TGFβ Affects Collagen Cross-Linking Independent of Chondrocyte Phenotype but Strongly Depending on Physical Environment

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ABSTRACT

Transforming growth factor beta (TGFβ) is often used in cartilage tissue engineering to increase matrix formation by cells with various phenotypes. However, adverse effects of TGFβ, such as extensive cross-linking in cultured fibroblasts, have also been reported. Our goal was to study effects of TGFβ on collagen cross-linking and evaluating the role of cellular phenotype and physical environment. We therefore used four different cell populations in two very different physical environments: primary and expanded chondrocytes and fibroblasts embedded in alginate gel and attached to tissue culture plastic. Matrix production, collagen cross-linking, and α-smooth muscle actin (αSMA) were analyzed during 4 weeks with or without 2.5 ng/mL TGFβ2. TGFβ2 did not affect collagen deposition by primary cells. In expanded cells, TGFβ2 increased collagen deposition. Chondrocytes and fibroblasts in monolayer produced more collagen cross-links with TGFβ2. In alginate, primary and expanded cells displayed an unexpected decrease in collagen cross-linking with TGFβ2. αSMA was not present in alginate cultures and barely upregulated by TGFβ2. Organized αSMA fibers were present in all monolayer cultures and became more pronounced with TGFβ2. This study demonstrates that the physical environment determined by the substrate used co-determines the response of cells to TGFβ. The presence of mechanical stress, determined with αSMA-staining, is probably responsible for the increase in collagen cross-linking upon addition of TGFβ.

INTRODUCTION

TGFβ PLAYS AN IMPORTANT role in cartilage repair. In tissue engineering studies, TGFβ is often used to re-differentiate expanded chondrocytes. Expansion, needed to obtain sufficient cell number for tissue engineering purposes, results in dedifferentiation and a more fibroblast-like phenotype of the cells. In combination with other growth factors and in a serum-free culture medium, TGFβ induces proteoglycan production and collagen type II gene expression by expanded chondrocytes.[1–3] In addition, adenoviral overexpression of TGFβ results in constitutive collagen type II expression by expanded chondrocytes.[4]

Next to redifferentiation of expanded chondrocytes, TGFβ is also used to induce a chondrogenic phenotype in bone marrow–derived mesenchymal stem cells resulting in the deposition of typical cartilage proteins such as aggrecan and gene expression of collagen type II.[5,6] Besides effects on cell phenotype, TGFβ induces production of cartilage matrix proteins. TGFβ leads to an increase in proteoglycan synthesis and content in healthy murine articular cartilage and protects against proteoglycan depletion in murine

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experimental osteoarthritis (OA).[7,8] Addition of TGFβ to chondrocytes in culture leads to various results: stimulation of proteoglycan and collagen production,[9–12] but also inhibition of proteoglycan deposition.[13,14] These results indicate differential effects of TGFβ1 and TGFβ2, which most likely depend on cell type, cell source, and culture substrate, but not necessarily on the isoform.[15] We have recently demonstrated that TGFβ downregulates the formation of collagen pyridinoline cross-link formation by articular chondrocytes in alginate culture.[13]

In contrast to the positive effects of TGFβ in cartilage repair, TGFβ is also the most potent inducer of unwanted collagen gene expression by fibroblasts resulting in excessive tissue deposition and eventually fibrosis.[16] Fibrosis is characterized by a high level of the pyridinoline collagen cross-links[17] and the formation of these cross-links is induced by the presence of either of the three isoforms of TGFβ.[18] Fibrosis is also seen *in vivo* in articular joints where injection of TGFβ leads to fibrosis of the synovium.[19] In addition, blockade of the TGFβ-pathway in murine experimental OA prevents against synovial fibrosis.[8]

Taken together, TGFβ has differential effects on cell behavior and extracellular matrix production and cross-linking. It seems that the effect is depending on cell phenotype, the physical environment determined by culture set-up, or a combination of these factors. The contribution of these factors to the differential effects of TGFβ has to be elucidated to be able to apply TGFβ to improve cartilage tissue engineering. This information will be useful for the choice of biomaterials to be used for cartilage tissue engineering. Our goal was to compare the effect of TGFβ on collagen biochemistry and especially collagen cross-linking in different cells cultured in different physical environments. To evaluate the influence of cell phenotype, we used different cells ranging from differentiated chondrocytes to fibroblasts. To evaluate the effect of the physical environment, the cells were cultured in two different environments: attached to plastic in monolayer, where they are under continuous cellular contractile stress, or embedded in alginate gel, where they cannot adhere and thus are free from mechanical loading.

EXPERIMENTAL PROCEDURES

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged 6–12 months. Primary fibroblasts were obtained from split skin biopsies of the skin covering the bovine joints. To isolate chondrocytes and fibroblasts, both the full-thickness slices of noncalcified articular cartilage and the split skin biopsies were subjected to pronase (2 mg/mL; Sigma, St. Louis, MO) digestion for 2 h followed by overnight collagenase B (1.5 mg/mL; Roche Diagnostics, Almere, The Netherlands) digestion. Depend-

ing on the culture conditions, the primary P0 or expanded P3 cells were either resuspended in 1.2% (w/v) low viscosity alginate (Keltone, Merck, San Diego, CA) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/mL to make alginate beads or cultured as monolayer on plastic in a concentration of 7500 cells/cm^2. Beads were made as described previously.[20] Beads were cultured in 75 µL/bead and monolayers in 2 mL/monolayer Dulbecco's modified Eagle's medium (DMEM) high glucose (GibcoBRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (Gibco-BRL), 50 µg/mL L-ascorbic acid 2-phosphate (Sigma), 50 µg/mL gentamicin, and 1.5 µg/mL fungizone (both Gibco-BRL). Cells were cultured with or without 2.5 ng/mL transforming growth factor beta2 (TGFβ2). TGFβ1 and TGFβ2 have similar effects on matrix production.[15] TGFβ3 is an important factor in chondrogenic differentiation of mesenchymal stem cells and less used when primary chondrocytes are cultured.[21,22] No differences were found in our previous study about the three isoforms and their effect on collagen cross-linking in fibroblast cultures.[18] Therefore, TGFβ2 was chosen based on previous results.[13] Culture medium was replaced three times a week.

Biochemical assays

Alginate beads or monolayers were digested overnight at 56°C in papain buffer (200 µg/mL papain in 50 mM ethylene diamintetraacetate (EDTA) and 5 mM L-cystein). The amount of DNA in each RNAse (Sigma)–pretreated papain-digested sample was determined using ethidium bromide (GibcoBRL) dye with calf thymus DNA (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP; lysylpyridinoline, LP) was performed by the methods of Bank et al.[23,24] The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

Gene expression analysis

For total RNA isolation, alginate beads were dissolved in 150 µL/bead 55 mM sodium citrate acid. Cell pellets of the alginate beads and monolayers were suspended in 1000 µL RNA-Bee™ (TEL-TEST, Friendswood, TX), and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). For glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*—forward: GTCAACGGATTTGGTCGTATTGGG; reverse: TGCCATGGGTGGAATCATATTGG; probe: Fam-TGGCGCCCCAACCAGCC-Tamra) and SRY-box containing

gene 9 (*SOX9*—forward: CAACGCCGAGCTCAGCA; reverse: TCCACGAAGGGCCGC, Fam-TGGGCAAGCTCTGGAGA CTTCTGAACG-Tamra), each cDNA sample was amplified using specific primers and specific probe (Eurogentec, Seraing, Belgium). Amplifications were done as described previously.[13] Data were analyzed using Sequence Detector Version 1.7 (Applied Biosystems, Foster City, CA) software and normalized for *GAPDH* expression.

Immunohistochemical staining for αSMA, type I collagen, and type II collagen

Staining for the presence and organization of αSMA was performed after 1 week of culture of primary chondrocytes and fibroblasts, to examine differences in mechanical stress between the monolayer and alginate beads. The additional effect of TGFβ2 on the organization of the αSMA fibers was also investigated. The presence of collagen types I and II was investigated after 4 weeks of culture. For all three stainings, three alginate beads were dissolved in 55 mM sodium citrate acid to prepare cytospins. Cells in monolayer were cultured on glass culture chambers (BD Biosciences, San Jose, CA) to stain them immunohistochemically. Cells with surrounding pericellular matrix from alginate beads were centrifuged on glass slides and fixed in cold acetone monolayers in cold 70% ethanol. The samples were incubated with monoclonal antibodies against αSMA (clone number 1A4, Sigma), type I collagen (ab6308; Abcam, Cambridge, UK), or type II collagen (1:100, II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) at room temperature for 1 h. This was followed by incubation with link and label from the link-label kit (BioGenex, San Ramon, CA) for 30 min. Freshly prepared neo-fuchsin substrate was used to achieve staining.

The collagen staining was scored for the number of cells positive for collagen types I and II per 100 cells in four fields of sight per specimen, resulting in a percentage of positive cells. An arbitrary scoring system from 1 to 5 was used, with 1 representing less than 10% of the cells positive, 2 representing 10–40% positive, 3 representing 40–60% positive, 4 representing 60–90% positive, and 5 representing more than 90% of the cells positive. A cell was considered positive when the matrix around the cell or the cytoplasm was stained pink.

Data analyses

The experiments were performed three times with three samples of seven beads or with three monolayers per experimental condition for biochemical analyses and one sample of 10 beads or one monolayer per experimental condition for gene expression analysis. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) software. All data are presented as mean ± standard deviation. Control groups without TGFβ2 and groups supplemented with TGFβ2 were compared with a Kruskall–Wallis *H*-test with a *post hoc* Mann–Whitney *U*-test. The effect of adding TGFβ2 in the alginate bead condition versus the monolayer for each cell type was compared with a two-way ANOVA with a *post hoc* Bonferroni test. The relative difference between the control group (set at 100%) and the TGFβ2 group was used for these calculations.

RESULTS

Cell features

The amount of DNA was determined at day 0 and after 28 days of culture to be able to compare matrix deposition between the conditions and examine the effect of the culture environment on cell proliferation. At day 0, 0.12 ± 0.03 μg DNA/bead and 0.23 μg DNA/monolayer was present. In all conditions, the amount of DNA increased or remained stable after 28 days of culture. This indicates that there was no cell death and even cell proliferation in most of the conditions. Overall, addition of TGFβ2 increased the total DNA amount ($p < 0.005$), although not always significantly and with exception of fibroblasts in alginate beads (Fig. 1A).

The total amount of isolated RNA was 101 ± 11 ng RNA per bead and $24,890 \pm 4904$ ng RNA per monolayer. This increased with the presence of TGFβ2 in every condition ($p < 0.05$) except for the fibroblasts in beads. Here, the amount of RNA was not significantly changed in the presence of TGFβ2.

Collagen matrix deposition

Addition of TGFβ2 to the primary chondrocytes in alginate beads had no effect on collagen deposition. A low amount of collagen was deposited in cultures of dedifferentiated chondrocytes, as well as primary and dedifferentiated fibroblasts in alginate beads. Addition of TGFβ2 increased the collagen deposition in P3 chondrocytes and fibroblasts in alginate beads ($p < 0.005$). Collagen deposition by primary chondrocytes and fibroblasts on plastic was high but not influenced by the presence of TGFβ2. Low amount of collagen was deposited in dedifferentiated P3 chondrocytes and P3 fibroblasts on plastic where addition of TGFβ2 increased collagen deposition ($p < 0.005$, Fig. 1B).

Matrices deposited by primary and expanded chondrocytes and fibroblasts in alginate beads had an average of 0.22 HP cross-links per collagen molecule. Matrices deposited by chondrocytes on plastic had a similar number of cross-links per collagen molecule in the control condition. Matrices deposited by fibroblasts on plastic had a relatively low number of HP cross-links per collagen molecule in the control condition. Addition of TGFβ2 to the culture medium resulted in a decrease of collagen cross-links in all alginate bead cultures and an increase of collagen cross-links in all cultures on plastic, independent of cell type ($p < 0.001$ for alginate bead vs. plastic, Fig. 1C).

Type of collagen produced

Chondrocytes cultured in alginate beads deposited mainly type II collagen (arbitrary score 4.25 ± 0.5, Fig. 2A) and less type I collagen (score of 1.5 ± 0.6, Fig. 2B) as determined by immunohistochemistry. Expanded chondrocytes in alginate beads had a trend toward more type I collagen than primary chondrocytes (score of 2 ± 0.5, not significant). However, after 28 days, type II collagen was the main type of collagen deposited (score 3.25 ± 1, $p < 0.05$ vs. collagen type I production) by the expanded chondrocytes as well. The primary and expanded fibroblasts in beads deposited almost no type II collagen and more type I collagen than the chondrocytes. Addition of TGFβ2 only increased the type II collagen deposition (score of 3.75 ± 0.5, $p < 0.05$ vs. control without TGFβ2) in the expanded chondrocytes, and no effect of TGFβ was seen in the other alginate bead conditions. Chondrocytes cultured on plastic deposited very little type II collagen (score 1.5 ± 0.6) and more type I collagen (score 3.75 ± 0.5) than the chondrocytes cultured in alginate beads. Dedifferentiated chondrocytes and both primary as well as expanded fibroblast deposited only type I collagen (score 4.75 ± 0.5, Fig. 2C) and almost no type II collagen (score 0.5 ± 0.6, Fig. 2D). After TGFβ addition, a trend toward more type II collagen was present in the primary chondrocytes on plastic (1.25 ± 0.5, not significant). No effect of TGFβ was seen in the other conditions.

Phenotype of the cells

After culture for 28 days, the gene expression for *SOX9* was examined to provide an indication of the phenotype of the cells. *SOX9* gene expression was the highest in primary chondrocytes cultured in alginate beads (P0 chondro beads). Chondrocytes cultured for three passages and subsequently in alginate beads (P3 chondro beads) and the primary chondrocytes cultured on plastic (P0 chondro monolayer) also expressed some *SOX9*, but not as much as the primary chondrocytes in alginate beads. The passage 3 chondrocytes on plastic and all the fibroblast conditions almost did not express the *SOX9* gene (Fig. 3). Addition of TGFβ2 reduced *SOX9* expression in the alginate bead and monolayer cultures of primary chondrocytes ($p < 0.005$) but increased it in the expanded chondrocytes in alginate beads ($p < 0.005$).

αSMA organization

In alginate beads, none of the chondrocytes had detectable αSMA, but some fibroblasts were positive for αSMA (Fig. 4A and C). In monolayer culture, positive αSMA staining without specific stress fibers was detectable in the majority of the chondrocytes (Fig. 4E). All fibroblasts were positive for αSMA as shown with a red staining, although also no clear stress fibers were visible (Fig. 4G). Addition of TGFβ2
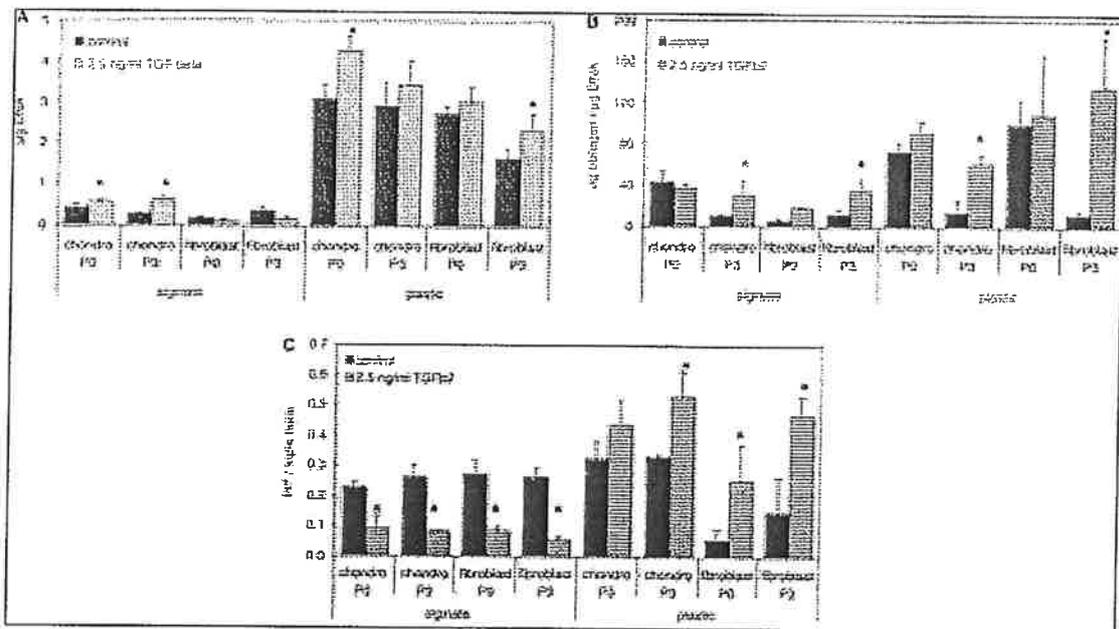

FIG. 1. Effect of 2.5 ng/mL TGFβ2 on **(A)** DNA content, **(B)** collagen deposition, and **(C)** number of collagen cross-links in cultures of primary (P0) and expanded (P3) chondrocytes and fibroblasts cultured in alginate beads or as monolayer on plastic for 28 days ($n = 6$). Asterisk (*) indicates significant difference ($p < 0.005$) from control without TGFβ2. Data are shown as mean ± SD.
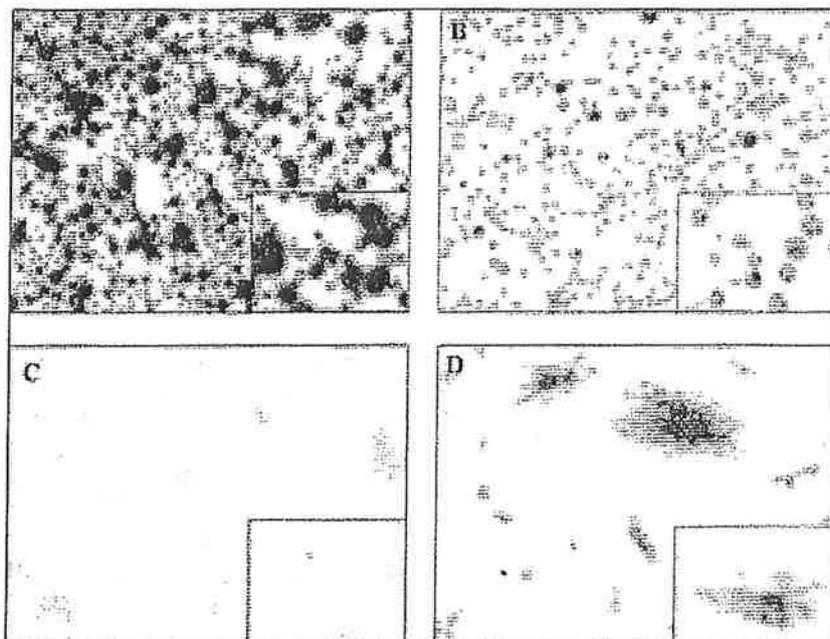
FIG. 2. Immunohistochemical staining for collagen type II (A, C) and collagen type I (B, D) of chondrocytes cultured for 28 days in alginate beads (A and B, here shown as cells with pericellular matrix on glass slides) and as monolayer on plastic (C, D). Positive cells are shown in red. Magnification is 100×. Color images available online at www.liebertpub.com/ten.

to chondrocytes and fibroblasts in alginate beads resulted in more αSMA and the intensity of staining was higher. However, no intracellular stress fibers of αSMA were visible (Fig. 4B and D). When the cells (chondrocytes and fibroblasts) were cultured on plastic, the presence of TGFβ2 induced αSMA with clearly visible organized stress fibers in all the cells as shown in Figure 4F and H.
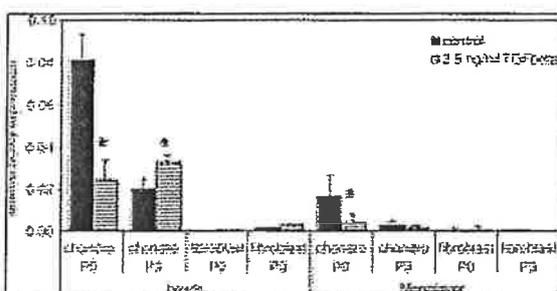

FIG. 3. Relative gene expression of *SOX9* by primary (P0) and expanded (P3) chondrocytes and fibroblasts cultured in alginate beads or in monolayer on plastic for 28 days (n = 6). The gene expression analysis was used to determine the phenotype of the cells after culture. Asterisk (*) indicates significant difference (p < 0.005) from control without TGFβ2. Data are shown as relative expression ± SD.

DISCUSSION

This study demonstrates that the effect of TGFβ on cross-linking of collagen is mostly determined by the physical environment determined by the culture substrate of the cells and less by the cell phenotype or the type of collagen produced. When chondrocytes are cultured in alginate beads, TGFβ2 leads to less collagen cross-links in the deposited matrix. In the monolayers on plastic on the other hand, addition of TGFβ2 leads to more collagen cross-links in the deposited matrix.

The results elucidate the discrepancy in our previous results. A study with skin fibroblast cultured on plastic showed that TGFβ increased collagen cross-link formation.[18] However, in a study with differentiated chondrocytes in alginate gel, we surprisingly found an inhibition of collagen cross-link formation by TGFβ.[13] These studies differed in cell type used as well as physical environment used. In the present study eight different combinations of cell type and physical environment were compared to evaluate what determines the effect of TGFβ on collagen cross-link formation.

One of the main physical differences between plastic and alginate culture is the contractile stress on the cell. The culture systems used were investigated for their effect on the contractile stress on chondrocytes and fibroblasts by staining the cells for the presence and organization of αSMA. It has previously been shown that when the culture
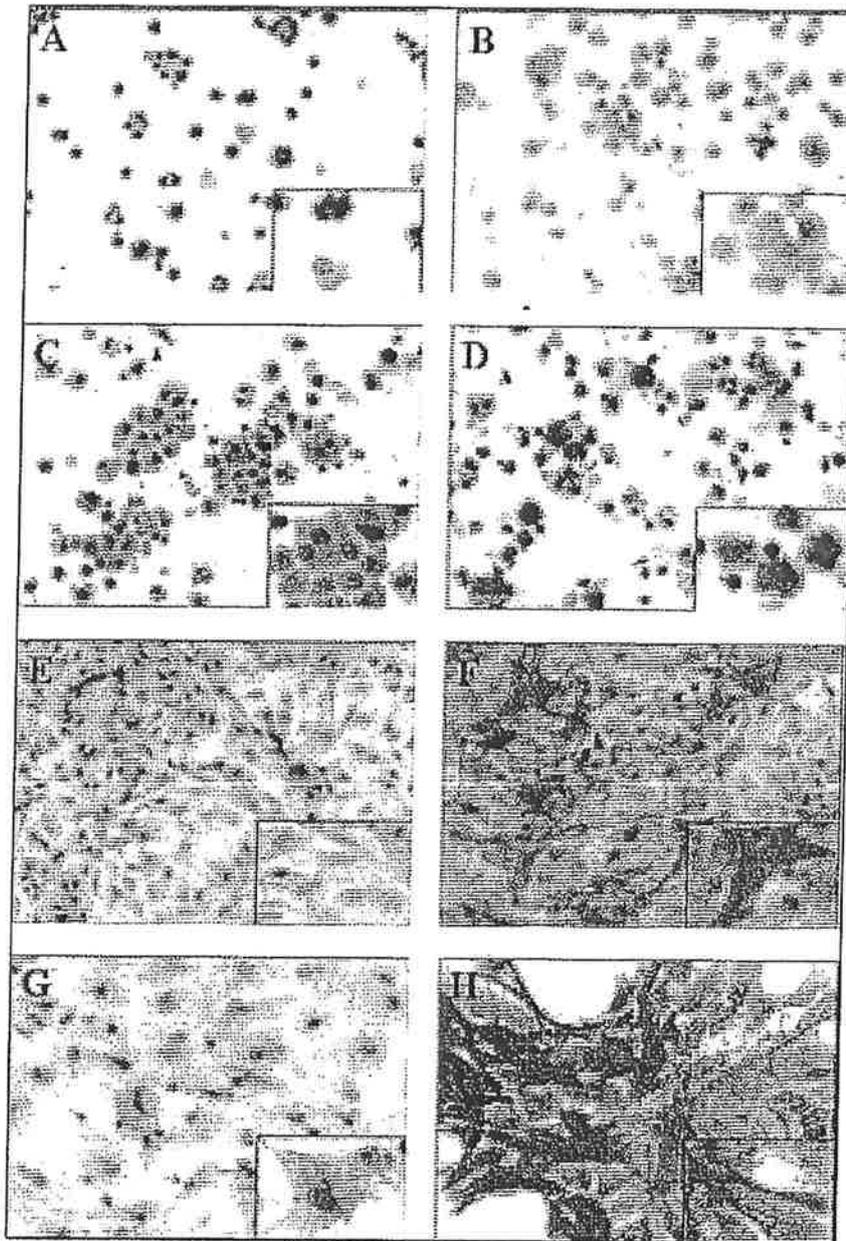
FIG. 4. Immunohistochemical staining for α-smooth muscle actin (αSMA) of primary P0 chondrocytes (**A, B, E, F**) and fibroblasts (**C, D, G, H**) cultured in alginate beads (**A–D**, here shown as cells with pericellular matrix on glass slide) or in monolayer on plastic (**E–H**) for 28 days. αSMA shown in red is upregulated in response to TGFβ2 (**B, D, F, H**) although not organized in spindles in the cells cultured in alginate beads (**B, D**). Cells cultured in monolayer in the presence of TGFβ2 have organized αSMA fibers (**F, H**). Magnification is 200× with a 1000× inset. Color images available online at www.liebertpub.com/ten.

substrate exerts force on a cell, more αSMA is present in the cell.[25-27] Inhibition of αSMA assembly reduces contraction of a collagen–glycosaminoglycan scaffold, indicating the importance of αSMA in the interaction between the cell and its environment.[28] This corresponds to our findings where αSMA is absent or poorly organized in the cells cultured in alginate beads, but clearly present in attaching cells cultured on plastic. It also confirms that cells cultured on plastic are

under continuous contractile stress as a result of anchorage to the substrate and cell–cell contact. Cells cultured in alginate beads do not experience this stress because of less or no cell–cell contact and the absence of anchorage to the culture substrate. In the culture systems we used, especially the monolayer on plastic, it is difficult to uncouple cell–cell contact and anchorage to the culture substrate and their effect on the organization of αSMA. The effect of other differences between alginate and plastic, like for instance differences in nutrient diffusion or cell viability, cannot be completely excluded. Considering the cell viability, the amount of DNA did not decrease from days 0 to 28 for any alginate condition. Together with the presence of RNA after 28 days of culture, this indicates the absence of cell death also in the fibroblasts cultured in alginate beads. We therefore believe that the effect seen in the alginate beads depends on the culture environment and not on cell viability and therefore a selection in the type of collagen deposited or phenotype. We can conclude that the culture environment greatly influences the effect of TGFβ on the collagen matrix.

To study the relation with cell phenotype, we determined gene expression for *SOX9* and immunohistochemical staining for collagen types I and II. *SOX9* gene expression and the type of collagen deposited are related to the phenotype of the cells but not to the effect of TGFβ on collagen cross-linking: in case of the alginate bead cultures, no collagen type II was present in the fibroblast cultures. Here, addition of TGFβ decreased the number of collagen cross-links. Collagen type II was also present in the P0 chondrocytes cultured on plastic. The deposition of collagen type II increased slightly together with an increase in collagen cross-links with the presence of TGFβ. On the other hand, our data indicate that the cellular contractile stress on the cells, as determined by αSMA production and organization, is related to the effect of TGFβ. We conclude from these data that the effect of TGFβ on collagen cross-linking is dependent on the physical environment of the cells. It was recently found that αSMA is a mechanosensitive protein that is recruited to stress fibers under high tension. This recruitment allows cells to exert more stress on the culture scaffold.[29] TGFβ further upregulates αSMA deposition and condensation. Previously, it was found that TGFβ induces αSMA in fibroblasts.[30,31] More recently, it is also seen in chondrocytes,[28,32] which corresponds with our findings, that cells incubated with TGFβ have more αSMA staining. We hypothesis that in conditions of high tensile stress on the cell, the presence of TGFβ further increases αSMA organization and leads to collagen cross-link formation to better resist the stress. Moreover, addition of TGFβ does not inevitably has to result in fibrosis. If tensile stress is prevented, TGFβ can be used to increase collagen and proteoglycan production without leading to fibrosis. Therefore, the intended effect of TGFβ in cartilage repair will depend on environmental conditions, such as mechanical environment and the scaffold used. Because collagen cross-linking has important implications

on mechanical properties of the repair tissue, this should be seriously considered when choosing a scaffold in tissue engineering procedures where TGFβ is often used to stimulate tissue formation.

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