Fibroblast Growth Factor Receptor 1-induced Differentiation of Endothelial Cell Line Established from tsA58 Large T Transgenic Mice

Shigeru Kanda,1 Eva Landgren, Magnus Ljungström*, and Lena Claesson-Welsh

Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 24 Uppsala [S. K., E. L., L. C-W.], and Department of Medical and Physiological Chemistry, Box 574, 751 23 Uppsala [M. L.], Sweden

Abstract

Angiogenesis of capillary endothelial cells includes at least four sequential cellular responses: digestion of basement membrane, migration, proliferation, and differentiation. To study differentiation of endothelial cells, we established a brain capillary endothelial cell line from H-2Kb-tsA58 transgenic mice. These cells are stable at 33°C and display endothelial cell-specific characters, such as expression of von Willebrand factor and binding sites for the lectin Bandeiraea simplicifolia, and uptake of acetylated-low density lipoprotein. We measured the effects of a panel of growth factors on cellular responses. A number of factors, such as hepatocyte growth factor, vascular endothelial growth factor, and platelet-derived growth factor (PDGF)-AA failed to induce biological responses. PDGF-BB, epidermal growth factor, and acidic and basic fibroblast growth factor (FGF) induced proliferation of the cells. Of all the factors tested, only acidic FGF and basic FGF induced differentiation of the cells, visualized as the formation of tube-like structures of cells grown in three-dimensional collagen gels. All factors were also analyzed for their effects on plasminogen activator (PA)-induction and migration of the cells. Transfected cells, expressing a chimeric receptor, composed of the extracellular part from the PDGF alpha-receptor and the intracellular part from FGF receptor-1, responded to PDGF-AA treatment with plasminogen activator induction, migration, and tube formation in collagen gels. These results indicate that FGF receptor-1 coupled to signal transduction pathways, leading to differentiation. This novel cell model offers the potential of detailed dissection of signal transduction pathways involved in the differentiation of endothelial cells.

Introduction

Angiogenesis is defined as the formation of new capillary blood vessels from preexisting vessels. This complex process plays a key role in development, ovulation, and wound healing, as well as in the progression of diseases, including arthritis, diabetic retinopathy, tumour growth, and metastasis (1). A number of different types of compounds have been implicated in the regulation of angiogenesis, but growth factors are believed to be key players in angiogenesis in vivo (2, 3). Receptors for growth factors are endowed with ligand-stimulatable tyrosine kinase activity. Binding of the growth factor leads to activation of the kinase, followed by autophosphorylation of the receptor molecules (4). Autophosphorylation serves to regulate kinase activity and to create binding sites for signal transduction molecules (5). Through different mechanisms, the Src homology 2 domain-containing signal transduction molecules become activated upon interaction with the receptor (6), creating signaling chains, which eventually become manifested as cellular responses. Different signal transduction molecules are believed to couple to different signaling chains, each giving rise to unique responses (7).

Angiogenesis can be divided into a series of sequential events (8–10), which can be summarized as follows. Focal degradation of the basement membrane occurs through up-regulation of protease activity in the endothelial cells lining the existing vessel (11). The endothelial cells form sprouts that invade the surrounding tissue, through the defects in the basement membrane (3, 12). Entire cells migrate into the tissue, while they proliferate, forming a cell bud. Gradually, the cells in the bud differentiate and form lumens through expansion of cytoplasmic vacuoles that fuse to give rise to a functional vessel (9). aFGF2, bFGF, and VEGF (also denoted as vascular permeability factor) are potent angiogenic growth factors in vivo and are known to stimulate the different angiogenic steps in vitro: induction of PA, migration, proliferation and, tube formation in collagen gels (2, 3, 13). Other growth factors, such as HGF (14), PDGF-BB (15), and EGF/TEF0 (16) are also known to be angiogenic in vivo. These factors exert their effect by specific binding to cell surface-expressed receptor tyrosine kinases (reviewed in Ref. 4). Only limited data are available on how signal transduction molecules binding to these receptors give rise to specific biological responses. It is conceivable that at least

---

1 To whom requests for reprints should be addressed. Phone: +46-18-551688; Fax: +46-18-506867.

2 The abbreviations used are: aFGF, acidic fibroblast growth factor; bFGF, basic FGF; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; FGF, FGF receptor; Flt-1, fms-like tyrosine kinase-1; Fik-1, fetal liver kinase-1; PA, plasminogen activator; SV40, simian virus 40; Ac-LDL, acetylated low density lipoprotein; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; BS-I, Bandeiraea simplicifolia-I; UEA-I, Ulex europaeus-I; BrdUrI, bromodeoxyuridine.
certain steps in the angiogenic process, such as differentiation to form lumen, involve signal transduction molecules only expressed in endothelial cells. No information is available on whether the different receptors would share the potential to directly activate such a signaling molecule.

To address these issues, we established a capillary endothelial cell line from brains of newborn transgenic mice expressing a temperature-sensitive variant (tsA58) of SV40 large T under control of the H-2Kb promoter (17). The capacity of a panel of growth factors to induce biological responses in vitro were examined using this cell line. Whereas several factors promoted growth and migration, only aFGF and bFGF were able to stimulate differentiation, visualized as tube formation in collagen gels. Expression of a chimeric receptor after transfection confirmed that initiation of signal transduction pathways downstream of the intracellular domain of FGFR-1 specifically mediated differentiation of the endothelial cells.

Results
Isolation and Purification of Mouse Brain Capillary Endothelial Cells. We were interested in studying growth factor-induced signal transduction leading to differentiation of endothelial cells. To establish a capillary endothelial cell line for this purpose, 2-week-old H-2Kb-tsA58 transgenic mice (17) were sacrificed, and after removal of white matter, brains were minced and capillary fragments were isolated by sequential filtration. After 5 days in culture at 33°C in the presence of IFN-γ, outgrowth of cells from the capillary fragments was evident. The resulting culture displayed a varied morphology, and at the 6th passage, about 30% of the cells were identified as glial cells (expressing glial acidic fibrillary protein), and about 3% of the cells were pericytes (expressing α-smooth muscle actin). The remaining population expressed von Willebrand factor (Fig. 1A) and efficiently internalized Dil-labeled Ac-LDL (data not shown). Efficient uptake of Ac-LDL has been recorded for endothelial cells and macrophages (18). In addition, binding of the endothelial specific lectins Bandeiraea simplicifolia (BS-I; Ref. 19) and Ulex europaeus (UEA-I; Ref. 20) was seen (data not shown); in the latter case, however, binding was evident only at 39°C. To purify the endothelial cells, BS-I-coated Dynabeads (21) were used, and the resulting culture appeared homogeneous, i.e., in a population of 1000 individually examined cells, all displayed endothelial cell characteristics.

Temperature-dependent Characteristics of tsA58 Endothelial Cells. The morphology of the purified endothelial cells was temperature dependent. As shown in Fig. 1B, cells cultured at 33°C were long and narrow, whereas at 39°C, the cells were flat. Nuclei of cells cultured at 33°C contained SV40 large T antigen (Fig. 1C), whereas very little expression was detected in cells kept at 39°C (data not shown). The growth rate of the cells was dramatically influenced by the culture conditions. Fig. 1D shows that cells kept at 33°C in the presence of IFN-γ grew continuously and rapidly. However, cell growth was contact inhibited, and the cells were unable to grow in soft agar at 33°C in the presence of IFN-γ, indicating that the temperature-regulated SV40 large T did not confer typical features of transformation. At 39°C in the absence of IFN-γ, the cells stopped dividing, and after 14 days under this condition, they gradually started to die. Thus, at 33°C, SV40 large T antigen expression mediated unlimited growth of the cells, which, however, retained endothelial cell characteristics, such as expression of endothelial cell markers and the ability to differentiate (see below). At this point, the cells were stable in culture at 33°C for more than 12 months, and the serial passage number passed 70.

Effect of Growth Factors on DNA Synthesis and Labeling Index of Endothelial Cells. A number of growth factors were analyzed with regard to their capacity to stimulate DNA synthesis in the murine brain endothelial cells at different temperatures. The factors were chosen based on their reported effects on angiogenesis. To demonstrate specificity, we used PDGF-AA, for which there are no or few receptors on primary brain capillary endothelial cells (22). The analysis was performed on cells kept without serum and IFN-γ, prior to stimulation. At 33°C, EGF, PDGF-BB and bFGF efficiently stimulated DNA synthesis (Table 1), measured as incorporation of [3H]-labeled deoxyuridine. PDGF-AA, HGF, VEGF, and TGF-β1 had no clear effect. At 39°C, only 20% FBS with ECGS stimulated DNA synthesis, and none of growth factors tested showed any stimulating effect on serum-starved cells (data not shown). Analysis of labeling index after stimulation with the different growth factors at 33°C gave results in agreement with those for the DNA synthesis; stimulation with EGF, PDGF-BB, and bFGF markedly increased labeling index (Table 1).

Effect of Growth Factors on Cell Migration. Cellular migration is an important feature in the in vivo function of endothelial cells. We analyzed the effects of different growth factors on migration of murine brain endothelial cells using the Boyden chamber technique (23). The ability of the cells to move from one side to the other of an 8-μm-thick nitrocellulose filter was assayed. To avoid the problem with collapse of the concentration gradient over this very thin filter and consequent lack of reproducibility, we performed the migration assays with growth factors on both sides of the filter. At 39°C, the cells were unable to migrate efficiently, independently of stimulus. At 33°C, EGF, bFGF, PDGF-BB, HGF, and TGF-β1 efficiently stimulated migration of the endothelial cells, whereas PDGF-AA and VEGF were without effect (Fig. 2).

Effect of Growth Factors on Induction of PA Activity. The first step in the angiogenic process could be degradation of the basement membrane of capillary vessels to allow invasion and migration of endothelial cells into the surrounding tissue. The protease activity of PA has been implicated in this process (24). Induction of PA activity by the different growth factors, in serum-starved cells in the absence of IFN-γ, was analyzed using a zymographic assay (Fig. 3). bFGF and EGF were found to stimulate PA activity, whereas TGF-β1 was strongly inhibitory. In HGF- and VEGF-treated cells, there was a partial reduction in PA activity, compared to control cells. Other growth factors were largely without effect (Fig. 3). The estimated molecular mass of PA induced by bFGF and EGF was 48 kDa. Inclusion of amiloride during the zymographic assays strongly inhibited the PA activity (data not shown). Both these parameters argue thaturoki-
Fig. 1. Characteristics of tsA58 mouse brain capillary endothelial cells. A, immunostaining for von Willebrand factor (factor VIII-related antigen) expression: a, control serum; b, von Willebrand factor-specific antiserum. ×112. B, monolayer of endothelial cells cultured in the presence of IFN-γ at 33°C (a) or at 39°C (b). ×76. C, indirect immunofluorescence staining of SV40 large T antigen in cells cultured at 33°C in the presence of 20 units/ml IFN-γ. ×306. D, growth curves of cells inoculated into 12-well plates at a density of 5 × 10⁴ cells/well and cultured at 33°C in the absence of 20 units/ml IFN-γ (○) or at 39°C in the absence of IFN-γ (●).
Table 1  Effects of growth factors on DNA synthesis and labeling index of mouse brain capillary endothelial cells

DNA synthesis was measured by incorporation of 125I-labeled deoxyuridine in the serum- and growth supplement-starved endothelial cells kept at 33°C, incubated with or without growth factors as described in "Materials and Methods." The effects of growth factors were tested using the following concentration intervals: EGF, 0.1–100 ng/ml; bFGF, 0.01–50 ng/ml; PDGF-AA, 0.1–100 ng/ml; PDGF-BB, 0.1–100 ng/ml; VEGF, 0.1–100 ng/ml; HGF, 0.1–200 ng/ml; and TGF-β, 0.01–20 ng/ml. The effects of concentrations giving maximal effect on DNA synthesis and labeling index are shown in the table. To determine labeling index, incorporation of BrdUrd in S-phase nuclei was visualized using anti-BrdUrd monoclonal antibody. Labeling index was expressed as percentage of labeled nuclei/total nuclei. Values are means ± SD for triplicate wells.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>DNA synthesis (cpm)</th>
<th>Labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2429 ± 76</td>
<td>9.4 ± 0.3</td>
</tr>
<tr>
<td>EGF 10 ng/ml</td>
<td>7010 ± 305</td>
<td>15.3 ± 0.8</td>
</tr>
<tr>
<td>bFGF 0.5 ng/ml</td>
<td>10987 ± 461</td>
<td>26.3 ± 4.6</td>
</tr>
<tr>
<td>PDGF-AA 50 ng/ml</td>
<td>2318 ± 91</td>
<td>11.2 ± 0.7</td>
</tr>
<tr>
<td>PDGF-BB 10 ng/ml</td>
<td>6550 ± 619</td>
<td>21.8 ± 1.6</td>
</tr>
<tr>
<td>VEGF 50 ng/ml</td>
<td>1873 ± 156</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>HGF 100 ng/ml</td>
<td>2237 ± 106</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>TGF-β1 10 ng/ml</td>
<td>2627 ± 182</td>
<td>10.8 ± 1.6</td>
</tr>
<tr>
<td>FBS 20%</td>
<td>11645 ± 470</td>
<td>26.1 ± 0.7</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of growth factors on migration (chemokinesis) of mouse brain capillary endothelial cells. The migration assay was performed using a Boyden chamber and an 8 μm filter. The cultured endothelial cells were harvested and suspended in Ham’s F12 medium containing 0.25% BSA. The cells were added to the upper wells at a density of 1.5 × 10⁴ cells/well, with or without growth factors. The same growth factors were added to the lower wells, and after 6 h incubation at 33°C, cells that had migrated into the lower wells were counted. The number of migrating cells in the absence of growth factor was set to 100%. Columns, from left to right: no addition; 20 ng/ml EGF; 10 ng/ml bFGF; 100 ng/ml PDGF-AA; 100 ng/ml PDGF-BB; 100 ng/ml VEGF; 100 ng/ml HGF; 50 ng/ml TGF-β1. EGF, bFGF, PDGF-AA, PDGF-BB, HGF, and VEGF were tested at concentration intervals of 1–100 ng/ml; TGF-β1 was tested at 1–50 ng/ml. The results from concentrations giving maximal migration are shown in the figure. Values are expressed as means for six wells, bars, SE.

nase was responsible for the PA activity (25). At 39°C, similar results were obtained, but with a less pronounced induction of PA activity in response to bFGF and EGF (data not shown).

Effect of Growth Factors on Tube Formation in Collagen Gels. We examined the capacity of the murine endothelial cells to differentiate into vessel-like structures in collagen gels in response to different growth factors. Cells were seeded in the middle of two collagen layers in the absence of serum and IFN-γ, and morphological changes induced by the different growth factors were analyzed after 24 h incubation at 33°C. As seen in Fig. 4, bFGF treatment allowed cells to form tube-like structures in the collagen gel. The same response was seen when cells were treated with aFGF (data not shown). This was in marked contrast to the morphology of untreated cells in collagen gels, as well as to cells treated with EGF, PDGF-AA, PDGF-BB, VEGF, HGF, and TGF-β1, which failed to stimulate endothelial cells to form tubes in the collagen gel. Occasionally, as seen in Fig. 4, treatment of cells with EGF, PDGF-BB, and PDGF-AA induced a different morphology than that seen in untreated cells, but these factors never gave rise to a more extensive response than that shown in the figure. At 39°C, the cells appeared fragile and did not always respond to bFGF; however, the pattern of responses was the same as for 33°C incubation, since only bFGF treatment led to tube formation (data not shown).

Fig. 5A shows a sagittal section of a tube in collagen gel containing bFGF-stimulated endothelial cells. Between the nuclei of two endothelial cells, a lumen can be seen. A confocal image of tube-forming, Dil-stained endothelial cells is shown in Fig. 5B. The stack of images was subjected to the Molecular Dynamics Image Space program called Slice Viewer. The program will display a main window with a "look through" model of the image stack as well as two subwindows with the sagittal cut of the look through model in either the x-z or y-z direction. It is thus possible to navigate in the main window and see the depth distribution of Dil-stained cells along the two axes. It was possible to observe a lumen within a cord of cells in many places and to follow a lumen along several lengths of cells. In Fig. 5B, positions where a lumen was readily identified are indicated by white arrow heads. These data support the notion that the bFGF-induced morphological changes of the brain endothelial cells in collagen gels represented the formation of tubes and were, therefore, a result of a differentiation process.

Growth Factor Receptor Expression on the Murine Endothelial Cells. To identify receptors for the different growth factors on the murine brain endothelial cells, in vitro kinase assays using immobilized receptor immunoprecipitates were performed. This assay was chosen because of its high sensitivity. Fig. 6A shows that the cells expressed functional EGF receptors, PDGF β receptors, FGF receptor-1 and c-met/HGF receptors, since ligand stimulation led to an increased phosphorylation of receptor proteins. A low level of FGFR-2 and FGFR-4 expression was also seen (data not shown). There was no detectable expression of FGFR-3 and the PDGF α-receptor. There are two types of receptors for VEGF, Fit-1, and Flik-KDR (26). We could not detect expression of Flik-1/KDR (data not shown). Although Fit-1 has the structural characteristics of a receptor tyrosine kinase, it fails to appreciably autophosphorylate in response to VEGF (see "Discussion"). Therefore, to detect expression of Fit-1
Fig. 3. Effects of growth factors on induction of PA activity in mouse brain capillary endothelial cells. After 24 h serum starvation, media conditioned by cells grown in the presence or absence of the different growth factors for 24 h at 33°C were collected and separated by SDS-PAGE. Different concentration ranges of the growth factors were tested (EGF, 1–50 ng/ml; bFGF, 0.5–100 ng/ml; HGF, 5–100 ng/ml; VEGF, 5–50 ng/ml; and TGF-β1, 1–20 ng/ml). The results from concentrations giving optimal responses are shown in the figure. After washing the polyacrylamide gel, the gel was put horizontally on top of an agarose gel containing casein and plasminogen. The stack was incubated for 2 days at 37°C. The band corresponding to PA activity was visualized as a lucent area in the white agarose gel and was photographed on a black paper. The densities of the PA bands were quantified using a software program (NIH Image, version 1.54). The density of the PA band in the sample from the conditioned medium without growth factor treatment was set to 100%. Right, the migration position of the 46 kDa PA band. The concentrations of the growth factors used were: EGF, 10 ng/ml; bFGF, 5 ng/ml; PDGF-AA, 50 ng/ml; PDGF-BB, 50 ng/ml; VEGF, 50 ng/ml; HGF, 100 ng/ml; and TGF-β1, 5 ng/ml.

on the brain endothelial cells, we used an anti-Flt-1 antibody for immunoprecipitation, followed by immunoblotting, which revealed a 190 kDa component in the absence, but not in the presence, of a peptide competing for the reactive site on the antibody (Fig. 6B). The predicted mass of Flt-1 is compatible with a migration rate corresponding to 190 kDa (26). We also used the Flt-1 antibody for immunoprecipitation from [35S]methionine-labeled cells (Fig. 6B). A band corresponding to the molecular mass of Flt-1 was seen when using the anti-Flt-1 antibody, but not when using preimmune antiserum, for immunoprecipitation. Apart from the 190-kDa component corresponding to Flt-1, both these analyses showed an abundant 150-kDa component immunoreactive with the Flt-1 antibody. The 150 kDa component, but not the 190-kDa component, was visualized using antiphosphotyrosine antibodies for immunoblotting of samples obtained from VEGF-stimulated cells (Fig. 6B). The relationship between these two components remains to be determined, but it is possible that the 150-kDa component arises as a result of proteolytic processing of the extracellular domain, similar to what has been shown for the related receptor, Flt-4 (27). It is an interesting perspective that the 150-kDa molecule represents an active form of Flt-1 that has gained the capacity to autophosphorylate and transduce signals for other responses than the ones examined in this work.

These data are in agreement with the pattern of growth factor-induced biological responses in the tsA58 endothelial cells. It was reported recently that VEGF stimulation of Fik-1/KDR leads to biological responses, whereas stimulation of Flt-1 was without effect (28). Therefore, the lack of responses to VEGF, as well as to PDGF-AA in our assays, can be explained by the lack of expression of the relevant receptor.

Transduction of Biological Responses by a PDGFR-α/FGFR-1 Chimeric Receptor. To identify the receptor capable of transducing the bFGF-induced biological responses in murine brain endothelial cells, we constructed a chimeric receptor, composed of the extracellular domain and transmembrane domain from PDGF α-receptor and the intracellular domain from FGFR-1. The latter receptor type was chosen, since it was the most abundantly expressed FGFR type on the endothelial cells. The resulting construct was transfected into the endothelial cells. After G418 selection, a stable cell line was obtained, which showed efficient uptake of Dil-Ac-LDL, and which expressed the PDGF-AA-stimulatable chimeric receptor (Fig. 7A). The capacity of this receptor to transduce the different biological effects was examined. As seen in Fig. 7, B–E, DNA synthesis, migration, induction of PA activity, and formation of tubes in collagen gel were clearly stimulated by PDGF-AA in cells expressing the chimeric receptors, although these responses were weaker than when bFGF was used to stimulate the endogenous FGFR receptors. We conclude that the FGFR-1 intracellular domain, through coupling to specific signal transduction pathways, mediated differentiation of the endothelial cells.
Discussion

In this report, we describe the characteristics of a murine brain endothelial cell line, which we established from brains of transgenic mice, expressing a temperature-sensitive variant of the SV40 early region, under control of the IFN-sensitive H-2 promoter. We show that FGFR-1 is able to transduce specific signals for differentiation of the endothelial cells. The cell line displays a number of endothelial cell specific markers, like expression of factor VIII-related antigen (von Willebrand factor), binding sites for the lectin BS-1, receptors for Ac-LDL, as well as binding sites for UEA-1 (only at 39°C). Using this cell line, which now has been in culture for more than 70 passages without signs of crisis, we have analyzed the effects of growth factors on a series of biological assays: protease induction, proliferation, migration, and differentiation. These assays were performed at 33°C. Apparently, the cells were not dedifferentiated at this temperature, and since the cells were less sensitive to manipulation at 33°C, the results were highly reproducible. Independent of temperature, similar results of growth factor treatment on migration, PA induction, and tube formation of the endothelial cells were obtained. In contrast, growth factor treatment failed to induce proliferation of serum-starved cells at 39°C.

We found that only bFGF (which could be substituted for by aFGF; data not shown), was able to stimulate cells to respond in all four assays. Both aFGF and bFGF lack classical signal sequences necessary for secretion (29), and it is unclear how the factors become released from the site of

Fig. 4. Effect of growth factors on tube formation of brain capillary endothelial cells in collagen gels. Cells were harvested and suspended in Ham’s F12 containing 0.25% BSA in the presence or absence of growth factors and inoculated onto the basal layer of collagen gels. Concentration ranges of growth factors tested were: EGF, 10–100 ng/ml; bFGF, 0.5–50 ng/ml; PDGF-AA, 10–100 ng/ml; PDGF-BB, 10–100 ng/ml; VEGF, 1–100 ng/ml; HGF, 1–200 ng/ml; and TGF-β1, 0.5–50 ng/ml. After attachment of the cells, medium was discarded, and a top layer of collagen gel was added and incubated overnight at 33°C. a, no addition; b, 5 ng/ml bFGF; c, 50 ng/ml EGF; d, 50 ng/ml PDGF-AA; e, 50 ng/ml PDGF-BB; f, 50 ng/ml VEGF; g, 100 ng/ml HGF; h, 10 ng/ml TGF-β1. ×50.
Fig 5. Characterization of the tube-like structure of endothelial cells in collagen gel. A, cells forming tube-like structures were frozen while still in the collagen gel, and sagittal cryostat sections were made. After fixation by ethanol, cells were stained with hematoxylin. Open arrowheads, nuclei of endothelial cells and a lumen (closed arrow) can be seen between the nuclei. ×360. B, analysis of Dil-stained bFGF-treated murine brain endothelial cells in collagen gels using confocal microscopy. The compiled image was acquired and treated using an Image Space program. The program will display a main window with a look through model of the image stack, as well as two subwindows, with the sagittal cut of the look through model in either the x-z or in y-z direction. White arrowheads, cell cords in which a lumen could be identified.

synthesis, which has challenged the concept of a role for FGFs in angiogenesis. Models for release of aFGF or bFGF in conjunction with temperature stress (30) or through disruptions in the plasma membrane (31) have been put forward. A role for aFGF and bFGF in angiogenesis is supported by data generated in vivo on stimulation of endothelial cell proliferation and vessel formation in the chick chorioallantoic membrane and rabbit cornea, as well as in vitro, on stimulation of endothelial cell proliferation, migration, and protease induction (2, 3). Using a chimeric receptor construct, we showed that FGFR-1-coupled signal transduction led to differentiation of murine brain endothelial cells in vitro, whereas a number of other growth factors for which receptors were expressed had no effect on differentiation of the cells. The weaker potency of induction of biological responses by the chimeric receptor, as compared when cells were stimulated with FGF, could possibly be due to that the stoichiometry of phosphorylation if the chimeric receptor was different from that of the endogenous FGFR-1. Another possibility is that other endogenous FGFRs such as FGFR-2 and FGFR-4, contributed to the FGF-induced responses. Together, our results indicate that FGFR-1 has the capacity to couple to a specific signal transducer(s) involved in generating the differentiation response. The activated FGFR-1 is known to bind and tyrosine phosphorylate phospholipase Cγ (32), but the biological significance of activation of phospholipase Cγ presently is unclear (33–35). Thus, our ongoing work on expression of truncated and point-mutated FGFR-1, in the murine brain endothelial cells, will make it possible to identify the structural requirement in the receptor for transduction of the differentiation response. Future work on the creation of transgenic mice carrying a “knock-in” of an FGFR-1 mutated at the critical site will show the in vivo relevance of this strategy.

A number of growth factors, for which we showed expression of functional receptors on the murine brain endothelial cell line, were examined for their effect in the different biological assays. EGF was able to stimulate growth, migration, and PA induction. PDGF-BB stimulated growth and migration, whereas HGF stimulated only migration. None of these factors alone (see Fig. 4), nor different combinations of EGF, PDGF-BB, and HGF added together (data not shown), induced differentiation of the endothelial cells. Different reports have, however, described that these factors can induce angiogenesis in vivo. It is conceivable that EGF, PDGF-BB, and HGF can support angiogenesis in vivo i.e., by stimulating one of several necessary steps, or by regulating expression of other growth factors or growth factor receptors. HGF has been shown to stimulate endothelial cells from adrenal cortex to undergo proliferation, migration, induction of PA ac-
Fig. 6. Growth factor receptor expression on the murine brain capillary endothelial cells. A, expression of EGF receptor, FGFR-1, PDGF β-receptor, and c-met/HGF receptor were analyzed using in vitro kinase assays following immunoprecipitation with specific anti-receptor antibodies, with or without stimulation of cells with the appropriate ligand. After precipitation of receptor proteins, [γ-32P]ATP was incorporated into proteins, and the samples were separated by SDS-PAGE, which was fixed, dried, and exposed. B, examination of Flt-1 expression by immunoblotting and metabolic labeling. Endothelial cells stimulated (+) or not (-) with 100 ng/ml VEGF, were lysed and immunoprecipitated with the anti-Flt-1 antibody. Samples were separated by SDS-PAGE, and proteins were transferred to nitrocellulose membrane. Immunoblotting was performed using the Flt-1 antibody, in the presence or absence of competing peptide, or using anti-phosphotyrosine antibodies. Immunoreactive proteins were detected by enhanced chemiluminescence. The endothelial cells were also metabolically labeled using [35S]methionine, lysed, and immunoprecipitated using nonimmune serum or the Flt-1 antiserum. The samples were analyzed by SDS-PAGE, and the dried gel was processed using an Image Analyzer (Fuji).

activity, and tube-like structure formation in collagen gels in vitro, whereas human omental endothelial cells were stimulated by HGF only to undergo proliferation (36). The varying effects of HGF on endothelial cells from different tissues might reflect that these endothelial cells may express subsets of receptor tyrosine kinases and thus be uniquely regulated. In addition, splice variants of the HGF receptor have been described. In mouse tissues, two major isoforms exist that differ by a 47-amino acid segment in the juxtamembrane domain. The isoforms have distinct signaling properties, and the p85 subunit of phosphatidylinositol 3'-kinase has been shown to interact much more efficiently with the shorter HGFR variant (37). In this context, it is noteworthy that PDGF-BB, but not HGF treatment of the brain-derived endothelial cells, induced phosphatidylinositol 3'-kinase activity. On the other hand, HGF treatment induced S6 kinase activity (data not shown).

We also examined the effects on the murine brain endothelial cells of TGF-β1, which is known as an inhibitor for angiogenesis in vitro (38). Using affinity cross-linking of 125I-labeled TGF-β1, all three types of TGF-β receptors could be detected on the murine brain endothelial cells (data not shown). TGF-β1 had no effect on the growth or tube formation of the cells, but it stimulated motility and inhibited the induction of PA activity. Expression of large T has been shown to lead to attenuation of the growth-inhibitory effect of
Fig. 7. Transduction of biological responses by the PDGFR-α/FGFR-1 chimeric receptor. A, in vitro kinase assay. Chimeric receptor expressing cells with or without stimulation with PDGF-AA were lysed, immunoprecipitated with anti-FGFR-1 antibody, subjected to kinase assay in the presence of [γ-32P]ATP, followed by SDS-PAGE and autoradiography. B, DNA synthesis. The tsA58 cells expressing the chimeric receptor were dose-dependently stimulated by PDGF-AA. DNA synthesis was measured as described in "Materials and Methods." bFGF stimulation was used as a positive control. C, migration. Migration, measured as described in the legend of Fig. 2, was stimulated by PDGF-AA. bFGF was used as a positive control. D, PA activity. Induction of PA activity in the endothelial cells expressing chimeric receptor was stimulated by PDGF-AA. The activity was measured as described in the legend of Fig. 3. The densities of the PA bands were 100% (no factor), 110% (PDGF-AA, 5 ng/ml), 147% (PDGF-AA, 50 ng/ml), and 360% (bFGF, 5 ng/ml), respectively. E, differentiation. Tube formation of the endothelial cells expressing chimeric receptor in collagen gel, performed as described in the legend of Fig. 4, was stimulated by PDGF-AA as well as by bFGF. ×152.
TGF-β (39). Since SV40 T antigen is expressed in the tsA58 cells, it may affect TGF-β-coupled signaling pathway for growth and migration of the cells, but apparently not for the inhibitory action on PA activity (40). This would indicate that the different inhibitory effects of TGF-β on growth, migration, and PA induction arise through different signal transduction pathways.

There are two receptors for VEGF, Flt-1 and Flik-1/KDR (41–43). Both receptors are expressed during development, but they are down-regulated to undetectable levels in adult brain endothelial cells, as assessed by in situ hybridization (44–46). Flik-1/KDR has an essential role in vasculogenesis during development (47) and is expressed on endothelial cells in glioblastomas but not in normal brain. This is in agreement with the fact that the cell line we have established from murine brain lacks expression of Flik-1/KDR. We could detect a certain level of Flt-1 expression; it is not clear why Flt-1, but not Flik-1/KDR, apparently becomes up-regulated during culture. It is alternatively possible that the cell line represents a subpopulation that was selected during establishment, maybe in response to inclusion of ECGS in the culture medium. Culture in the presence of VEGF in the establishment of new endothelial cell lines will be tested for the potential selection of Flik-1/KDR-expressing cells. VEGF stimulation of Flik-1/KDR-expressing cells leads to autophosphorylation of the receptor, migration of cells, and proliferation (28, 45). Flt-1, on the other hand, fails to autophosphorylate appreciably and to transduce biological responses, and the role of this receptor is unclear (28, 48). Thus far, we have failed to record a response to VEGF in murine brain endothelial cells, as expected from the expression pattern of VEGF receptors on these cells. However, in response to VEGF stimulation, we detected an abundant tyrosine-phosphorylated species of 150 kDa, which cross-reacted with Flt-1 antibodies. Identification of the molecular nature of this component could be critical for understanding signal transduction by Flt-1.

Materials and Methods

Growth Factors. Recombinant human EGF was a gift from Amgen, Inc. (Thousand Oaks, CA). Human recombinant PDGF-AA and PDGF-BB were gifts from Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Human recombinant bFGF was purchased from Farmitalia Carlo Erba, Italy. aFGF was a gift from Dr. Ralf F. Pettersson (Ludwig Institute for Cancer Research, Stockholm, Sweden). Human recombinant HGF was purchased from R&D Systems (Minneapolis, MN). Human recombinant VEGF165 was a gift from Dr. D. Gospodarowicz (Chiron Corp., Emeryville, CA). Human recombinant TGF-β1 was a gift from Kirin Brewery Company (Tokyo, Japan).

Isolation and Purification of Capillary Endothelial Cells from H-2Kb-tsA58 Transgenic Mouse Brain. Mouse brain capillaries were isolated from five 2-week-old H-2Kb-tsA58 transgenic mice (Charles River Laboratories, Wilmington, MA). The mice were killed by decapitation, and the brains were removed using sterile technique. After removing cerebellum, arachnoid membrane, and white matter, brain tissue was minced and homogenized by 10 strokes in a Dounce homogenizer with a loose pestle. Then, the homogenate was filtered through a 250 μm-pore nylon mesh, and after washing the mesh with PBS, remaining capillary fragments were collected and incubated into fibronectin-coated culture dishes and incubated for 2 h at 33°C in Ham’s F12 medium (GIBCO-BRL). Thereafter, capillary fragments were treated with collagenase type VIII (Sigma Chemical Co., St. Louis, MO; 1 mg/ml) for 5 min at 37°C to digest basement membranes. After washing in PBS, fragments were cultured at 33°C in a growth medium composed of Ham’s F12 containing 20% FBS (GIBCO-BRL), 150 μg/ml ECGS (Sigma), 10 ng/ml EGF, 5 μg/ml insulin (from bovine pancreas, Sigma), and 20 units/ml recombinant mouse INF-γ (Genzyme, Cambridge, MA). The culture was continued; after the 6th passage, cells were harvested by trypsinization, and endothelial cells were purified using lectin-coated Dynabeads (Dyna1, Oslo, Norway; Ref. 21) with minor modification. Briefly, tosylactivated Dynabeads were coated with BS-1 lectin and mixed with harvested cells at a ratio of 50 beads: 1 cell and incubated for 10 min at 4°C with gentle mixing. Then the rosetted cells were washed five times using a magnet, and the bead-cell mixture was resuspended in Ham’s F12 containing 20% FBS and 0.01 μM 1-O-methyl-α-L-galactopyranoside and incubated for 10 min at room temperature to dissociate cells from the beads. The beads were removed using a magnet, and the recovered cells were cultured in growth medium at 33°C.

Immunofluorescence Staining. All procedures were performed at room temperature (21°C). Cultured cells grown in fibronectin- and gelatin-coated Lab Tek chambers were washed with PBS, fixed with acetone, and incubated with 5% normal goat serum. After that, the cells were incubated with antiserum to a monoclonal fibronectin IgG antibody (DakoPatts; 1:200 dilution), anti-human 3-1-smooth muscle actin IgG (DakoPatts; 1:100 dilution), a monoclonal anti-VEGF receptor antibody, anti-factor VIII-related antigen antiserum (Behring Institute; 1:100 dilution), anti-SV40 large T antigen antiserum (a gift from Dr. David Lane, Dundee University, Dundee, Scotland; 1:100 dilution), or nonimmune serum as a negative control, for 60 min. After washing in PBS, the cells were incubated with biotinylated antirabbit or mouse IgG antibodies for 60 min, then washed again and incubated further with FITC-labeled streptavidin for 30 min. After a final wash, the cells were covered with glycerin and observed in a fluorescence microscope. To identify binding of lectins, fixed cells were incubated with FITC-labeled BS-1 or UEA-1 (Sigma) for 60 min, and after washing, cells were covered with glycerin and observed. The human foreskin fibroblast cell line, AG1521 (Human Genetic Mutant Cell Repository, Camden, NJ), was used as a negative control.

Uptake of Ac-LDL. To identify endothelial cells, Ac-LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL; Paesel + Lorei, Frankfurt, Germany) was added to the growth medium at a concentration of 1 μg/ml, and the culture was continued at the indicated temperature (18). After 4 h, the cells in Lab Tek chambers were washed and fixed with 10% buffered formalin. After washing in PBS, cells were covered with glycerin, and observed in a fluorescence microscope. AG1521 cells were used as a negative control.

Growth Curve Determination. The cells maintained at 33°C in growth medium were inoculated into fibronectin- and gelatin-coated wells of 12-well plates at a density of 1.3 × 10⁴ cells/cm² and cultured at 33°C or 39°C in growth medium with or without 20 units/ml mouse IFN-γ. The medium was changed every other day; on the indicated day, the cells were trypsinized, and the number of the cells was determined using a hemacytometer.

DNA Synthesis and Labeling Index. The cells were inoculated into fibronectin- and gelatin-coated wells of 24-well plates at a density of 1.7 × 10⁴ cells/cm² in the growth medium, and the culture started at 33°C or 39°C. On the next day, the medium was changed to Ham’s F12 containing 0.25% BSA and 20 units/ml aprotinin to synchronize the cell cycle. Two days later, the medium was changed to fresh serum-free medium, and growth factors were added. The cells were incubated for 18 h in the case of 33°C culture or 22 h for 39°C culture. Then, 1 μg/ml BrdU-labeled deoxyuridine was added, and incubation continued for 4 h. After aspiration of medium containing labeled deoxyuridine, cells were fixed in 10% trichloroacetic acid and lysed with 1 N NaOH. The incorporation of 15N-labeled deoxyuridine into DNA was measured in a gamma counter. For the assay of labeling index, 10 μg/ml BrdU was added, and the cells were washed and cultured for 4 h. The cells were then washed once, resuspended in culture medium at 70% ethanol at 4°C for 20 min. After washing, 4 N HCl was added, and the samples were incubated for 20 min and then washed 5 times with PBS. Anti-BrdU monoclonal antibody (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) was added to the cells and incubated for 60 min at room temperature. The cells were washed three times with PBS and then incubated with FITC-conjugated mouse IgG for 30 min. Labeled nuclei with BrdU were visualized by incubation with diamobenzidine solution, and counterstaining of whole nuclei was performed with a light hematoxylin staining. Over 1000 endothelial cell nuclei were counted in each well, and the labeling index was expressed as percentage of the number of labeled nuclei/total nuclei.
Migration Assay. Migration (chemokinesis) was measured using a Boyden chamber, essentially as described (23). A Costar nucleopore filter (8 μm pore) was coated with type I collagen and fibronectin. Endothelial cells were harvested and resuspended at a cell density of 4 × 10^5 cells/mL in Ham’s F12 containing 0.25% BSA. Fifty μl of the cell suspension, with or without growth factors, was added to the upper chambers and incubated for 24 h at 37°C in 5% CO_2. After incubation, the membranes were removed, fixed in methanol, washed with water, and stained with Giemsa solution. After drying, the stained filters were visualized and quantified using an automated image analyzer (Nihon Denshi, Tokyo, Japan).

PA Activity. Cells were cultured into fibronectin- and gelatin-coated wells of 12-well plates and cultured in growth medium at 37°C. When cells reached confluency, the medium was changed to Ham’s F12 containing 0.25% BSA and serum-free conditions were maintained at 37°C or 39°C. On the next day, the medium was discarded, and fresh Ham’s F12 containing 0.25% BSA, with or without growth factors, was added to the cells, and incubation was continued for another 24 h at the same temperature. At the second day, the medium was removed from the plate, and the plates were washed three times with PBS containing 0.1% bSA. The plates were incubated under 5% CO_2 for 24 h at 37°C or 39°C. After incubation, the cells were detached with trypsin and counted using a hemocytometer.

Tube Formation Assay. Tube formation was performed according to the method described previously (50) with minor modifications. Type I collagen with 10× Ham’s F12 and 2% FBS was added to the wells of 12-well plates. After gelation, the cells suspended in Ham’s F12 containing 0.25% BSA were added to the wells containing the collagen gel. After gelation, the collagen gel was removed and cultured in growth medium for 12 h. After incubation, the cells were detached with trypsin and counted using a hemocytometer.

Histological Analysis of Tube-like Structures in Collagen gels. The tubes in the collagen gel were frozen in dry ice/acetone. The frozen gel was cut into small pieces, which were put in Tissue Tek OCT compound (Miles, Inc., Elkhart, IN) and then frozen again. After making 8 μm-thick sagittal cryosections, samples were fixed with 95% ethanol and stained with hematoxylin solution. After mounting, sections were examined using light microscopy.

Confocal Microscopy. Cells growing in 12-well dishes were stained with DIII from Molecular Probes, Inc. (Eugene OR) as follows. A 1× stock solution of DIII was made in 99.5% EIOH. The cells were incubated with 20 μM DIII diluted in growth medium for 15 min at 37°C, and then after washing twice with Ham’s F12 medium, the cells were cultured overnight in growth medium. The following day, the endothelial cells were stimulated with 10 ng/ml bFGF to induce tube formation, as described above. After 2 h stimulation, the cells were fixed with 10% buffered formalin for 8 h at room temperature and stored at 4°C until analysis. To analyze the three-dimensional structure, a Molecular Dynamics 2001 inverted confocal microscope was used. DIII was excited by the 568 nm line of the Krypton/Argon laser and the emission filter was an E505LP. The Nikon PlanApo 10× 0.45 NA objective, together with a ×2.0 electronic zoom, was used to capture stacks of 70 images, each 512 × 512 pixels.

Antibodies. Anti-EGF receptor antibody and antiphosphotyrosine monoclonal antibody, PY20, were purchased from Transduction Laboratories (Lexington, KY). Anti-Fit-1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PDGFR α-receptor, PDGFR-β (51), and anti-PDGFR β-receptor, PDGFR-3 (52), antibodies have been described previously. Anti-FGF-1 IgG was raised and affinity purified as described (53). Anti-mouse c-met/HGF receptor antisemur (54) was a kind gift from Dr. K. Michael Weidner (Max-Debrueck-Centrum, Berlin, Germany), and antisemur against FGFR-4 (55) was a generous gift from Dr. K. Altaloo (Molecular and Cancer Laboratory, University of Helsinki, Helsinki, Finland).

In vitro Tube Assay. Cultures of endothelial cells in 10-well plates were cultured in Ham’s F12 containing 0.25% BSA and 2 units/ml aprotinin for 18 h at 37°C. After stimulation with different ligands at 37°C for 8 h, cells were rinsed with ice-cold PBS and lysed in Triton X-100 lysis buffer [20 mM Tris HCl (pH 7.5), 150 mM NaCl, 10% glycerin, 1% Triton X-100, 5 mM EDTA, 0.5 mM Na_3VO_4, 200 units/ml aprotinin, and 1 mM DTI]. After a 15-min centrifugation at 15,000 g in the cold, the supernatant was used for immunoprecipitation with specific antibodies against growth factor receptors. The immune complex was collected using protein A-Sepharose CL-4B; then the beads were washed and resuspended in kinase buffer [20 mM HEPES (pH 7.5), 10 mM MgCl_2, 2 mM MnCl_2, 0.05% Triton X-100, and 1 mM DTI], and in vitro phosphorylation was performed in the presence of [γ-32P]ATP for 10 min on ice. The reactions were stopped by addition of 2× sample buffer [8% SDS, 0.4% Triton X-100 (pH 8.8), 1 x sucrose, 10 mM EDTA, 0.02% bromophenol blue, and 4% 2-mercaptoethanol]. The samples were heated at 95°C for 4 min and analyzed through SDS-gel electrophoresis in 5–12% gradient polyacrylamide gels. After fixation in methanol-acetic acid, the gel was treated with 1% KOH for 30 min at 55°C, fixed again, dried, and exposed on XAR films (Fuji).

Molecular and Immunoprecipitation. Endothelial cells were labeled in methionine-free MCDB 104 medium supplemented with 100 μCI/ml of [35S]methionine (Amersham) for 3 h at 37°C. After labeling, the cells were washed, lysed in lysis buffer [20 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 10 mM EDTA, 30 mM pyrophosphate, 1 mM PMSF, and 100 units/ml aprotinin] and immunoprecipitated with anti-Fit-1 antibody or nonimmune serum, followed by adsorption to protein A-Sepharose CL-4B. After extensive washing, labeled receptor protein was eluted from the beads by heating in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis in 5–12% gradient gels. After fixation, the gel was treated with Amplify (Amersham), dried, and exposed.

Immunoprecipitation and Immunoblotting. Confusion cultures in 15-cm dishes were cultured at 33°C in Ham’s F12 containing 0.25% BSA and 2 units/ml aprotinin for 18 h. The cells were stimulated with 100 ng/ml VEGF_165 for 8 min at 37°C, and after washing, cells were lysed in NP40 buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1% glycerin, 1% NP40, 0.1 mM Na_3VO_4, 100 units/ml aprotinin, 1 mM PMSF, and 2.5 mM EDTA] and immunoprecipitated with anti-Fit-1 antibody, followed by adsorption to protein A-Sepharose CL-4B. After washing, the samples were eluted from beads by heating in sample buffer and then separated by SDS-polyacrylamide gel electrophoresis in 5–12% gradient gels. After electrophoretic transfer onto a nitrocellulose membrane (Hybond-C; Amersham), the blot was probed with anti-Fit-1 antibody or PY20. Alternatively, peptide-blocked antibody was used. For this purpose, 50 μg of the Fit-1 peptide used for immunization (amino acids 1348–1367) was incubated with 20 μg of the Fit-1 antibody at 4°C for 18 h. Antibody incubation was followed by incubation with horseradish peroxidase-conjugated antirabbit IgG or antimouse IgG and detection through enhanced chemiluminescence (ECL; Amersham).

Plasmids. cDNAs for FGFR-1 (53) and PDGFR-α (56) were subcloned into the pALTER vector (Promega Corporation, Madison, WI), and site-directed mutagenesis was performed using the Altered Site Plus Mutagenesis system (Promega Corp.). Point mutations that created a cleavage site for HindIII at position 1195–1200 of the FGFR-1 and 1776–1781 of the PDGFR-α were then introduced into the respective insert with oligonucleotides prepared using a Pharmacia LKB Gene Assembly Plus Synthesizer. The chimeric PDGFR-α/FGFR-1 was constructed by cleaving the FGFR-1 and PDGFR-α cDNAs with HindIII and SalI, followed by ligation of the fragment containing the entire extracellular part of PDGFR-α to that corresponding to the intracellular part of FGFR-1. All mutations and constructs were confirmed by nucleotide sequencing. The chimeric receptor cDNA was inserted into the eukaryotic expression vector pcDNA1/new (Invitrogen).
Transfection. Circular plasmid DNA, purified by CsCl gradient centrifugation, was introduced into mouse brain capillary endothelial cells using electroporation, as described by Westermark et al. (57). Selection of transfected cells was initiated after 48 h by adding G418 sulfate (GIBCO-BRL) at 0.3 mg/ml to the culture medium. Clones were picked after 2 weeks and scored for positive clones by in vitro kinase assay using the PDGF α-receptor antisem.

Acknowledgments
We appreciate the generosity of Drs. K. Michael Weidner and Walter Birchmeier for providing the antibody against mouse c-met/HGF receptor; Dr. Karl Alltalo for the anti-FGF-4 antisem; Dr. Kohei Miyaizono for the antisem against types I, II, and III receptors for TGF-β; Dr. David Lane for antisem against SV40 large T antigen; Kirin Brewery Company for recombinant human TGF-β1; Amgen, Inc. for recombinant human EGF; Dr. Denis Goszcadowicz for recombinant VEGFα; Dr. Ralf F. Pettersson for aFGF; and Dr. Petra Franzén for iodinated TGF-β1. We also thank Drs. Mitsuyasu Kato and Monica Hamansson for advice on isolation of capillary fragments from murine brain, Dr. Koutaro Yokote for advice on the migration assay, Dr. Jorma Keski-Oja for advice on PA assay, Jill Sandström for excellent technical assistance, Ingeláed Schlirer for secretarial assistance, and Drs. Mark Noble, Carl-Henrik Heldin, and Bengt Westermark for fruitful discussions.

References


