cytoplasma en een contractie van de endotheelcel Door deze contractie ontstaan openingen tussen de cellen (te zien op electronen microscopisch niveau), waardoor de passage toeneemt. Dit effect wordt beschreven voor histamine (via een H1-receptor), thrombine en een calcium ionofoor.

Een verhoging van de concentratie van cyclisch AMP in de cel leidt tot een afname van de doorlaatbaarheid. Dit effect wordt beschreven voor forskoline en isobutyl methyl xanthine (in hoofdstuk 3 en 4) en voor isoproterenol en iloprost (hoofdstuk 4). Dit effect gaat samen met een verandering in de actine-myosine configuratie in de cel. Het effect van isoproterenol kan geblokkeerd worden door een β-adrenerge blokker. Nor-epinephrine heeft slechts een relatief klein effect op de cyclisch AMP concentratie in de cel, terwijl het wel de doorlaatbaarheid aanzienlijk verlaagt. Dit suggereert dat nor-epinephrine ook via een ander mechanisme de doorlaatbaarheid kan verlagen.

Gedurende de studie zijn twee opmerkelijke effecten gevonden op humaan endotheel. Zowel TNF als PMA, beide beschreven in de literatuur als veroorzakers van lekkage door dierlijk endotheel, verlagen de doorlaatbaarheid door humaan endotheel. Het effect van TNF is slechts zeer tijdelijk, terwijl het effect van PMA minimaal enkele uren duurt (hoofdstuk 5 en 6, respectievelijk). Het effect van beide stoffen wordt alleen gevonden op humaan navelstreng-endotheel en niet op humaan aorta endotheel. Hoewel TNF de productie stimuleert van prostacycline in primaire cellen, en prostacycline de cAMP concentratie in de cel kan verhogen, lijkt dit niet de verklaring van de verminderde passage. Ook in de aanwezigheid van aspirine kan TNF de doorlaatbaarheid verlagen, terwijl geen effect van TNF op de cAMP concentratie wordt gevonden.

Hoewel TNF en PMA niet de doorlaatbaarheid van monolagen van humane aorta cellen beïnvloeden, lijkt dit niet te wijten te zijn aan een algemene ongevoeligheid voor deze stoffen: beide stoffen beïnvloeden de produktie van plasminogeen activatoren (hoofdstuk 6).
In hoofdstuk 7 worden de effecten van lipoproteinen en vetzuren op de doorlaatbaarheid van het endotheel besproken. Alle geteste lipoproteinen verlagen tijdelijk de permeabiliteit van de humane navelstreng endotheelcellen. Dit effect kan vooralsnog niet toegeschreven worden aan een cyclisch AMP of protein kinase C afhankelijk proces. Hoewel op korte termijn de meeste vetzuren zorgen voor een (minimale) afname van de passage, blijkt dat na langdurige incubatie met deze vetzuren (behalve arachidonzuur), de passage toeneemt.
In hoofdstuk 8 worden de belangrijkste resultaten besproken en bediscussieerd. Er wordt nogmaals op gewezen dat hoewel sommige mechanismen om de passage te beïnvloeden gelijk zijn voor dierlijk en humaan endotheel, er ook enkele belangrijke verschillen zijn.

ABBREVIATIONS

apo	Apolipoprotein
Ca	Calcium
cAMP	cyclic Adenosine 5'-Monophosphate
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Ethylenebis(oxyethylenenitrilo) Tetraacetic Acid
HDL	High Density Lipoproteins
HMF	High Molecular Weight Fraction
HRP	Horse-Radish Peroxidase
HS	Human Serum
HSA	Human Serum Albumin
IBMX	Isobutyl Methyl Xanthine
IL	Interleukin
KCN	Kaliumcyanide
LDL	Low Density Lipoproteins
LMF	Low Molecular Weight Fraction
LPDS	Lipoprotein Depleted Serum
LPS	Lipopolysaccharide
NBCS	Newborn Calf Serum
PBS	Phosphate-Buffered Saline
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PP	Polypropylene
PTFE	Polytetrafluoroethylene
SD	Standard Deviation
TEER	Transendothelial Electrical Resistance
TNF	Tumour Necrosis Factor
VLDL	Very Low Density Lipoproteins

Curriculum Vitae

Op 25 juni 1962 werd ik te Zutphen geboren. Na succesvol lagere en middelbare school doorlopen te hebben, behaalde ik in 1980 mijn diploma Atheneum-B aan het Marnix-College te Haarlem. In datzelfde jaar begon ik met mijn studie biologie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen "B2" behaalde ik in september 1983 en het doctoraalexamen in juni 1986. Het doctoraalexamen omvatte het hoofdvak mathematische biologie (Instituut voor Theoretische Biologie i.s.m. Primatencentrum T.N.O. te Rijswijk) en het bijvak immunologie (vakgroep Nierziekten, Academisch Ziekenhuis Leiden).
Van 1 juli 1986 tot en met 1 juli 1990 ben ik werkzaam geweest op het Gaubius Instituut T.N.O. te Leiden. Hier heb ik binnen de sectie "pathofysiologie van het endotheel" o.l.v. dr. V.W.M. van Hinsbergh het onderzoek verricht waarvan het resultaat in deze dissertatie is beschreven.
Sinds 1 september 1990 ben ik werkzaam aan de Erasmus Universiteit, Medische Faculteit, afd. Biochemie II, te Rotterdam.