Activation of Nuclear Receptor Nur77 by 6-Mercaptopurine Protects Against Neointima Formation

Nuno M.M. Pires, MSc*; Thijs W.H. Pols, MSc*; Margreet R. de Vries, BSc; Claudia M. van Tiel, PhD; Peter I. Bonta, MD; Mariska Vos, BSc; E. Karin Arkenbout, MD, PhD; Hans Pannekoek, PhD; J. Wouter Jukema, MD, PhD; Paul H.A. Quax, PhD; Carlie J.M. de Vries, PhD

Background—Restenosis is a common complication after percutaneous coronary interventions and is characterized by excessive proliferation of vascular smooth muscle cells (SMCs). We have shown that the nuclear receptor Nur77 protects against SMC-rich lesion formation, and it has been demonstrated that 6-mercaptopurine (6-MP) enhances Nur77 activity. We hypothesized that 6-MP inhibits neointima formation through activation of Nur77.

Methods and Results—It is demonstrated that 6-MP increases Nur77 activity in cultured SMCs, which results in reduced [^{3}H]thymidine incorporation, whereas Nur77 small interfering RNA knockdown partially restores DNA synthesis. Furthermore, we studied the effect of 6-MP in a murine model of cuff-induced neointima formation. Nur77 mRNA is upregulated in cuffed arteries, with optimal expression after 6 hours and elevated expression up to 7 days after vascular injury. Local perivascular delivery of 6-MP with a drug-eluting cuff significantly inhibits neointima formation in wild-type mice. Locally applied 6-MP does not affect inflammatory responses or apoptosis but inhibits expression of proliferating cell nuclear antigen and enhances protein levels of the cell-cycle inhibitor $p27^{Kip1}$ in the vessel wall. An even stronger inhibition of neointima formation in response to local 6-MP delivery was observed in transgenic mice that overexpressed Nur77. In contrast, 6-MP does not alter lesion formation in transgenic mice that overexpress a dominant-negative variant of Nur77 in arterial SMCs, which provides evidence for the involvement of Nur77-like factors.

Conclusions—Enhancement of the activity of Nur77 by 6-MP protects against excessive SMC proliferation and SMC-rich neointima formation. We propose that activation of the nuclear receptor Nur77 is a rational approach to treating in-stent restenosis. (***Circulation.*** **2007;115:493-500.)**

Key Words: muscle, smooth ■ receptors, cytoplasmic and nuclear ■ restenosis ■ stents ■ transcription factors

Postangioplasty restenosis is a common complication after percutaneous coronary interventions and is mainly caused by excessive proliferation of vascular smooth muscle cells (SMCs).[1] As a result, the coronary artery narrows again, which severely reduces beneficial effects of the intervention. Even though the introduction of drug-eluting stents has considerably decreased restenosis incidence, postangioplasty restenosis remains a persistent problem.[2–4]

Clinical Perspective p 500

The phenotypic modulation of SMCs from quiescent differentiated cells into activated proliferative cells plays a key role in restenosis. In our search for genes involved in such phenotypic changes, we have revealed induction of Nur77 gene expression.[5] Furthermore, we showed that Nur77 is expressed in SMCs in the diseased human vessel wall but not in healthy vessels.[6] Paradoxically, overexpression of Nur77 in SMCs leads to a quiescent SMC phenotype, and mice that overexpress Nur77 in arterial SMCs are protected against SMC-rich lesion formation.[6] Therefore, we propose Nur77 as a potential target for treatment of restenosis.

Nur77 (NR4A1, TR3, NGFI-B) is highly homologous to Nurr1 (NR4A2) and NOR-1 (NR4A3), and together they form the nuclear receptor subfamily 4 group A (NR4A) of nuclear hormone receptors.[7] The NR4A transcription factors are implicated in diverse cellular events, such as apoptosis, differentiation, and proliferation.[8,9] Nuclear receptors consist of an N-terminal activating function-1 domain, a DNA-binding domain, and a ligand-binding domain at the C-terminus.[10] At this time no traditional ligands are known to activate Nur77-like factors through interaction with their ligand-binding domains, which designates Nur77, Nurr1, and

Received March 14, 2006; accepted November 27, 2006.

From the Gaubius Laboratory, TNO–Quality of Life (N.M.M.P., M.R.d.V., P.H.A.Q.), Leiden; the Departments of Cardiology (N.M.M.P., J.W.J.) and Surgery (P.H.A.Q.), Leiden University Medical Center, Leiden; and the Department of Medical Biochemistry (T.W.H.P., C.M.v.T., P.I.B., M.V., E.K.A., H.P., C.J.M.d.V.), Academic Medical Center, Amsterdam, the Netherlands.

*N.M.M. Pires and T.W.H. Pols contributed equally to this work.

Correspondence to Dr Carlie J.M. de Vries, Department of Medical Biochemistry, Academic Medical Center, K1-116, Meibergdreef 15, 1105AZ Amsterdam, The Netherlands. E-mail c.j.devries@amc.uva.nl

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.106.626838

NOR-1 as orphan receptors. Recently, however, it has been shown that 6-mercaptopurine (6-MP) increases Nur77 transactivation via its activating function-1 domain without direct binding to Nur77.[11] 6-MP is currently used for the treatment of leukemia, whereas azathioprine, the pro-drug of 6-MP, is prescribed at relatively low doses as a chronic immunosuppressive drug in inflammatory bowel disease as well as after organ transplantation.[12–14]

A well-defined mouse model of neointima formation consists of placement of a nonconstrictive perivascular cuff around the femoral artery.[15] Previously we showed that the nonconstrictive perivascular cuff may be constructed from a polymeric formulation suitable for controlled drug delivery.[16] This drug-eluting cuff simultaneously induces reproducible intimal hyperplasia and allows confined delivery of drugs to the cuffed vessels. In the present study, drug-eluting cuffs were applied to evaluate the local effect of 6-MP on neointima formation. We demonstrate that enhancement of the activity of Nur77 by 6-MP inhibits SMC proliferation and protects against SMC-rich neointima formation. These observations assign the nuclear receptor Nur77 as a potential target to prevent (in-stent) restenosis.

Methods

SMC Culture

Human SMCs were explanted from umbilical cord arteries. Cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Breda, the Netherlands) with 10% (v/v) fetal bovine serum (FBS) with penicillin and streptomycin (Invitrogen). Cells were used at passages 5 to 7. SMCs were characterized with a monoclonal antibody and directed against smooth muscle α-actin (1A4; DAKO, Glostrup, Denmark), and SMCs demonstrated uniform fibrillar staining. To determine cellular viability, cells were washed with phosphate-buffered saline and subsequently incubated in medium in the presence of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Diagnostics, St. Louis, Mo). After 2 hours, medium was discarded, formazan crystals were dissolved in isopropanol, and optical density was measured at 590 nm.

Apoptosis was induced by incubating cells for 24 hours in medium with 0.25 μmol/L staurosporine (Sigma). Subsequently, SMCs were fixed and stained with Hoechst dye, and the relative number of apoptotic nuclei was determined.

Transfection Experiments and Luciferase Assay

Cells were electroporated according to the Amaxa method (Amaxa, Cologne, Germany) with Nucleofector reagent for SMCs. In each transfection, 0.5 to 1.0×10^6 cells were used, and 3.5 μg Nur77-reporter plasmid with 1.5 μg renilla luciferase plasmid, which contains the thymidine kinase promoter, was used to correct for cell number and transfection efficiency. The Nur77-reporter plasmid contained the Nur response element of the pro-opiomelanocortin (POMC) promoter in triplicate with the −34/+63 minimal promoter of POMC gene.[17] Twenty-four hours after transfection, cells were incubated with 6-MP (Sigma) for 24 hours, and luciferase activity was assayed with the Dual Luciferase Reporter System (Promega, Madison, Wis).

DNA Synthesis Assay

SMCs were seeded in 24-well plates at 1 to 4×10^4 cells/well and reached 60% to 70% confluence after 24 hours. SMCs were made quiescent by incubation for 24 hours in FBS-free medium. 6-MP was dissolved in dimethylsulfoxide and applied 1 hour before FBS stimulation. SMCs were stimulated for 24 hours with 10% (v/v) FBS, and subsequently cells were labeled for 18 hours with 0.25 μCi/well [methyl-^{3}H]thymidine (Amersham Biosciences, Buckinghamshire, UK). Incorporated [^{3}H]thymidine was precipitated for 30 minutes at 4°C with 10% (wt/vol) trichloroacetic acid, washed twice with 5% (wt/vol) trichloroacetic acid, and dissolved in 0.5N NaOH (0.5 mL/well), and radioactivity was measured by liquid-scintillation count.

Small Interfering RNA Experiments

The following small interfering RNA (siRNA) sequences were used: Nur77 siRNA, 5′-CAGUCCAGCCAUGCUCCUCdTdT-3′, as described previously[18]; control siRNA, 5′-CAGACGAGCCUUGCUCGUCdTdT-3′ (Ambion, Austin, Tex). Five micrograms of siRNA was transfected into 0.5 to 1×10^6 SMCs with Nucleofector reagent for SMCs (Amaxa) according to the manufacturer's recommendations. Total mRNA was isolated 5 days after transfection with the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, Calif). Subsequent cDNA synthesis was performed with the iScript cDNA synthesis kit (Biorad, Hercules, Calif). Real-time polymerase chain reaction (PCR) was performed with SYBR green mix (Biorad) in the MyIQ System (Biorad). Primers for Nur77 were as follows: forward, 5′-GTTCTCTGGAGGTCATCCGCAAG-3′; reverse, 5′-GCAGGGACCTTGAGAAGGCCA-3′. As a control for equal amounts of first-strand cDNA in different samples, we corrected for Ribosomal Phosphoprotein (P0) mRNA levels, which were determined with the following primers: forward, 5′- TCGACAATGGCAGCATCTAC-3′; reverse, 5′-ATCCGTCTCCACAGACAAGG-3′.

Drug-Eluting Cuffs

Poly(ε-caprolactone)–based drug-eluting cuffs were manufactured as previously described.[16–18] Briefly, 6-MP was dissolved at different concentrations in blended, molten drug–polymer mix, and cuffs were designed to fit around the mouse femoral artery. Drug-eluting cuffs are shaped as longitudinally cut cylinders with an internal diameter of 0.5 mm, an external diameter of 1.0 mm, a length of 2.0 mm, and a weight of ≈5.0 mg.[16] Drug-eluting cuffs were loaded with 0.5% and 1% (w/w) 6-MP, and the in vitro release profiles were determined for a 4-week period as described before (n=5 per group).[16] 6-MP showed a sustained and dose-dependent release. Total release values at 4 weeks were 11.3±2.3 μg (46.3%) and 30.0±3.5 μg (58.7%) for the 0.5% and 1% 6-MP–eluting cuffs, respectively.

Femoral Artery Cuff Murine Model

All animal studies were approved by the Academic Medical Center (Amsterdam, the Netherlands) institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government. Wild-type FVB mice, transgenic mice that expressed the full-length Nur77 gene (Nur77), or mice that expressed a dominant-negative variant of Nur77 (ΔTA) (the latter 2 strains were under control of the SM22α promoter, which directs the expression of transgenes specific to SMCs) in an FVB background were used for experiments.[6] Male mice that were 10 to 12 weeks old were fed a standard chow diet. At the time of surgery, mice were anesthetized with an intraperitoneal injection of 5 mg/kg midazolam (Roche, Basel, Switzerland), 0.5 mg/kg medetomidine (Orion, Helsinki, Finland), and 0.05 mg/kg fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and loosely sheathed with a nonconstrictive cuff.[15] Either an empty control cuff or a 6-MP–eluting cuff (0.5% or 1% w/w) was used (n=6 per group).

Nur77 mRNA Expression in Cuffed Mouse Femoral Artery

Male wild-type mice underwent femoral artery cuff placement as described above. Animals were euthanized at different time points after surgery (0, 6, 24, 48, and 72 hours, and 7 days), with 4 mice per group. Femoral arteries were isolated, harvested, and snap-frozen. Total RNA was isolated with the RNeasy Fibrous Tissue Mini-Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. cDNA was made from all RNA samples with Ready-To-Go

reverse transcription PCR beads (Amersham Biosciences, Uppsala, Sweden).

Intron-spanning primers and probes were designed to hybridize with murine Nur77 cDNA (sense, 5′-GGGCATGGTGAA-GGAAGTTGT-3′; antisense, 5′-AGGCTGCTTGGGTTTTGAAG-3′; probe, 5′-CCGCCCTTTTAGGCTGTCTGTCCG-3′) with Primer Express 1.5 (Applied Biosystems, Foster City, Calif). Hypoxanthine phosphoribosyltransferase was assayed to correct for cDNA input. For each time point, reverse transcription PCR was performed in duplicate. Data are presented as fold-induction of Nur77 mRNA expression in injured over noninjured vessels.

Quantification and Histological and Immunohistochemical Analysis of Lesions in Cuffed Femoral Arteries

Mice were euthanized at indicated time points after cuff placement. The thorax was opened and a mild pressure-perfusion (100 mm Hg) with 4% formaldehyde in 0.9% NaCl (v/v) was performed for 5 minutes by cardiac puncture. After perfusion, femoral arteries were harvested, fixed overnight in 4% formaldehyde, dehydrated, and embedded in paraffin. Serial cross sections (5 μm thick) for histological analysis were used throughout the entire length of the cuffed femoral artery. All samples were routinely stained with hematoxylin-phloxine-saffron. Weigert's elastin staining was used to visualize elastic laminae. Ten equally spaced cross sections were used in all mice to quantify intimal lesions. Image analysis software (Leica Qwin, Wetzlar, Germany) was used to measure the total cross-sectional medial area between the external and internal elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.[15] Standard biotin-streptavidin procedures were used for immunohistochemistry to detect mouse leukocytes (CD45, 1:200; BD Pharmingen, San Diego, Calif), proliferating cell nuclear antigen (1:100; Calbiochem, San Diego, Calif), or $p27^{Kip1}$ (1:100; Novocastra, Newcastle on Tyne, UK). Biotin was detected with streptavidin–horseradish peroxidase conjugates (DAKO), and sections were subsequently developed with amino-ethylcarbazole and hydrogen peroxide. Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) with an in situ cell death detection kit (Roche). The relative number of positive cells was counted.

Statistical Analysis

Data are reported as mean±SEM and were analyzed with the nonparametric Mann-Whitney *U* test (SPSS 14.0 for Windows, SPSS Inc, Chicago, Ill). Two-sided probability values were tested against adjusted type I error rates with Bonferroni-Holm step-down of the family-wise α error. Statistical significance is indicated with an asterisk (*) in the table and figures.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

6-MP Enhances Nur77 Activity in Cultured SMCs

It has been shown that 6-MP enhances the transcriptional activity of Nur77 in C2C12 mouse skeletal muscle cells, whereas 6-MP does not affect Nur77 activity in CV-1 monkey kidney fibroblast cells.[11,19] To investigate whether 6-MP increases Nur77 transcriptional activity in vascular cells, human SMCs were transduced with lentivirus encoding Nur77, which resulted in expression in 85% to 90% of the infected cells and increased Nur77 mRNA levels compared with mock-infected SMCs (data not shown). Immunofluorescence of transduced SMCs revealed Nur77 protein overexpression located in the nucleus (data not shown). SMCs that overexpressed Nur77 were subsequently electroporated with the firefly luciferase reporter construct, which contains the

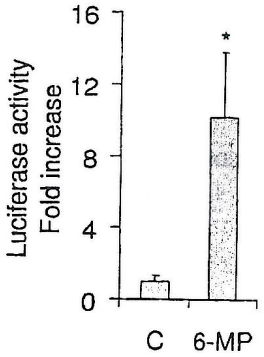

Figure 1. 6-MP enhances Nur77 activity in cultured SMCs. The transcriptional activity of Nur77 was monitored by measuring luciferase activity in SMCs that expressed Nur77 transfected with a Nur77 reporter-luciferase construct, which contained the POMC-derived Nur response element. Cells were cultured in the absence of (C) or for 24 hours in the presence of 6-MP (n=4, mean±SEM). *Statistically significant.

palindromic Nur response element (Nur77 response element from the POMC promoter) sequence ($TGATATTTn_6AAA-TGCCA$)[17] to monitor Nur77 transcriptional activity in combination with the thymidine kinase–renilla luciferase construct as a control for transfection efficiency. Incubation of SMCs for 24 hours with 50 μmol/L 6-MP resulted in a 10-fold increase in Nur77 activity ($P=0.021$, Figure 1), comparable with the induction observed in C2C12 cells. Our data indicate that 6-MP robustly enhances Nur77 activity in cultured SMCs.

6-MP Inhibits Proliferation of SMCs: Involvement of Nur77

To study whether 6-MP modulates SMC proliferation, we investigated DNA synthesis of cultured human SMCs in the presence of increasing concentrations of 6-MP. As expected, 6-MP inhibits DNA synthesis in SMCs ($P=0.001$ for 25 μmol/L and $P=0.001$ for 50 μmol/L 6-MP; Figure 2A). To assess the specific contribution of Nur77 in this process, Nur77 expression was knocked down by siRNA in human SMCs. Transfection with siRNA directed against Nur77 or with control siRNA results in downregulation of FBS-induced Nur77 mRNA levels in the siNur77-transfected cells ($P=0.021$), as determined by real-time reverse transcription PCR (Figure 2B). [^{3}H]Thymidine incorporation is significantly higher in cells in which Nur77 is knocked down by gene-specific siRNA in comparison to SMCs transfected with control siRNA. To visualize the relative effect of 6-MP on DNA synthesis in SMCs transfected with siNur77 RNA or with control siRNA, we expressed [^{3}H]thymidine incorporation as a percentage of control condition (Figure 2C). DNA synthesis is inhibited by 6-MP for 61% when SMCs are transfected with control siRNA, whereas the effect of 6-MP is significantly less in SMCs transfected with Nur77-specific siRNA because only 41% inhibition of DNA synthesis is observed ($P=0.021$ at 25 μmol/L and $P=0.021$ at 50 μmol/L 6-MP). These data clearly demonstrate that 6-MP inhibits DNA synthesis in SMCs at least partly through activation of Nur77.

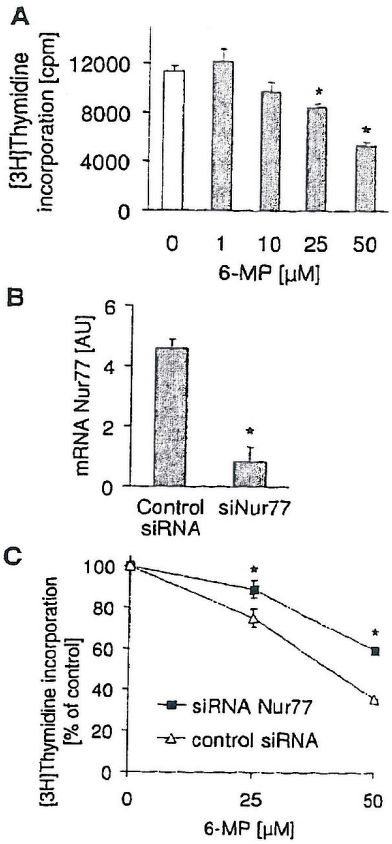

Figure 2. 6-MP inhibits proliferation of cultured SMCs: involvement of Nur77. A, DNA synthesis of SMCs grown in medium with vehicle (control, □) or indicated concentrations 6-MP (□). DNA synthesis was assayed by [^{3}H]thymidine incorporation. At 25 μmol/L or 50 μmol/L 6-MP, DNA synthesis is reduced (mean±SEM, n=8). B, Nur77 mRNA expression is reduced in SMCs transfected with siNur77 in comparison to SMCs transfected with control siRNA. mRNA levels were determined by real-time reverse-transcription PCR, and cDNA content of the samples was corrected for P0 expression (mean±SEM, n=4). C, The effect of 6-MP on DNA synthesis was determined by [^{3}H]thymidine incorporation in SMCs transfected with control siRNA (△) or with siNur77 (■). 6-MP inhibits DNA synthesis less effectively in siNur77-transfected cells than in control siRNA-transfected cells, which demonstrated that the inhibitory effect of 6-MP is at least partly mediated through activation of Nur77 (mean±SEM, n=4). *Statistically significant.

6-MP Is Not Cytotoxic to SMCs and Does Not Induce Apoptosis

To verify whether 6-MP is cytotoxic, quiescent SMCs were incubated for 24 hours with increasing concentrations of 6-MP, and viability of the cells was determined with a standard MTT assay. The number of viable cells was reduced in response to staurosporine (35% reduction, P=0.021), whereas 6-MP did not affect cellular viability (Figure 3A), which indicates that under these conditions 6-MP has no cytotoxic effect on human SMCs.

To investigate whether 6-MP induces apoptosis in SMCs, the cells were cultured for 24 hours with increasing concentrations of 6-MP. As a positive control, staurosporine was shown to induce apoptosis in SMCs (50±2%). Clearly, no

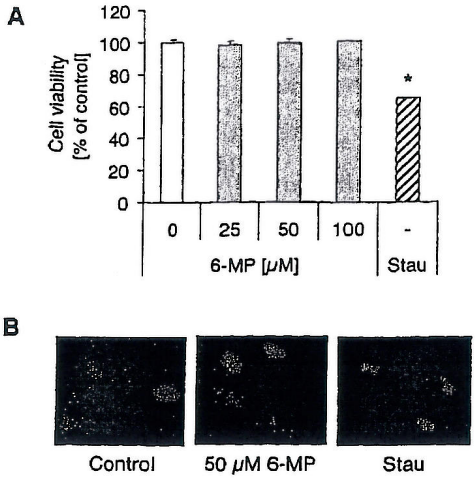

Figure 3. 6-MP is not cytotoxic to SMCs and does not induce apoptosis. A, Viability of SMCs incubated for 24 hours with vehicle (control, □), 6-MP (□) or staurosporine (Stau, ▨). Viability of cells was determined by MTT assay and expressed as a percentage of control (mean±SEM, n=3). *Statistically significant. B, SMCs were incubated for 24 hours with vehicle, 6-MP, or Stau. Nuclei were subsequently stained using Hoechst dye. Only Stau induces apoptosis and reduces cell viability.

evidence was found that 6-MP affects cell death under these conditions (3±1%) in comparison to control cells (4±1%) (Figure 3B).

Nur77 Is Expressed During the Process of Neointima Formation

To assess the potential inhibitory effect of 6-MP on SMC-rich lesion formation in vivo, the well-established murine model of cuff-induced neointima formation was applied.[15] We studied Nur77 mRNA expression during neointima formation and observed that Nur77 mRNA expression is upregulated after cuff placement, with optimal expression 6 hours after vascular injury (189±26-fold increase; Figure 4). Nur77 mRNA expression is enhanced up to 7 days after surgery in comparison to noncuffed vessels (13.4±1.1-fold increase). Given that Nur77 mRNA transcripts are regulated on vascular injury strictly dependent on conditions and time, it is conceivable that Nur77 plays a role in the process of neointima formation.

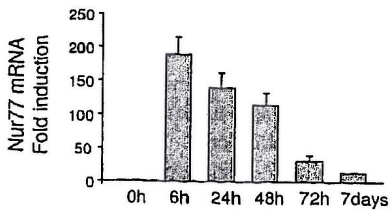

Figure 4. mRNA expression of Nur77 in the vessel wall after cuff injury. At different time points after cuff placement (6 hours to 7 days), cuffed vessel segments were harvested and assayed for Nur77 mRNA content. The mRNA expression levels are indicated as the relative expression in comparison to sham-operated vessels (mean±SEM, n=6). Nur77 mRNA is upregulated as early as 6 hours after injury and remains elevated for up to 7 days.

Quantitative Morphometry and Immunohistochemical Analysis of Mouse Femoral Lesions After Cuff Placement in Wild-Type Mice for 7 Days

		6-MP–Eluting Cuff	
	Control	0.5% (*P*)	1% (*P*)
Intimal area, $\times 10^3$ μm^2	3.9±0.5	3.0±0.5 (0.346)	2.0±0.3 (0.014)*
Medial area, $\times 10^3$ μm^2	11.9±0.8	10.3±0.8 (0.099)	9.0±0.9 (0.086)
Total vessel area,† $\times 10^3$ μm^2	38.6±4.7	37.1±4.7 (0.906)	34.0±4.0 (0.668)
Intima-media ratio	0.33±0.04	0.28±0.05 (0.288)	0.21±0.05 (0.045)*
Proliferating cell nuclear antigen$^+$ cells, %			
Intima	21.3±1.8	18.1±2.8 (0.465)	6.4±0.8 (0.006)*
Media	22.3±4.5	20.4±3.0 (1.000)	7.0±1.9 (0.008)*
CD45$^+$ cells, %			
Intima	22.8±5.1	21.0±2.1 (1.000)	30.2±4.7 (0.150)
Media	13.3±2.6	9.3±1.0 (0.195)	14.0±1.9 (0.775)
TUNEL$^+$ cells, %			
Intima	0.3±0.1	0.2±0.1 (0.233)	0.2±0.1 (0.798)
Media	0.6±0.2	0.7±0.2 (0.906)	0.9±0.4 (0.794)

Values are shown as mean±SEM (n=6 per group). TUNEL indicates terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling.

*Statistically significant according to Mann-Whitney *U* test with Bonferroni-Holm correction as compared to control.

†Total vessel area comprises the surface within the external elastic lamina, which includes the lumen.

6-MP Inhibits Proliferation of SMCs in Early Cuff-Induced Lesions

To evaluate the effect of 6-MP on cuff-induced neointima formation in vivo, we used a drug-eluting cuff loaded with increasing concentrations of 6-MP, which allowed restricted, local, perivascular delivery of compounds to the cuffed vessel segment.[16] The effect of 6-MP was initially evaluated in wild-type animals, and we compared lesions induced by control cuffs with lesions in cuffs containing 0.5% or 1% 6-MP within 7 days. Morphometric analysis of the lesions revealed that the intimal area was significantly reduced in 1% 6-MP cuffs (see the Table). No changes were observed in total vessel area or medial surface; consequently, the intima-media ratio was also reduced in response to local 6-MP delivery. To understand the mechanism that underlies the effect of 6-MP, we characterized the effect of 6-MP on inflammation, apoptosis, and cellular proliferation. Immunohistochemistry with anti-CD45 antibodies was performed to quantify leukocyte accumulation in the media and neointima. No differences were observed in relative leukocyte numbers within the cuffed vessel segments on perivascular delivery of increasing 6-MP concentrations as compared with control-cuffed femoral arteries. TUNEL staining showed that local 6-MP delivery has no significant effect on the number of apoptotic cells in the media or in the intimal region as compared with arteries treated with control cuffs (Table). It has been shown, especially in early lesions 5 to 7 days after vascular injury, that the number of proliferating cells in the vessel wall is relatively high.[20] For that reason, we used proliferating cell nuclear antigen staining to quantify cellular proliferation in these early lesions and observed in femoral arteries with a control cuff that 22.3±4.5% of the cells were positive in the medial and 21.3±1.8% in the intimal regions. In line with the data on lesion size, 1% 6-MP–eluting cuffs led to a profound inhibition of medial (68.6%) and intimal (71.3%) cellular proliferation (Table).

Effect of 6-MP on Cuff-Induced Neointima Formation in WT, Nur77-Transgenic, and ΔTA-Transgenic Mice

To reveal involvement of Nur77 in the 6-MP–mediated effects after vessel injury, we analyzed lesion formation in wild-type and transgenic mice that expressed full-length Nur77 cDNA in the arterial wall. Microscopic analysis of cuffed femoral artery segments revealed that after 4 weeks a concentric neointima was formed in mice administered an empty control drug-eluting cuff in both wild-type and Nur77-transgenic mice. Animals administered a 6-MP–eluting cuff showed reduced lesion formation (Figure 5A). Morphometric analyses revealed significant inhibition of cuff-induced neointima formation in vessel segments, locally treated with the 1% 6-MP concentration, in both wild-type (P=0.016) and Nur77-transgenic mice (P=0.007, Figure 5B). Wild-type animals treated with 0.5% 6-MP–eluting cuffs did not show a decrease in neointima formation (P=0.32), whereas the same 6-MP concentration substantially reduced neointima formation in Nur77-transgenic mice (P=0.015). No changes were observed in medial areas of the cuffed femoral arteries (data not shown). Consequently, a similar dose-dependent decrease was seen in intima-media ratios of 6-MP–treated Nur77-transgenic mice: control cuff, 0.75±0.11; 0.5% 6-MP, 0.47±0.05 (P=0.04); 1% 6-MP, 0.30±0.06 (P=0.003). Again, intima-media ratios of cuffed arteries in wild-type mice were only significantly decreased in the 1% 6-MP cuffs: control cuff, 1.10±0.16; 0.5%, 0.75±0.05 (P=0.15); 1%, 0.51±0.03 (P=0.008).

To further establish functional involvement of Nur77 in 6-MP–mediated effects on neointima formation, 6-MP–elut-

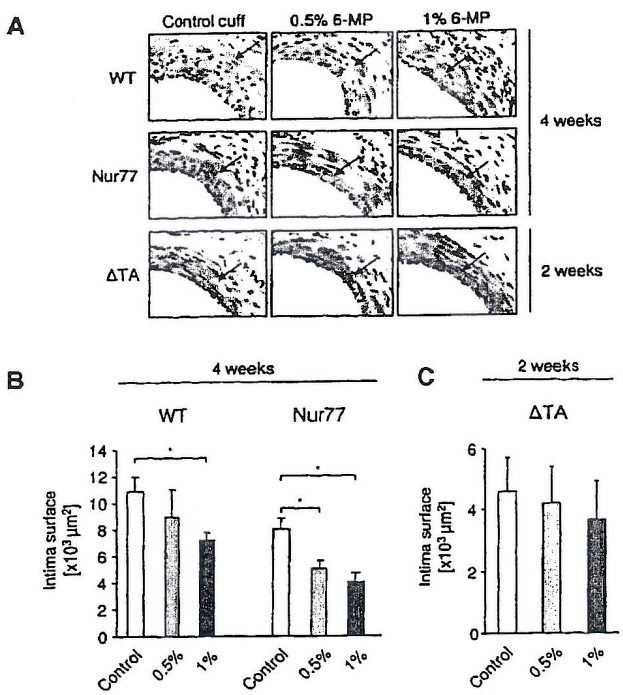

Figure 5. The effect of local 6-MP delivery on neointima formation. A, Representative cross sections of femoral arteries of wild-type mice, transgenic mice that expressed full-length Nur77 cDNA (Nur77), or mice that expressed ΔTA, with cuffs that contained different amounts of 6-MP. The cuffed vessel segments of wild-type and Nur77-transgenic mice were analyzed by hematoxylin-phloxine-saffron staining after 4 weeks and of ΔTA-transgenic mice after 2 weeks (magnification ×400; arrows indicate the internal elastic lamina). B, Morphometric analyses of cuffed vessel segments revealed total intimal area in wild-type and Nur77-transgenic mice 4 weeks after placement of cuffs. Cuffs contained either no (control), 0.5%, or 1% 6-MP. C, Total intimal area in cuffed femoral arteries in ΔTA transgenic mice 2 weeks after placement of cuffs that contained either no, 0.5%, or 1% 6-MP (mean±SEM, n=6). *Statistically significant.

ing cuffs were placed around the femoral artery of transgenic mice that expressed ΔTA, which inhibits the activity of all 3 Nur77-like factors. Previously, we have shown that SMC-rich lesions develop relatively quickly after carotid artery ligation in ΔTA-transgenic mice.[6] In line with these data, we also observed enhanced lesion formation in the currently applied femoral artery cuff model, which results in almost fully occlusive lesions within 4 weeks (data not shown). To reliably evaluate the effect of 6-MP–eluting cuffs in ΔTA-transgenic mice, we analyzed neointima formation after 2 weeks, when lesions are comparable in size to those in wild-type and Nur77 mice after 4 weeks (Figure 5A). Morphometric analyses of intimal areas revealed that local delivery of 6-MP in ΔTA-transgenic mice did not change media thickness and had no significant effect on neointima formation either in the 0.5% (P=0.46) or in the 1% (P=0.37) 6-MP–eluting cuff (Figure 5C).

Previously we showed that Nur77 enhances the expression of the cell-cycle inhibitor $p27^{Kip1}$, both in cultured SMCs and in organ cultures of carotid arteries.[6,21] To further ascertain that cellular proliferation rather than apoptosis is changed

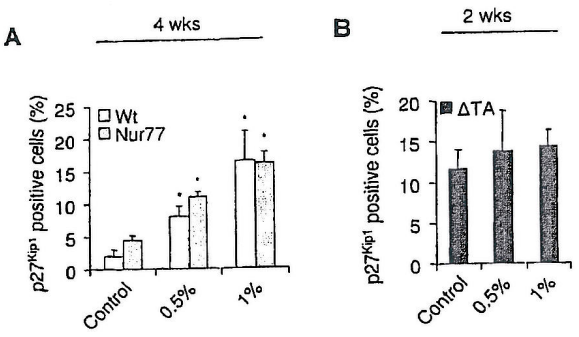

Figure 6. The effect of Nur77 and 6-MP on vascular $p27^{Kip1}$ expression. A, Immunohistochemical analysis of cuffed vessel segments of wild-type and Nur77-transgenic mice after 4 weeks revealed increased relative expression $p27^{Kip1}$ in the media in response to local delivery of 6-MP (0.5% or 1% 6-MP–eluting cuffs). B, The lesions of ΔTA-transgenic mice were analyzed after 2 weeks and showed no change in relative $p27^{Kip1}$ expression in response to 6-MP (mean±SEM, n=6). *Statistically significant.

when Nur77 activity is enhanced, we extended our present data with quantitative analyses on $p27^{Kip1}$ protein expression in sections of cuffed arteries in wild-type and Nur77- or ΔTA-transgenic mice (Figure 6). 6-MP enhances the expression of this cell-cycle inhibitor, which corresponds to reduced lesion formation in both wild-type and Nur77 mice. The lesions in ΔTA mice were analyzed after 2 weeks because lesions develop much more quickly in the mice in which the transcriptional activity of Nur77 is inhibited. In ΔTA mice, 6-MP no longer affects vascular $p27^{Kip1}$ expression, which corresponds with unchanged lesion size.

Altogether, these data are in line with the in vitro observations and demonstrate that 6-MP inhibits cuff-induced neointima formation that involves activation of Nur77.

Discussion

In the present study, we investigated whether 6-MP protects against restenosis through activation of the nuclear receptor Nur77. Our data clearly demonstrate that activation of Nur77 by 6-MP inhibits SMC proliferation, a hallmark of restenosis. In addition, with the use of a murine model of neointima formation, we provide evidence that supports the hypothesis that activation of Nur77 by 6-MP inhibits neointima formation.

Wansa and colleagues[11] have shown that 6-MP activates Nur77 in murine myoblast C2C12 cells, whereas Ordentlich et al[19] did not observe enhanced Nur77 activity in response to 6-MP in CV-1 cells. These data suggest that Nur77 is not activated by 6-MP in all cell types, which may be explained by the fact that 6-MP does not affect the activity of Nur77 through direct interaction but rather influences interaction of (unknown) coactivators and/or corepressors with Nur77-like factors.[22] Consequently, the relative expression levels of such presumed cofactors in distinct cell types may determine the effect of 6-MP on Nur77-like factor activation. In this report, we show that 6-MP does enhance Nur77 activity in human SMCs. Moreover, SMCs transfected with siRNA against Nur77 show less effect of 6-MP on proliferation in comparison to SMCs transfected with control siRNA. These data

clearly demonstrate involvement of Nur77 in 6-MP–mediated inhibition of human SMC proliferation.

We substantiated these in vitro observations in a well-defined mouse model, in which we showed that perivascular delivery of 6-MP inhibits neointima formation. Moreover, mice that overexpressed Nur77 in the arterial vessel wall and received 6-MP locally showed additional inhibition of lesion formation in comparison to 6-MP–treated wild-type mice. In addition, transgenic mice that overexpressed the dominant-negative form of Nur77 in the vessel wall, which inhibits transcriptional activity of all Nur77-like factors, did not show an effect of 6-MP on neointima formation. The latter experiments unambiguously demonstrated that 6-MP inhibits neointima formation through activation of Nur77-like factors.

We and others have shown that not only Nur77 expression but also Nurr1 and NOR-1 expression are enhanced in activated SMCs.[5,6,23,24] For NOR-1, it has been demonstrated that gene-specific anti-sense oligonucleotides decrease SMC proliferation.[23] These data support a proliferative function of NOR-1, which is in contrast to the effect of Nur77 on SMCs. So far, the function of Nurr1 in SMC proliferation has not been elucidated. 6-MP is known to enhance the activity of all 3 members of the NR4A subgroup of nuclear hormone receptors.[11] In the present study, we observed a decrease in DNA synthesis of SMCs in the presence of 6-MP. Nur77 knockdown by a specific siRNA does not abolish the effect of 6-MP entirely, which indicates that the remaining effect of 6-MP may be attributed to residual Nur77 activity and/or to 6-MP–mediated Nurr1 activation.

Azathioprine was one of the first immunosuppressive drugs applied for prophylaxis of acute organ transplant rejection, inflammatory bowel disease, and rheumatoid arthritis.[12–14] Azathioprine is the pro-drug that is converted in vivo initially into 6-MP and subsequently into thioguanosine nucleotides that, at high doses, may interfere with nucleotide metabolism in proliferating cells.[25] The dose at which azathioprine is applied chronically in transplantation protocols and inflammatory bowel disease does not invoke systemic cytostatic effects and is even compatible with normal pregnancy.[26] Over the years, cyclosporin A has replaced azathioprine as a drug in these pathologies; however, patients treated with cyclosporin A are at relatively high risk for cardiovascular disease.[27,28] Consequently, the prescription of azathioprine currently is undergoing a revival in clinical practice, with beneficial effects on lipid profiles, extent of plasma low-density lipoprotein oxidation, and fibrinolytic parameters.[29–31] Our study demonstrates that 6-MP also has a favorable effect directly on the vessel wall, notably at sites of local injury when Nur77-like factors are induced. At this point, we assume that in the drug-eluting cuff experiments, the amount of 6-MP, which is locally released, does not provoke any systemic effects on lipid profiles and fibrinolytic markers. This is supported by the observation that 6-MP does not affect neointima formation in transgenic ΔTA mice.

In conclusion, we have shown that activation of Nur77 by local 6-MP delivery in a mouse restenosis model does not affect inflammatory cell influx or apoptosis of vascular cells. Significantly, 6-MP–mediated Nur77 activation reduces SMC proliferation and protects against neointima formation. We therefore propose that activation of the nuclear receptor Nur77 by 6-MP or by other activators/agonists is a rational approach to treat (in-stent) restenosis.

Sources of Funding

N.M.M. Pires, T.W.H. Pols, and Dr Jukema are supported by the Netherlands Heart Foundation grants 2001-T-32, 2003B199, and 2001-D0-32, respectively. Drs Quax, de Vries, Arkenbout, and Bonta are supported by the Molecular Cardiology Program of the Netherlands Heart Foundation (M 93.001, 93.007), and Dr van Tiel is supported by NWO-Research Institute Diseases of the Elderly grant 948-00-006. This work emanates from the European Vascular Genomics Network, which is supported by the European Union FP6 Network of Excellence LSHM-CT-2003-503254.

Disclosures

None.

References

1. Komatsu R, Ueda M, Naruko T, Kojima A, Becker AE. Neointimal tissue response at sites of coronary stenting in humans: macroscopic, histological, and immunohistochemical analyses. *Circulation*. 1998;98:224–233.
2. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, Caputo RP, Kereiakes DJ, Williams DO, Teirstein PS, Jaeger JL, Kuntz RE. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med*. 2003; 349:1315–1323.
3. Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT, Turco M, Caputo A, Bergin P, Greenberg J, Popma JJ, Russell ME. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *N Engl J Med*. 2004;350:221–231.
4. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. *Int J Cardiol*. 2005; 99:9–17.
5. de Vries CJ, van Achterberg TA, Horrevoets AJ, ten Cate JW, Pannekoek H. Differential display identification of 40 genes with altered expression in activated human smooth muscle cells: local expression in atherosclerotic lesions of smags, smooth muscle activation-specific genes. *J Biol Chem*. 2000;275:23939–23947.
6. Arkenbout EK, de Waard V, van Bragt M, van Achterberg TA, Grimbergen JM, Pichon B, Pannekoek H, de Vries CJ. Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation*. 2002;106:1530–1535.
7. Maruyama K, Tsukada T, Ohkura N, Bandoh S, Hosono T, Yamaguchi K. The NGFI-B subfamily of the nuclear receptor superfamily. *Int J Oncol*. 1998;12:1237–1243.
8. Hsu HC, Zhou T, Mountz JD. Nur77 family of nuclear hormone receptors. *Curr Drug Targets Inflamm Allergy*. 2004;3:413–423.
9. Winoto A, Littman DR. Nuclear hormone receptors in T lymphocytes. *Cell*. 2002;109:S57–S66.
10. Giguere V. Orphan nuclear receptors: from gene to function. *Endocr Rev*. 1999;20:689–725.
11. Wansa KD, Harris JM, Yan G, Ordentlich P, Muscat GE. The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. *J Biol Chem*. 2003;278:24776–24790.
12. Estlin EJ. Continuing therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of methotrexate, 6-mercaptopurine and 6-thioguanine. *Cancer Treat Rev*. 2001;27: 351–363.
13. Gearry RB, Barclay ML. Azathioprine and 6-mercaptopurine pharmacogenetics and metabolite monitoring in inflammatory bowel disease. *J Gastroenterol Hepatol*. 2005;20:1149–1157.
14. McGeown MG, Douglas JF, Donaldson RA, Hill CM, Kennedy JA, Loughridge WG, Middleton D. Ten-year results of renal transplantation with azathioprine and prednisolone as only immunosuppression. *Lancet*. 1988;1:983–985.
15. Quax PH, Lamfers ML, Lardenoye JH, Grimbergen JM, de Vries MR, Slomp J, de Ruiter MC, Kockx MM, Verheijen JH, van Hinsbergh VW. Adenoviral expression of a urokinase receptor targeted protease inhibitor

inhibits neointima formation in murine and human blood vessels. *Circulation*. 2001;103:562–569.
16. Pires NM, van der Hoeven BL, de Vries MR, Havekes LM, van Vlijmen BJ, Hennink WE, Quax PH, Jukema JW. Local perivascular delivery of anti-restenotic agents from a drug-eluting poly(epsilon-caprolactone) stent cuff. *Biomaterials*. 2005;26:5386–5394.
17. Philips A, Maira M, Mullick A, Chamberland M, Lesage S, Hugo P, Drouin J. Antagonism between Nur77 and glucocorticoid receptor for control of transcription. *Mol Cell Biol*. 1997;17:5952–5959.
18. Kolluri SK, Bruey-Sedano N, Cao X, Lin B, Lin F, Han YH, Dawson MI, Zhang XK. Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. *Mol Cell Biol*. 2003;23:8651–8667.
19. Ordentlich P, Yan Y, Zhou S, Heyman RA. Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *J Biol Chem*. 2003;278:24791–24799.
20. Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. *J Clin Invest*. 2000; 105:293–300.
21. de Waard V, Arkenbout EK, Vos M, Mocking AI, Niessen HW, Stooker W, de Mol BA, Quax PH, Bakker EN, VanBavel E, Pannekoek H, de Vries CJ. TR3 nuclear orphan receptor prevents cyclic stretch-induced proliferation of venous smooth muscle cells. *Am J Pathol*. 2006;168: 2027–2035.
22. Wansa KD, Muscat GE. TRAP220 is modulated by the antineoplastic agent 6-mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors. *J Mol Endocrinol*. 2005;34:835–848.
23. Martinez-Gonzalez J, Rius J, Castello A, Cases-Langhoff C, Badimon L. Neuron-derived orphan receptor-1 (NOR-1) modulates vascular smooth muscle cell proliferation. *Circ Res*. 2003;92:96–103.
24. Martinez-Gonzalez J, Badimon L. The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. *Cardiovasc Res*. 2005;65:609–618.
25. Tidd DM, Paterson ARP. A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res*. 1974;34:738–746.
26. Polifka JE, Friedman JM. Teratogen update: azathioprine and 6-mercaptopurine. *Teratology*. 2002;65:240–261.
27. Hollander AAMJ, van Saasse JLCM, Kootte AMM, van Dorp Wt, van Bockel HJ, van Es LA, van der Woude FJ. Beneficial effects of conversion from cyclosporin to azathioprine after kidney transplantation. *Lancet*. 1995;345:610–614.
28. Sutherland F, Burgess E, Klassen J, Buckle S, Paul LC. Post-transplant conversion from cyclosporin to azathioprine: effect on cardiovascular risk profile. *Transplant Int*. 1993;6:129–132.
29. van den Dorpel MA, Ghanem H, Rischen-Vos J, Man in't Veld AJ, Jansen H, Weimar W. Conversion from CsA to azathioprine treatment improves LDL oxidation in kidney transplant recipients. *Kidney Int*. 1997;51:1608–1612.
30. van den Dorpel MA, Levi M, Ijzermans JN, ten Cate JW, Weimar W. Beneficial effects of conversion from CsA to AZA on fibrinolysis in renal transplant recipients. *Arterioscler Thromb Vasc Biol*. 1999;19: 1555–1558.
31. Miller LW. Cardiovascular toxicities of immunosuppressive agents. *Am J Transplant*. 2002;2:807–818.

CLINICAL PERSPECTIVE

Percutaneous coronary intervention is a routine intervention for treating coronary artery disease. A complication of this procedure is restenosis, which is caused by excessive proliferation of smooth muscle cells in response to the local vascular injury provoked by percutaneous coronary intervention. Drug-eluting stents that contain sirolimus or paclitaxel have been proven to prevent in-stent restenosis; however, these drugs may adversely affect vascular tissue histology. Other strategies to locally and selectively inhibit proliferation of intimal smooth muscle cells may be favorable. In previous studies, it has been shown that the nuclear receptor Nur77 inhibits formation of smooth muscle cell–rich lesions and promotes endothelial cell survival. Moreover, the activity of the transcription factor Nur77 is enhanced by 6-mercaptopurine. On the basis of this knowledge, we hypothesized that 6-mercaptopurine may inhibit in-stent restenosis in a Nur77-dependent manner. The data presented in the present study demonstrate that enhancement of the activity of Nur77 with 6-mercaptopurine protects against smooth muscle cell proliferation and prevents lesion formation in a mouse model. These data support consideration of Nur77 as a target for the prevention and treatment of in-stent restenosis.