Hyaluronan is a glycosaminoglycan of the extracellular matrix. In tumors and during chronic inflammatory diseases, hyaluronan is degraded to smaller fragments, which are known to stimulate endothelial cell differentiation. In this study, we have compared the molecular mechanisms through which hyaluronan dodecasaccharides (HA12), and the known angiogenic factor, fibroblast growth factor 2 (FGF-2), induce capillary endothelial cell sprouting in a three-dimensional collagen gel. The gene expression profiles of unstimulated and HA12- or FGF-2-stimulated endothelial cells were compared using a microarray analysis approach. The data revealed that both FGF-2 and HA12 promoted endothelial cell morphogenesis in a process depending on the expression of ornithine decarboxylase (Odc) and ornithine decarboxylase antizyme inhibitor (Oaiz) genes. Among the genes selected up-regulated in response to HA12 was the chemokine CXCL1/GRO1 gene. The notion that the induction of CXCL1/GRO1 is of importance for HA12-induced endothelial cell sprouting was supported by the fact that morphogenesis was inhibited by antibodies specifically neutralizing the CXCL1/GRO1 protein product. HA12-stimulated endothelial cell differentiation was exerted via binding to CD44 since it was inhibited by antibodies blocking CD44 function. Our data show that hyaluronan fragments and FGF-2 affect endothelial cell morphogenesis by the induction of overlapping but also by distinct sets of genes.

Angiogenesis, i.e. the formation of new blood vessels, is a central event during several physiological processes, such as embryogenesis and wound healing, as well as during pathological conditions, such as diabetic retinopathy, rheumatoid arthritis, and tumor progression (1). To form new vessels, endothelial cells have to break their junctions with adjacent endothelial cells, migrate, proliferate, and finally re-establish their cell-cell junctions, leading to formation of new capillaries. Stimulation of endothelial cells by angiogenic factors, such as vascular endothelial cell growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), and interactions of adhesive endothelial cell surface receptors with extracellular matrix components are important during angiogenesis; blocking of the integrin αvβ3 interactions to vitronectin caused regression of newly formed capillaries (2).

CD44 is a ubiquitously distributed transmembrane adhesion receptor and a major receptor for the glycosaminoglycan hyaluronan (3, 4). Several studies have reported the presence of CD44 on endothelial cells (5, 6). CD44-hyaluronan interactions have been implicated in inflammation and tumorigenesis (for reviews, see Refs. 7 and 8) and are essential for cellular migration and adherence. Hyaluronan in its native state is a high molecular mass polymer, but during tissue damage, inflammatory diseases, and aggressive forms of tumors, hyaluronan fragments are formed by the action of hyaluronidases and reactive oxygen species (9, 10). Mixtures of hyaluronan fragments of 4–25 disaccharides and hyaluronan fragments of defined size, e.g. hyaluronan dodecasaccharides (HA12), have been shown to induce angiogenesis in chicken chorioallantoic membrane and wounds (11, 12), as well as in cultures of capillary endothelial cells grown in a three-dimensional collagen gel (6).

Hyaluronan and hyaluronan fragments mediate their cellular functions through binding to specific cell surface receptors, such as CD44 and RHAMM (receptor for hyaluronan-mediated motility (13)). Such interactions result in the activation of signaling cascades (13, 14). Hyaluronan fragments have been shown to induce an up-regulation of early response genes (15) and a CD44-mediated activation of protein kinase C, as well as the Raf-1, MEK-1, and ERK1/2 signaling pathway in endothelial cells (16). In an attempt to investigate how hyaluronan fragments affect the differentiation of endothelial cells at the molecular level, we have studied the gene expression profile of endothelial cells in response to HA12 stimulation by using a microarray approach. In addition, the effect of HA12 on the induction of sprout structures of endothelial cells grown in a three-dimensional collagen gel was compared with that of the angiogenic factor FGF-2.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal anti-mouse CXC chemokine antibody (rat IgG2a, Clone 124014) was purchased from R&D Systems.
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(Minneapolis, MN). Antibodies against mouse CD44 (KM114, rat IgG1) were obtained from the hybridoma cell line TIB-242 (American Type Culture Collection, Manassas, VA); antibodies were purified from conditioned media of the hybridoma cell line. Rat IgG3 and rat IgG2 served as isotype controls for antibodies against mouse CXC chemokine CXCL1/ GRO1 and CD44, respectively, and were bought from R&D Systems. Fatty acid-free bovine serum albumin (BSA), gelatin, and fibronectin from human plasma were purchased from Sigma. Fetal bovine serum, trypsin-EDTA, Ham’s F-12 medium, and phosphate-buffered saline without Ca2+ and Mg2+ were purchased from Sigma. Human plasma, Fetal bovine serum, trypsin, collagenase, elastase, hyaluronidase, and saline were purchased from Sigma. Fetal bovine serum, trypsin, collagenase, elastase, hyaluronidase, and saline were purchased from Sigma. RIBI Life Sciences; OSI Luminex ScanArray 4000 scanner, and images, formed by superimposing Cy3 and Cy5 images for each slide, were analyzed using the QuantArray software (PerkinElmer Life Sciences). Statistical analysis of the triplicate data sets was performed using GeneSpring 7.1 (Silicon Genetics). Raw data were normalized by non-linear Lowess normalization. Regulated genes were selected for each time point, based on the average ratio value > 1.8 for up-regulated genes and <0.56 for down-regulated genes. In addition, regulated genes had to be expressed at all three arrays out of three and with a t test value for the ratios within replicates corresponding to probability lower than 0.05. Functional classification of the predominantly regulated genes was performed manually based on searches in NCBI Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi), GeneCards (nciarray.nci.nih.gov/cards/), and NCBI PubMed.

**Real-time PCR**—Total RNAs (2 μg/20-μl reaction volume) at 1, 4, and 12 h of cell culturing were reverse-transcribed (SuperScript II RNase, Invitrogen) to create cDNA templates to quantify the expression profile of some genes also by real-time PCR. The PCR was performed by qPCR™ core kit for SYBR™ Green I (Eurogentec) according to manufacturer’s instructions with some modifications. Briefly, for each PCR reaction, 10 μl of cDNA transcription reaction (corresponding to 100 ng of RNA template) was mixed with gene-specific primer sets (forward and reverse each, 300 ng for the detection of target genes and 200 ng for the detection of GAPDH gene), SYBRGreenI (1/66 000), MgCl2 (3.5 mM), dNTPs (200 μM), Hot Goldstar enzyme (0.025 units/μl), and nuclease-free water (Ambion) to adjust the total reaction volume up to 20 μl. The primers were designed using a Primer Express™ software (Applied Biosystems). PCR reactions were performed in a Chromo 4 real-time PCR machine (MDS Analytical Technologies). The reactions were amplified for 40 cycles at an annealing temperature of 60 °C using an ABI PRISM 7000 sequence detection system (SDS software; Applied Biosystems). The results were exported to Excel, and the relative expression levels of each gene were calculated by a relative standard curve-based method. GAPDH was used as an endogenous control for the relative quantification of the target messages. The primer concentration optimization and the absence of nonspecific products was confirmed by performing dissociation curve analysis, which resulted in single products at specific melting temperatures. For each transcript, a standard curve was run that showed a good real-time PCR efficiency (slope variation: −2.6 to −3.2) in the investigated ranges of the reverse-transcribed cDNA input (0–100 ng of RNA template) with high linear correlation coefficients (r 2).

**Statistical Analysis**—Statistics were carried out using StatView 5.0. Comparisons between two data groups were performed with unpaired Student’s t test. Statistical significance error was set to less than 5%.

**RESULTS**

**Gene Expression Profiling of Capillary Endothelial Cells**—The effect of hyaluronan fragments on the differentiation of endothelial cells growing in three-dimensional collagen gels, in comparison with known angiogenic molecules such as FGF-2, was determined. Capillary endothelial cells were cultured in the absence or presence of 100 μg/ml HA12 or 5 ng/ml FGF-2 for various periods of time, and the length of the tubuli-like structures was measured, as described under "Experimental Procedures." As shown in Table 1A, FGF-2- and HA12-stimulated cells formed about 2- and 1.5-fold longer tubuli-like structures, respectively, when compared with unstimulated cells, peaking after 12 h of stimulation. Based on these data, the gene expression profile in response to HA12 or FGF-2 during different phases of endothelial cell morphogenesis was determined at the molecular level by microarray analysis. RNA was isolated after 1, 4, and 12 h of stimulation by HA12 or FGF-2, and the transcriptional response was evaluated according to the scheme depicted in Fig. 1B. The analysis revealed that several genes were induced or suppressed distinctly by either HA12 or
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For, forward; Rev, reverse.

| Target gene | Primer sequence | Amplicon | T<sub>m</sub> °C | GenBank accession number |
|-------------|-----------------|----------|-----------------|------------------------|
| Has1        | For, 5'-GCCCTCCCTCTCCTCCTGT-3' | 73       | 82              | D82964                 |
| Has2        | For, 5'-TACATGCTGCTACCTCGCTGTTT-3' | 77       | 78              | U96965                 |
| Has3        | For, 5'-TCTAGTGTTAGCATACCAGTTTTT-3' | 78       | 76              | U96968                 |
| CXCL1/GRO1  | For, 5'-AAGAACATCCAGAGCT-TAGT-3' | 69       | 83              | NM_008176              |
| CD44s       | For, 5'-CAGACTCTGTGCCAGTGTTT-3' | 69       | 81              | AJ251594               |
| CD44v10     | For, 5'-TTTCACTGAGCCATTCCGAGAT-3' | 85       | 81              | AJ251594               |
| GAPDH       | For, 5'-GGCTAGTGACCTCGTCCTGT-3' | 171      | 85              | NM_008084              |

*<sup>T</sup><sub>m</sub> (melting temperature), the maximum value for the dissociation curve, which is a useful tool to verify a specific amplicon product.

FGF-2, but there were also genes affected both by HA12 and by FGF-2 (see Supplemental Tables II–IV).

**Genes Regulated by HA12 but Not FGF-2**—Stimulation of mouse brain capillary endothelial cells growing in three-dimensional collagen matrices by HA12 led to the distinct up-regulation of 179 genes and down-regulation of 210 genes (see Supplemental Table II). Some of the most markedly differentially expressed genes, of known or not yet known importance during endothelial cell differentiation, are depicted in Table II. The examination of gene expression ratios between stimulated and unstimulated cells at defined points during endothelial cell morphogenesis revealed a distinct pattern. In the set of up-regulated genes, the CXC chemokine ligand 1 gene (CXCL1; also designated as growth-regulated oncogene 1, GRO1; melanoma growth stimulatory activity, MGSA (20)) was up-regulated more than 3-fold after 1 h of stimulation by HA12 and then returned to baseline expression during the time course of endothelial cell morphogenesis. The encoded protein belongs to the CXC family of chemokines. The mouse CXCL1/GRO1 protein and its human homolog, the CXCL8/IL8 protein, have been implicated in inflammation, proliferation of tumor cells, migration, and angiogenesis (21–23). HA12 also regulated the production of other cytokines. The IL17d gene, encoding interleukin 17D, which stimulates the production of other cytokines including IL6 and IL8 (24), was markedly induced during active sprout formation. A marked suppression of the leukemia inhibitory factor (LIF) gene was observed; LIF belongs to the cytokine IL6 family and has been shown to inhibit endothelial cell proliferation, an important step of angiogenesis (25). It appears that endothelial cells, in response to hyaluronan fragments, secrete chemokines. We found the induction of CXCL1/GRO1, possibly leading to an autocrine stimulation of endothelial cells via CXCR2 receptors, to be particularly interesting and decided to explore this further in relation to HA12-induced endothelial differentiation (see further below).

Among the genes up-regulated at 4 h, there were genes known to play a central role in post-translational events (Abce1) and genes that stimulate entrance into the cell cycle (Fos). Other genes most likely involved in intracellular signaling cascades (Stbx4, Wdr12) were also up-regulated by HA12. The majority of up-regulated genes were seen during active morphogenesis of HA12-stimulated endothelial cells (12 h; Table II and supplemental Table II). Among them, a marked and distinct up-regulation (more than 3-fold induction) was observed of genes encoding the transcription factors Nfyb and Pknox1; Nfyb and Pknox1 stimulate the transcription of various genes such as type 1 collagen (26) and urokinase (27), respectively. Other up-regulated genes encode proteins involved in connections of membrane proteins and the cortical cytoskeleton (Vil2 gene; ezrin); in metabolism (Fhp2 gene; and in fructose biphosphatase 2) and cortical actin binding (Myob1; myosin 1b). Thus, the proteins induced upon HA12 stimulation of endothelial cells have broad roles in cell growth and homeostasis as well as in modulation of extracellular matrix and reorganization of the cytoskeleton.

In addition to the LIF gene, a series of other genes known to be involved in a multitude of cellular events was distinctly suppressed in HA12 stimulated cells, including the ribosome-binding protein 1 (Rbpb1) gene, which is markedly down-
regulated at all stages of endothelial cell differentiation. Other suppressed genes were the integrin \(\beta 7\) gene (\(\text{Itgb7}\)) and laminin \(\gamma 1\) gene (\(\text{Lamc1}\)), which are involved in cell-cell and cell-matrix interactions. During active morphogenesis of endothelial cells, at 12 h of HA12 stimulation, a marked down-regulation of \(\text{Rasa1}\) and \(\text{Rgs 12}\) genes (members of regulator of G-protein signaling family that act as GTPase-activating proteins), as well as of \(\text{Ptpn11}\) and \(\text{Ptpra}\) (protein tyrosine phosphatases), was notable. Furthermore, genes involved in cellular growth and division (\(\text{Ets2}\), \(\text{Ier3}\), \(\text{Ddx5}\)) were also suppressed. Genes Regulated by Both HA12 and FGF-2—Stimulation of capillary endothelial cells with HA12 or FGF-2 led to a total of 93 up-regulated and 308 down-regulated genes in common (Supplemental Table III). The majority of genes were induced during the intensive formation of tubuli-like structures at 12 h of stimulation (Table III). However, the expression of the \(\text{Icsbp1}\) gene, a member of the interferon regulatory transcription factor (IRF) family, a transcription factor critical for both early differentiation and final maturation of dendritic cells (28), remained elevated from 1 to 12 h. Notably, the gene for another member of the IRF family, \(\text{Irf1}\) gene, was up-regulated after 12 h of stimulation of endothelial cells with HA12 or FGF-2. Thus, it appears that the induction of IRF members occur during all the phases of endothelial cell differentiation in response to both FGF-2 and HA12 stimulation. The \(\text{Arl6ip}\) gene, which encodes a protein that protects cells from apoptotic inducers such as UV radiation and serum starvation (29), was also induced in response to HA12 or FGF-2. Among the genes induced after long stimulation by HA12 or FGF-2 (12 h) was the ornithine decarboxylase (\(\text{Odc}\)) gene (Table III). The \(\text{Odc}\) protein is a key enzyme in the synthesis of polyamines that are essential for the growth and differentiation of the cells and has been shown to be angiogenic. The polyamines feedback-regulate \(\text{Odc}\) by the induction of an antizyme, which binds to \(\text{Odc}\), inhibits its activity, and promotes its degradation (30, 31). \(\text{Odc}\) is overexpressed in several tumors and is associated with tumor growth and angiogenesis by suppression of type XVIII collagen and endostatin (32). In this context, it is interesting that another strongly up-regulated gene was the \(\text{Oazin}\) gene, an ornithine decarboxylase antizyme inhibitor, the encoded protein of which can release enzymatically active \(\text{Odc}\) from antizyme suppression (31). Thus, the coordinated up-regulation of \(\text{Odc}\) and \(\text{Oazin}\) genes may lead to an increase in \(\text{Odc}\) activity in endothelial cells stimulated by HA12 or FGF-2; the possible role of \(\text{Odc}\) in endothelial cell differentiation was therefore further explored (see below). Other genes induced by both HA12 and FGF-2 were \(\text{Mdm2}\) and \(\text{CcnG1}\), which encode a ubiquitin protein ligase and cyclin G1, respectively; they promote the proteasomal degradation of p53 (33, 34).
Several genes were also down-regulated in cultures of endothelial cells stimulated either with FGF-2 or with HA12. One example was the \textit{Col3a1} gene, which encodes collagen III that is expressed in blood vessels and appears together with collagen I in thickened intima of atherosclerotic lesions and most likely contributes to plaque growth and narrowing of arterial lumen (35). Another notable down-regulated gene was the \textit{Svil} gene, the encoded protein of which, supervillin, is an F-actin-binding protein implicated in migration, adhesion, and signal transduction to the cell nucleus (36). Additional markedly commonly suppressed genes encode the fibroblast growth factor receptor (\textit{Fgfr1}), protein kinases involved in various signaling events (\textit{Rock1}, \textit{Ttk}, \textit{Stk11}, \textit{Map4k4}, \textit{Ikbkb}), tissue inhibitor of MMP3 (\textit{Timp3}), fibronec- tin (\textit{Fn1}), platelet-derived growth factor receptor-\(\beta\) (\textit{Pdgfrb}), and growth arrest protein (\textit{Gas1}), which blocks the entry to S phase.

\textit{Genes Regulated by FGF-2 but Not HA12—}Gene array hybridization of FGF-2-stimulated cells versus unstimulated cells revealed up-regulation of 205 genes and down-regulation of 337 genes (Supplemental Table IV); the most profoundly modulated genes from each category are depicted in Table IV. Several genes encoding pleiotropic molecules were early up-regulated more than 2-fold in response to FGF-2 at different stages of endothelial cell differentiation. For example, these genes include the \textit{PitdInb} gene, which encodes a protein that binds phospholipids; the \textit{Pleur} gene, which encodes the urorosinease receptor and is involved in regulation of angiogenesis (37); the \textit{Gja1} gene, which encodes a protein involved in FGF-2-dependent modulation of gap-junctional intercellular communication in endothelial cells and fibroblasts from diabetic individuals (38); the \textit{Egr1} gene, which encodes a key transcription factor involved in vascular pathophysiology (39); and the \textit{Pecam} gene, which encodes the CD31 antigen expressed at intercellular junctions of endothelial cells.

Notably, after 4 h of stimulation, an induction of the \textit{CD44} gene, which encodes the CD44 protein that is a hyaluronan receptor and is involved in a variety of biological functions, and an induction of the \textit{Ugdh} gene, which encodes UDP-glucose dehydrogenase involved in the biosynthesis of glycosaminoglycans, for example hyaluronan and heparan sulfate, were detected. During active morphogenesis after 12 h of stimulation, several genes regulating cellular metabolism (\textit{Txnip}) and signaling events (\textit{Tnfrsf12a}, \textit{Sef}-pending \textit{Epha2}) and genes the function of which is still unknown in endothelial differentiation (\textit{Mgea5}, \textit{Bcas3}) were up-regulated. A notable down-regulation was observed of genes involved in the induction of apoptosis (\textit{Casp12}) but also of genes involved in cell survival by suppressing caspases (\textit{Bcl2l}, \textit{Igtp}). Furthermore, genes encoding proteins that promote the ubiquitination and degradation of proteins (\textit{Cul3} and \textit{Fbxo3} genes) or are associated with microtubule motor activity in the presence of ATP (\textit{Kif11}) were also markedly suppressed. In addition, several genes with a potential to modulate the matrix structure and a large number of genes, the function of which is still elusive, were strongly down-regulated.

\textit{HA12- and FGF-2-induced Endothelial Cell Differentiation Affect Differentially HAS and CD44 Gene Expression—}The interactions between extracellular matrix components, growth factors, and cell surface receptors regulate the molecular mechanisms participating in endothelial cell differentiation (40). Given the differential regulation of hyaluronan-synthesizing enzymes by various cytokines and growth factors (41), which consequently results in changes of the extracellular matrix composition, we examined the expression of \textit{HAS1}, \textit{HAS2}, and \textit{HAS3} levels by real-time PCR. Furthermore, based on the knowledge that CD44-
hyaluronan interactions trigger changes in cellular functions, we have also investigated the expression levels of CD44s and CD44v10 molecules during the course of sprout formation. Fig. 2 shows that HA12-induced differentiation of brain capillary endothelial cells triggered a considerable increase of *Has2* mRNA after 4 h of stimulation, which was sustained also during active endothelial cell morphogenesis at 12 h. Furthermore, the *Has2* transcript was also up-regulated, in particular after 4 h of stimulation with FGF-2. Notably, HA12-induced endothelial cell differentiation induced an about 2-fold increase of *Has3* mRNA at 4 h after treatment over untreated control cells, whereas FGF-2 had no effect. The expression levels of *Has1* mRNA were hardly detected in the cells. FGF-2 stimulation resulted in a constitutive induction of both CD44s and CD44v10 mRNAs over the experimental time course, whereas HA12 stimulation did not affect the transcripts noticeably. These results indicate that not only growth factors, such as FGF-2, but also hyaluronan fragments trigger up-regulation of *Has2*, and to a lesser extent, *Has3*.

Validation of CXCL1/GRO1 Expression by Real-time PCR—
To verify the marked and distinct early CXCL1/GRO1 gene expression detected by microarray during the HA12-induced sprouting of endothelial cells, we examined its mRNA expression by real-time PCR. As shown in Fig. 3, a powerful induction of the CXCL1/GRO1 transcript (about 10-fold) was detected in mouse brain capillary endothelial cells growing in a three-dimensional collagen gel already after 1 h of stimulation by FIG. 2. Real-time PCR to determine *Has* and CD44 mRNAs in endothelial cells stimulated with HA12 and FGF-2. Mouse capillary brain endothelial cells grown in a three-dimensional collagen gel were stimulated with HA12 and FGF-2, and RNAs were prepared at the indicated times and subjected to reverse transcriptase-PCR as described under “Experimental Procedures.” Changes in mRNA levels are relative to GAPDH mRNA level. Data are the mean ± S.D. of two different experiments. White bars, unstimulated cells; gray bars, FGF-2-stimulated cells; black bars, HA12-stimulated cells.
HA12; after longer culture treatment, the gene levels decreased to control levels. Cultures stimulated with FGF-2 exhibited, after 1 h of stimulation, an early up-regulation of \( \text{CXCL1/GRO1} \) (about 2-fold) followed by a decline at 4 h and return to up-regulation by 12 h. Thus, \( \text{CXCL1/GRO1} \) gene expression is induced by at least two different mechanisms, a HA12-dependent early and powerful up-regulation, and a lower but sustained up-regulation by FGF-2.

Inhibition of HA12-induced Endothelial Cell Differentiation by Anti-CXCL1/GRO1 Antibodies—To examine whether the early up-regulation of the \( \text{CXCL1/GRO1} \) gene in cell cultures stimulated with HA12 (Table II and Fig. 3) leads to corresponding secretion of the \( \text{CXCL1/GRO1} \) protein and whether this is involved in endothelial cell sprouting, we treated endothelial cells undergoing morphogenesis with neutralizing anti-CXCL1/GRO1 antibodies. As assessed by measuring the length of the tubular structures formed, anti-CXCL1/GRO1 antibodies completely suppressed the HA12-induced differentiation of endothelial cells, but not the FGF-2-induced cell morphogenesis, when compared with cultures treated with control IgG (Fig. 4).

These data suggest that the up-regulation of the \( \text{CXCL1/GRO1} \) gene and the subsequent production of the \( \text{CXCL1/GRO1} \) protein are necessary for the HA12-induced differentiation of capillary endothelial cells.

Suppression of FGF2- and HA12-induced Endothelial Cell Differentiation by DFMO—Our finding that Odc and Oazin genes are up-regulated by FGF-2 and HA12 is of interest given the importance of Odc and Oazin activities in endothelial cell differentiation (30, 31). We therefore investigated the effect of the Odc inhibitor, \( \text{DFMO} \), on differentiation of endothelial cells grown in a three-dimensional collagen gel upon stimulation with FGF-2 or HA12. As shown in Fig. 5, stimulation with FGF-2 or HA12 induced differentiation of the cells when cultured in the absence, but not when cultured in the presence, of DFMO. DFMO prevented tubulogenesis in FGF-2-stimulated cells (#, \( p < 0.0001 \)) or HA12-stimulated cells (##, \( p = 0.0013 \)). Thus, up-regulation of Odc and Oaz activities is important for the HA12- and FGF-2-induced sprouting of endothelial cells.

Blocking of CD44 Suppresses HA12-induced but Not FGF2-induced Endothelial Cell Differentiation—Given that brain capillary endothelial cells express the hyaluronan receptor CD44 (Rahmanian et al. (6)), we investigated whether CD44 is involved in endothelial cell differentiation induced by HA12 or FGF-2. The addition of KM114 antibodies (which block the binding of hyaluronan to CD44 receptors (42)) to endothelial cells grown in collagen gels had no effect on FGF-2-induced cell differentiation but completely suppressed the HA12-induced
sprout formation (Fig. 6). These findings suggest that CD44 is the predominant hyaluronan receptor through which HA12 induces morphogenesis of brain capillary endothelial cells.

**DISCUSSION**

Among the key events associated with angiogenesis are changes in the synthesis and degradation of extracellular matrix. A general concept emerging from recent studies is that degradation products of extracellular components possess other biological functions than their precursor molecules (11, 43). Since considerable changes in the levels and molecular mass of hyaluronan molecules have been detected in tissues during the progression of cancer, wounding, and inflammation, we have studied how hyaluronan fragments affect endothelial cell differentiation at the molecular level.

Our finding that HA12 and FGF-2 affect a large number of genes in common further strengthens the notion that hyaluronan fragments are important during endothelial cell differentiation. After the classical experiment on the chick chorioallantoic membrane demonstrating that hyaluronan fragments of 4–25 disaccharides in length induce the formation of new blood vessels (11), several studies have assessed hyaluronan fragments as an angiogenic factor (44) and signaling molecule (15). Studies on activated macrophages have shown that hyaluronan fragments induce the production of chemokine genes (MIP-1, RANTES, and CXCL1/GRO1), the functions of which are crucial in initiating and maintaining the inflammatory response (45). Increased production of hyaluronan has been correlated to numerous pathophysiological situations, including poor prognosis of tumor patients, poor host defense by tumor-adjacent
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fibroblasts, and progression to chronic inflammation (46–48). In this study, we showed that defined hyaluronan fragments of six disaccharides induced endothelial sprouting through up-regulation of the mouse chemokine CXCL1/GRO1 since blocking antibodies completely suppressed the HA12-induced capillary morphogenesis (Fig. 4). This novel finding suggests that the angiogenic effect of HA12 is mediated by up-regulation of CXCL1/GRO1, which binds to the putative CXC chemokine receptor CXCR2. Several studies support CXCR2 as the receptor mediating the angiogenic effects of CXC chemokines including CXCL1/GRO1 and CXCL8/IL8. Its importance in mediating angiogenesis has been demonstrated in vivo using CXCR2−/− mice in a cornea assay of angiogenesis (23). Recently, it has been reported that activation of CXCR2 in endothelial cells by either CXCL8/IL8 or CXCL1/GRO1 leads to a translocation of the small G-protein Rac to the plasma membrane (49), which activates p21-activated kinase and causes phosphorylation of myosin II followed by changes in actin distribution and retraction of endothelial cell margins (50). Interestingly, we observed that HA12 induces more than 2-fold up-regulation of the VHL gene, which encodes ezrin that is associated with activated CD44 and participates in cell shape changes, adhesion, motility, endocytosis/exocytosis, and signal-transduction pathways. Activation of CD44 is intimately associated with its ability to interact with hyaluronan and/or hyaluronan fragments, which triggers the association of its cytoplasmic domain with actin cytoskeleton via binding to ezrin (8). Furthermore, HA12 induced the expression of the myosin 1b gene (Myo1b, Table II). Myosin 1b belongs to the myosin I family of proteins and participates in a variety of cellular processes including membrane fusion/vesicle scission (51). Taken together, CD44 activation upon HA12 binding may trigger ezrin-dependent signaling events leading to the induction of CXCL1/GRO1 production and subsequent CXCR2 activation, which results in retraction of endothelial cells, a phenomenon observed during angiogenesis.

In this study, we have compared the effects of HA12 stimulation on gene regulation in brain capillary endothelial cells with those of FGF-2. These cells do not express VEGF receptors, which mediates a strong angiogenic effect; in other systems, hyaluronan fragments have been shown to synergize with VEGF in the induction of angiogenesis (52). It is interesting to note that several of the genes induced by HA12 have also been shown to be induced by VEGF in myometrial endothelial cells including the genes for CXCL1/GRO1, Fbp2, a fructose bisphosphatase enzyme specific for gluconeogenesis, Wdr12, a member of the WD repeat protein family, and Nktr, which is present on the surface of natural killer cells (53) (Table II). Thus, the similar and synergistic effects of HA12 and VEGF on tubulogenesis may be due to common regulation of the CXCL1/GRO1 gene as well as regulation of other genes, the angiogenic functions of which are not well characterized.

Another interesting observation is that HA12 induces Has2 and to a lesser extent Has3 transcripts during active endothelial cell sprouting (Fig. 2). Only proliferating endothelial cells synthesize hyaluronan (54). This observation suggests that stimulation of endothelial cells by hyaluronan fragments leads to increased synthesis of hyaluronan, which after depolymerization can enhance the angiogenic effects.

Our finding that HA12 and FGF-2 regulate overlapping sets of genes in endothelial cells further demonstrate the importance of both growth factors and matrix molecules in angiogenesis. One general concept that is emerging from these studies is that targeting of multiple receptors could be a successful approach in angioproliferative diseases such as infantile heman-giomas and proliferative diabetic retinopathy, as well as anti-angiogenic therapy of tumors.
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