The functional state of endothelial cells (quiescent or angiogenic) goes along with the expression of specific small leucine-rich repeat proteoglycans (SLRPs). Resting endothelial cells in large blood vessels as well as confluent endothelial cells in vitro do not synthesize decorin, but they express biglycan, which becomes up-regulated when endothelial cells start to migrate (1). Similar observations were made in EA.hy 926 cells, a cell line derived from human umbilical vein endothelial cells (2). However, when endothelial cells form capillaries in collagen lattices, they express decorin (1). This could be observed in vivo in human capillary endothelium during inflammation-induced angiogenesis, as well as in vitro in a co-culture model with EA.hy 926 cells and rat fibroblasts grown on a floating collagen lattice to mimic the situation during angiogenesis in vivo (2).

Decorin is the most well known member of the SLRP family. These small proteoglycans are characterized by core proteins containing several leucine-rich repeat motifs flanked by cysteine-rich clusters. As proteoglycans they are linked to at least a single glycosaminoglycan chain. Decorin is almost ubiquitously expressed and has been implicated in the control of collagen fibril formation and the regulation of TGF-β activity (for review, see Refs. 3 and 4). More recently it has been shown to influence directly the behavior of several different types of cells. For example, it can interact with members of the ErbB receptor family on tumor cells and lead to a more differentiation phenotype of these cells (5, 6). In endothelial cells decorin induction initiates the alignment of cells in cord-like structures on top of a collagen matrix or leads to the formation of endothelial cell-lined cavities inside the collagen matrix (2). These morphological changes are accompanied by an induction and activation of specific matrix metalloproteinases (MMP-1, MMP-2, MMP-9, and MMP-14) (7). In addition, it could be shown that endothelial cells that synthesize decorin escape apoptosis (2). All these changes were only observed in decorin-synthesizing cells, independent of the mode of decorin induction. Thus decorin production could be induced either by co-culture with fibroblasts or by infection of the cells with a replication-deficient adenovirus containing the human decorin cDNA, indicating that decorin is instrumental in the formation of capillary-like structures in collagen lattices.

The physiological regulation of decorin synthesis in different cell types is still a matter of discussion, as the same cytokine can have opposite effects on decorin synthesis; TGF-β has been shown to up-regulate decorin in mesangial cells but to down-regulate the proteoglycan in most other cell types (8, 9). Besides TGF-β, several other cytokines have been implicated in the regulation of decorin synthesis on a transcriptional as well as on a translational or posttranslational level (IL-1, tumor necrosis factor (TNF)-α, TGF-α, epidermal growth factor, etc.; Ref. 4). However, no cytokine that has been associated with angiogenesis (VEGF, basic fibroblast growth factor, TNF-α, IL-1, and TGF-β) could be identified as an inducer of decorin synthesis in endothelial cells (2). As decorin induction in endothelial cells in vivo is observed during inflammation (2, 10) cytokines released during this process are potential candidates for its induction. One of these cytokines is IL-6. It can react with a variety of cells by a trimeric receptor complex that consists of a membrane-bound
for inhibition studies with rhodocetin the integrin inhibitor was added in the indicated concentrations together with the interleukins, and the rest of the experiment was carried out as described.

For the analysis of different matrices tissue culture plastic was coated with fibrillar collagen, acid-soluble collagen, and collagen type VI (1 mg/ml, prepared according to Trueb and co-workers (17)). In addition, cell culture dishes were coated with growth factor-reduced Matrigel (BD Biosciences), which was additionally extracted over night with serum-free medium, before EA.hy 926 cells were plated. The cells were stimulated, metabolically labeled, and harvested as described above. Immunohistochemistry—Immunostaining of collagen gels was performed as described previously (2), except that in addition to chondroitin ABC lyase (2 h, 37 °C) in 5% bovine serum albumin, 10% goat serum (Sigma) in phosphate-buffered saline, proteinase XXIV (0.005%, 10 min, 37 °C, Sigma) was used to unmask epitopes.

**RESULTS**

**Induction of Decorin mRNA in Endothelial Cells by IL-10 and IL-6**—In previous experiments we excluded a large number of growth factors and cytokines associated with angiogenesis as inducers of decorin synthesis in endothelial cells (2). In this study IL-6 and IL-10 were tested because both are released during inflammation in vivo. In addition, IL-10 has been shown previously to induce decorin mRNA synthesis in fibroblasts (12). Semiquantitative reverse transcription-PCR showed that IL-10 can dose dependently increase decorin mRNA production in endothelial cells in a concentration range between 12.5 and 50 ng/ml. The expression of decorin mRNA doubled after 8–12 h and reached a maximum at 16 h (n = 3, result not shown). Therefore, mRNA was harvested after 16 h in the following experiments. In several experiments (at least n = 3) IL-10 (50 ng/ml) led to a 5-fold increase in amplification, while higher doses had no further effect or reduced specific mRNA synthesis (Fig. 1A). IL-6 also caused a 5-fold increase in decorin mRNA levels, but the highest decorin expression was achieved with 10 ng/ml. Higher concentrations of IL-6 reduced decorin mRNA levels (Fig. 1B). Using both cytokines at optimal concentrations a stronger effect on decorin mRNA expression was observed (Fig. 2). These results indicate that IL-10 and IL-6 may influence decorin mRNA synthesis by at least partially different pathways. The addition of cytokines to endothelial cells cultured on a fibrillar collagen matrix led to a reduced increase in the expression rate (13–29%) compared with uncoated dishes (Fig. 2). This reduction is very likely due to increased unspecific binding of cytokines to the collagen coat compared with the plastic surface.

**Decorin Protein Synthesis**—Immunohistochemical studies showed that EA.hy 926 cells in collagen gels start to synthesize decorin protein after stimulation with IL-10 and IL-6 (Fig. 3). After 6 days of culture treated cells with IL-10 (Fig. 3A) or the combination of IL-10 and IL-6 (result not shown) showed tubular structures surrounded by decorin, while untreated cells neither showed any staining for decorin nor any formation of capillary-like structures (Fig. 3B). A comparable effect due to IL-6 stimulation was temporally delayed (Fig. 3C). Thus decorin core protein was detectable at day 12 of the incubation period.
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**Fig. 1.** Dose response of IL-10 and IL-6 on the decorin mRNA expression in EA.hy 926 cells. IL-10 (A) or IL-6 (B) was added at different concentrations to $1.5 \times 10^5$ EA.hy 926 cells grown on plastic. After 16 h RNA was isolated, and a semiquantitative real time reverse transcription-PCR was performed. Augmentation of decorin (DCN) mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression and compared with control samples (ΔΔCt method). The values ± S.E. in each panel are the averages of at least three independent experiments. The maximal augmentation was achieved with 50 ng/ml IL-10 and 10 ng/ml IL-6, respectively. The solid line shows the linear regression between 0 and 50 ng/ml IL-10 and 0 and 12.5 ng/ml IL-6, respectively.

**Fig. 2.** Influence of combined IL-10/IL-6 application on the expression of decorin mRNA in EA.hy 926 cells. Samples were processed as described in Fig. 1. The stimulation of decorin (DCN) mRNA synthesis could be enhanced further by combined IL application compared with single application of IL-10 (50 ng/ml) and IL-6 (10 ng/ml), respectively. Black bars, plastic dishes; gray bars, collagen-coated dishes. Collagen coating of cell culture plastic reduced the effect of the cytokines on decorin stimulation.

(Fig. 3D). These results indicate that the two cytokines can induce decorin synthesis in this angiogenesis model in cell culture. In the next series of experiments we attempted to immunoprecipitate decorin from the medium and the cell layer of endothelial cells growing on plastic dishes as used in routine cell culture. Even when these cells were actively synthesizing decorin mRNA as shown in Fig. 1 no decorin could be found by immunoprecipitation (Fig. 4A). Therefore, we tested various conditions (different time points, concentrations, and types of labeled metabolites; different concentrations and combinations of interleukins; and fibroblast conditioned medium), but translation of decorin mRNA into core protein and posttranslational modification to proteoglycan were not observed. Only when EA.hy 926 cells were seeded on a collagen type I matrix similar to the matrix in the angiogenesis model synthesis of decorin could be detected (Fig. 4A). An increased effect of the combination of IL-10 and IL-6 was also observed on the level of protein...
FIG. 5. Immunoprecipitations of decorin from IL-6- and IL-10-treated primary human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated as described for EA.hy 926 cells in Fig. 4A. Cells either cultured on plastic (lanes 1, 2, 6, and 7) or on collagen-coated dishes (lanes 3, 4, 8, and 9) were treated with a combination of IL-10 (50 ng/ml) and IL-6 (10 ng/ml) for 16 h (lanes 2, 4, 7, and 9). Decorin was immunoprecipitated, and half of each sample was digested with chondroitin ABC lyase (lanes 6–10) to show the core protein. Radiolabeled decorin from human fibroblasts was applied as positive control (lanes 5 and 10). Only primary endothelial cells cultured on a collagen matrix synthesize decorin in response to IL-10/IL-6.

synthesis (Fig. 4A). To test whether protein synthesis like mRNA synthesis is dependent on the amount of interleukin in the medium, dosages from 25 to 100 ng/ml IL-10 and from 5 to 20 ng/ml IL-6 were added to endothelial cells growing on a collagen matrix. Immunoprecipitations of metabolically labeled decorin showed that there is no dose dependence on the amount of interleukin added. Under conditions when cells are cultured on fibrillar collagen and a 5- to 6-fold increase in the mRNA level is observed, translation is induced. Higher concentrations of interleukins, which do not lead to a further mRNA increase on plastic (see Fig. 1), still induce the same increase in decorin translation on collagen-coated dishes. These results demonstrate that the collagen matrix has two effects on decorin induction: a positive effect on translation of decorin (Fig. 4B) and a negative effect on mRNA transcription because of sequestration of the interleukins (Fig. 2). In addition, these results prove that the interaction of endothelial cells with their surrounding matrix is of crucial importance for decorin protein synthesis in endothelial cells.

To investigate whether this behavior is specific for EA.hy 926 cells, primary human umbilical vein endothelial cells were stimulated with IL-6 and IL-10 under similar conditions. Immunoprecipitations of metabolically labeled decorin showed that also primary cells are dependent on the collagen matrix for decorin protein synthesis (Fig. 5).

Matrix Requirements for Decorin Synthesis—To further investigate the requirements for the stimulation of protein synthesis different types of coatings were compared. Neutralized collagen type I from rat tail tendon or from calf skin (2), which forms a fibrillar matrix, supported decorin synthesis. However, a coat of the same collagen in its acid-soluble form had no effect (Fig. 6A). Also a coat of monomeric collagen type VI did not have any effect. However, growth factor-reduced Matrigel, which by itself had no effect on decorin synthesis, could support interleukin-stimulated decorin translation (Fig. 6A). These data show that the interaction with a fibrillar matrix of collagen type I or a network-forming matrix of collagen type IV, laminin, and entactin is necessary for the induction of decorin protein synthesis.

To analyze the cellular interactions, antibodies to α1 and α2 integrins were applied to endothelial cells plated on a fibrillar collagen type I matrix. In previous experiments (using 50 μg/ml–0.5 ng/ml) a concentration of antibody was determined that does not lead to cell detachment and apoptosis within 24 h (result not shown). Addition of 5 ng/ml antibodies directed against α1 or α2 integrin subunits at the time of cell seeding and during incubation with the cytokines led both to a reduction in decorin synthesis (Fig. 6B) compared with culture conditions without antibodies or isotypic control antibodies. Antibodies to α1 integrin inhibited decorin synthesis by 70%, while antibodies to α2 integrin led to 85% inhibition compared with the control. These results were corroborated by incubation with the integral inhibitor rhodocetin, which abolished decorin synthesis completely at a concentration that was shown previously to inhibit cell adhesion by about 50% (15). Rhodocetin blocks collagen binding of α2β1 integrin in a arginine-glycine-aspartic acid-independent manner and presumably antagonizes α2β1 integrin signaling by binding to the A-domain of the α2 subunit proximal to the collagen binding site (18). These data indicated that α2β1 integrin as well α1β1 integrin is involved in the induction of decorin translation in endothelial cells.

DISCUSSION

This study shows that IL-6 and IL-10, two cytokines that are released during inflammation and wound healing, can induce decorin synthesis in endothelial cells. Decorin synthesis has been observed previously in endothelial cells in different cell culture models of angiogenesis (2, 19) and also in vivo (2, 10). Two independent in vivo investigations showed that the inflammatory component is of special importance, as decorin was only found in newly formed capillaries in granulomatous tissue or in neovessels in temporal arteries but not in capillaries of the ovary in different phases of follicle and corpus luteum formation.

Both IL-6 and IL-10 are released during inflammation and wound healing. The importance of IL-6 has been emphasized...
we show that it is also functional on endothelial cells. As 
showing that the role of collagen-binding integrins
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a positive feedback loop is initiated. This positive feedback loop increases the expression of decorin, which in turn leads to the inhibition of fibroblast proliferation. The interaction between fibroblasts and endothelial cells is mediated by collagen-binding integrins, which facilitate the formation of a functional network of cells. The positive feedback loop is essential for maintaining the balance between proliferation and migration in the tissue.

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Recently by wound healing studies in the IL-6-null mouse. Without challenge the mouse does not show any obvious phenotype, but during wound healing leukocyte infiltration and re-epithelialization as well as angiogenesis and collagen accumulation are reduced (20). In contrast, IL-10 has been associated with angiogenic processes. Thus an increase in angiogenesis was observed in the IL-10-null mouse in the hindlimb ischemia model. This increase in angiogenesis was associated with a prolonged up-regulation of VEGF and increased matrix metalloproteinase activity (21). However, these findings in the constitutive IL-10-null mice cannot rule out that a regulated induction of IL-10 expression during inflammation-induced angiogenesis may be necessary to down-regulate VEGF expression and to induce decorin synthesis in endothelial cells. Furthermore the constitutive lack of IL-10 may induce other mechanisms that are not involved in angiogenesis under normal circumstances.

The influence of IL-6 and IL-10 on the metabolism of proteoglycans has not yet been analyzed in detail. In synovial cells IL-6 increased the synthesis of versican, a large hyaluronan-binding proteoglycan, but changes in other proteoglycans were not observed (22). In chondrocytes the total proteoglycan content was reduced by IL-6 (23). However, the IL-6-null mouse showed a stronger reduction in proteoglycan content in an arthritis model than the wild type mouse (24) indicating that IL-6 has not only inhibitory effects on proteoglycan synthesis. Even less is known about the effect of IL-10. Yamamoto and co-workers (12) found a stimulation of decorin expression in fibroblasts, and van Roon and co-workers (25) described a direct stimulation of cartilage proteoglycans. In this study we show for the first time that IL-6 and IL-10 both induce decorin in vascular endothelial cells. The concentrations that are necessary to induce decorin synthesis are similar to the ones used in previous studies (12, 22). These concentrations exceed the concentration found in human plasma. However, plasma concentrations may not reflect the actual concentration of cytokine molecules, which are present close to the endothelial cell surface during inflammation, because both IL-6 and IL-10 can be bound and concentrated by heparan sulfate proteoglycans in the vicinity of or on the surface of endothelial cells (26, 27). The less than additive effect of the combined addition of IL-6 and IL-10 on decorin expression may be due to the fact that both interleukins are using intracellularly the JAK/STAT pathway (19, 28), and both can activate STAT3 and STAT1, which may be limiting for a further increase of decorin transcription. This possibility needs to be investigated.

The importance of the surrounding matrix for the induction of decorin can be clearly seen by the fact that decorin protein synthesis are simultaneously induced during endothelial tube formation. Furthermore we showed by using inhibitory antibodies as well as the integrin inhibitor rhodocetin that integrin signaling in endothelial cells is necessary for decorin protein synthesis. Rhodocetin is a specific snake venom inhibitor specifically directed against the α1β1 integrin. Its effects against tumor invasion and other αβ integrin-integrin-related functions in fibrosarcoma cells have been shown recently (29). Here we show that it is also functional on endothelial cells. As Matrigel contains ligands for the α1β1 and αβ integrins as well, it also could induce decorin translation. Our data emphasizes the role of collagen-binding integrins α1β1 and αβ in angiogenesis that has been revisited recently (30). Previous studies by Senger and co-workers (31, 32) have demonstrated that both collagen-binding integrins play a key role in vessel formation, presumably by cross-talking with VEGF signaling. Our results show that these integrins may regulate neovascularization in yet another way. After the primary induction of angiogenesis by e.g. VEGF endothelial cells may be induced to migrate and degrade the extracellular matrix (MMP-1 and MMP-14 expression). However, when this primary stimulus is reduced the endothelial cells would encounter intact fibrillar collagen. If the contact with fibrillar collagen occurs simultaneously with a cytokine stimulus (IL-6 and IL-10) decorin synthesis would be induced. The newly synthesized decorin again could control collagen fibrillogenesis as well as MMP-1 and MMP-14 synthesis (7), which could lead to further migration into the direction of the stimulus but also to a feedback inhibition of decorin synthesis. These data directly show the complex interactions between matrix/cytokine/matrix-modifying enzymes. They further demonstrate that in a complex process like angiogenesis determination of mRNA expression alone is not enough to predict the synthesis and availability of a functional protein.

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Interleukin (IL)-6 and IL-10 Induce Decorin mRNA in Endothelial Cells, but Interaction with Fibrillar Collagen Is Essential for Its Translation
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