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Differential mitogenic responses of human macrovascular and microvascular endothelial cells to cytokines underline their phenotypic heterogeneity

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Abstract. A variety of growth factors promote the complex multistep process of angiogenesis. The mitogenic activity of vascular endothelial growth factors (VEGFs) and placental growth factors (PlGFs), known as cytokines acting predominantly on endothelial cells, was tested on human umbilical vein endothelial cells (HUVEC) and microvascular endothelial cells (MIEC) and compared with the potency of the universally acting basic fibroblast growth factor (FGF-2). The cells were seeded at different cell numbers and incubated with various doses of growth factors for a period of 24–72 h in culture medium ± serum. Proliferation was determined by measuring the optical density after staining the cells with the tetrazolium salt WST-1.

VEGF_{121} and VEGF_{165} increased the number of HUVEC and MIEC at low and high seeding densities various doses and incubation times. The efficiency of FGF-2 was less pronounced at high seeding densities of the cells under serum-free conditions. PlGF-1 and PlGF-2 stimulated mitogenesis on HUVEC only at low cell numbers and after a short incubation time by 125 ± 3% and 102 ± 5% ($P < 0.001$), respectively. Longer incubation times with the lower seeding density in the absence of FCS did not induce a significant stimulatory effect of the PlGFs. MIEC responded stronger to all growth factors. In particular under serum free conditions, PlGF-1 and PlGF-2 effectively stimulated cell proliferation by 247 ± 54% ($P < 0.01$) and 288 ± 40% ($P < 0.05$) at low cell numbers, and by 81 ± 13% ($P < 0.05$) and 49 ± 13% ($P < 0.01$), respectively, at high cell numbers. The addition of fetal calf serum caused a reduced proliferative response of all growth factors on both cell types related to the controls. In conclusion, MIEC and HUVEC differ in their proliferative response to VEGFs, PlGFs and FGF-2.

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INTRODUCTION

Angiogenesis, the formation of new blood vessels, plays an important role in many physiological processes. A variety of peptide growth factors, in particular the heparin-binding growth factors, promote this complex multistep process, which includes endothelial cell proliferation *in vitro* and *in vivo* (Kumar *et al.* 1998). The only growth factors known to act almost exclusively on endothelial cells (besides their reactivity on placental trophoblast) are the vascular endothelial growth factors (VEGFs) and the placental growth factors (PlGFs) (review Neufeld *et al.* 1999, review Torry *et al.* 1999). VEGFs and PlGFs are members of a dimeric growth factor subfamily with homologous sequences to platelet derived growth factor (Ferrara *et al.* 1992; Terman *et al.* 1992; DiSalvo *et al.* 1995). VEGFs and PlGFs exist as multiple, alternatively spliced isoforms that may constitute various homodimers as well as heterodimers via interchain disulphide bridge formations (Ferrara *et al.* 1991; Hauser & Weich 1993; Maglione *et al.* 1993). The free soluble protein isoforms VEGF_{121}, VEGF_{165}, PlG$F_{131/149}$ (PlGF-1) and PlG$F_{201/219}$ (PlGF-3) are assumed to induce mitosis of endothelial cells. The highly basic proteins VEGF_{145}, VEGF_{189}, VEGF_{206} and PlG$F_{152/170}$ (PlGF-2) are bound to heparin-containing proteoglycans on the cell surface, basement membrane or extracellular matrix and rather act as vascular permeability factors (Ferrara *et al.* 1991; Hauser & Weich 1993; Maglione *et al.* 1993; Cao *et al.* 1997). VEGF mRNA and protein are expressed in diverse cell types of mesenchymal and epithelial origin of several organs (Ferrara *et al.* 1991), whereas the expression of PlGF mRNA and protein is mainly restricted to placental tissues and human umbilical vein endothelial cells (HUVEC) (Hauser & Weich 1993; Khalig *et al.* 1999; Torry *et al.* 1999).

The high affinity receptors for VEGFs are KDR (kinase insert domain-containing receptor) and flt-1 (Fms-like tyrosine kinase) proteins. The localization of KDR and flt-1 mRNA is restricted to vascular endothelial and trophoblast cells (Neufeld *et al.* 1999; Torry *et al.* 1999). KDR is regarded as the major regulator of vasculogenesis and angiogenesis (Millauer et al. 1993; Cunningham *et al.* 1999a,b) and transduces signals for mitogenicity, chemotaxis, actin reorganization, mobilization of intracellular Ca^{2+} and changes of gross morphology of the cell. Flt-1 does not signal any of these effects in response to VEGF, despite its ability to bind VEGF with higher affinity than KDR (Waltenberger *et al.* 1994). PlGFs bind with high affinity to flt-1, but not to KDR receptors and appear to have weak growth stimulatory effects on endothelial cells.

Most of the studies into mitogenic effects of endothelial growth factors have been carried out on HUVECs, but they produced varying results. Low doses of VEGF_{165} were shown to induce a three- to fourfold increase in the final cell count of HUVEC, whereas the major isoform of the PlGF family, PlGF-2, caused only a 10–20% growth stimulation at high concentrations (Park *et al.* 1994). Other studies reported about a 40% lower efficiency of PlGF-2 as compared to VEGF (Sawano *et al.* 1996). PlGF-1 seemed to have negligible effects on HUVEC proliferation when cells were exposed to PlGF-1 for a long period of time (3–7 days) in the presence of high serum concentrations (Park *et al.* 1994; Sawano *et al.* 1996; Cao *et al.* 1997). In contrast, short-term exposure (48 h) to PlGF-1 at low serum concentrations resulted in a dose dependent increase in HUVEC proliferation, but with significantly weaker mitogenic potency than VEGF_{165} (Ziche *et al.* 1997). In very recent studies PlGF-1 and -2 induced the migration of endothelial cells, but had, if any, either weak stimulating or even inhibiting effects on proliferation (Migdal *et al.* 1998; Khalig *et al.* 1999).

Endothelial cells are morphologically and functionally heterogeneous with the greatest differences between those from the macro- and microcirculation as documented in a variety of tissues (review Garlanda & Dejana 1997). In the human full term placenta this heterogeneity

was reflected by different antigenic properties and glycosylation patterns between macro- and microvascular endothelial cells (Lang *et al.* 1993, 1994). The mitogenic activity of endothelial growth factors is tissue-specific and differs with the location of the endothelial cell (Millauer *et al.* 1994; Waltenberger *et al.* 1994; Seetharam *et al.* 1995). Human dermal microvascular endothelial cells (MIEC) and HUVEC differ in their dose–response characteristic towards VEGF (Gupta *et al.* 1997).

Different experimental conditions are likely to account for opposing effects of growth factors on the *same* cell type, as was shown on HUVEC, and results on cells from the macro- and microcirculation cannot be compared at all. The diversity of parameters obviously affecting the activity of endothelial cell mitogens calls for a detailed and systematic analysis. The present study tested the hypothesis that different experimental conditions account for the inconsistent data. Therefore, we concentrated on well-known confounding factors, which have varied among the studies, i.e. concentration of cytokines, presence or absence of serum, duration of incubation as well as seeding density. Because of the well known heterogeneity of endothelial cells we compared the proliferative effects of the most frequently used VEGF-and PlGF isoforms on HUVEC and MIEC. In addition the universally acting basic fibroblast growth factor (FGF-2), an 18 kd heparin binding protein monomer, was included in the study because it stimulates the proliferation of a variety of cell types regardless of their origin from the macro- or microcirculation (for review see Szebenyi & Fallon 1999).

MATERIALS AND METHODS

Cell culture

HUVEC and MIEC were purchased from BioWhittaker-Clonetics (Verviers, Belgium) and cultured in BioWhittaker-Clonetics complete medium® containing 5% (v/v) dialysed fetal bovine serum, bovine brain extract (18 μg/ml), rhEGF (10 ng/ml), hydrocortisone (1.0 μg/ml), gentamycin (50 mg/ml) and amphotericin B (50 ng/ml). All experiments were carried out with the same serum batch. Cells were only used up to the fifth passage to avoid phenotypic drift. BioWhittaker-Clonetics basal medium® (modified MCDB 131 formulation) without supplements but with gentamycin and amphotericin B was used for the proliferation assays.

Characterization of the cells

For each passage the identity of the endothelial cells was tested with monoclonal von Willebrand factor antibody (Dakopatts, Glostrup, Denmark) and Ulex europaeus lectin (Sigma, Taufkirchen, Germany). Both are standard markers for endothelial cells. HUVEC and MIEC were grown and stained on chamber slides (Lab-Tek, Nalgene Nunc International, Naperville, USA). At confluence, the chamber slides were washed in HBSS (Life Technologies Gibco, Wien, Austria), fixed in acetone for 3 min at room temperature and stained as previously described (Lang *et al.* 1993).

The cells were further characterized by measuring LDL uptake: acetylated low density lipoprotein labelled with 1,1′-dioctadecyl – 3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, Stoughton MA, USA) was diluted to 10 μg/ml in the culture medium, added to the living cells and incubated for 4 h at 37 °C. The culture medium was removed and the cells were washed in PBS (three times). The cells were fixed in 3% formaldehyde/PBS for 20 min at room temperature, rinsed in distilled water (5 s) and mounted in glycerol/PBS (90%/10%). Stained slides were examined using a Zeiss Axiophot microscope. According to these measurements more than 99% of the cells were viable endothelial cells at seeding.

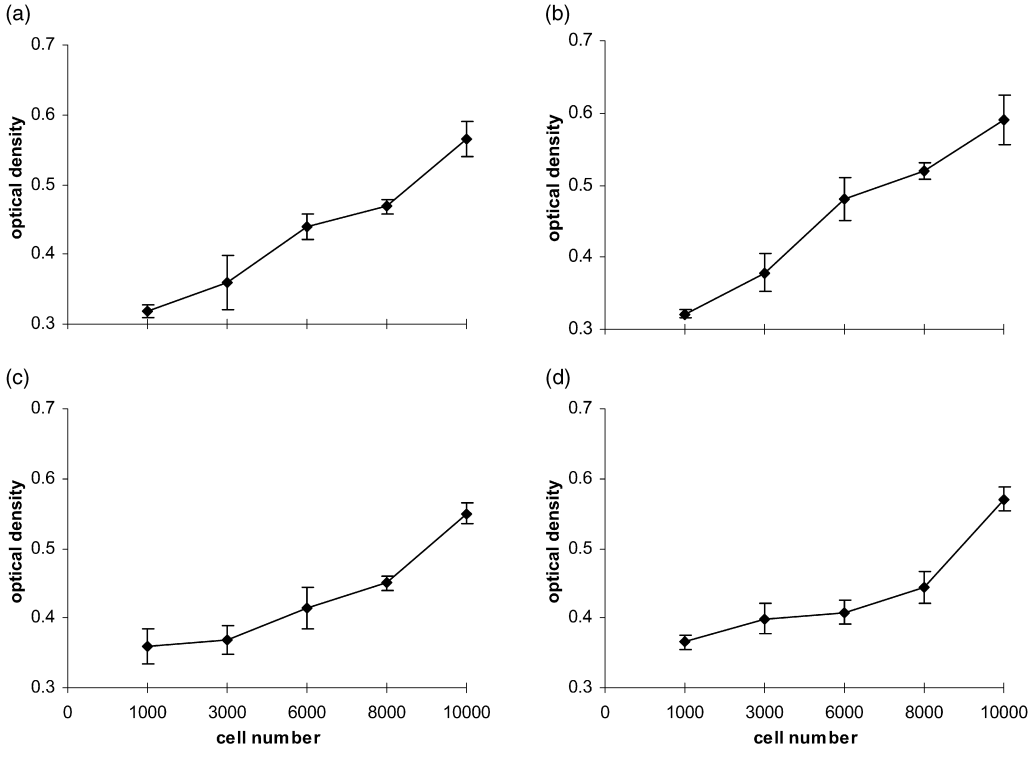

Figure 1. Correlation between the number of HUVEC (a, b) and MIEC (c, d) with the optical density of the WST-1 reaction product measured after 24 h in basal medium with FCS (a, c) and complete medium with FCS (b, d).

Cell proliferation assay

HUVEC and MIEC were seeded at a density of 3×10^3 and 6×10^3 cells/well on 96 well microtiter plates, which had been precoated with 1% (v/v) gelatin (Sigma) in HBSS for 1 h at 37 °C. Two hundred microlitres basal medium were added with or without 2.5% fetal calf serum and either without (control) or with 1, 10, 50, 100 ng/ml of VEGF_{121}, VEGF_{165}, PlGF-1, PlGF-2 and FGF-2 (Strathmann Biotech, Hannover, Germany). The concentration of the cytokines was within the range applied in previous experiments (Park *et al.* 1994; Sawano *et al.* 1996; Cao *et al.* 1997; Ziche *et al.* 1997; Migdal *et al.* 1998).

The mitogenic activity of the growth factors was determined by a colourimetric assay based on formazan dye formation (WST-1, Boehringer Mannheim, Mannheim, Germany), which directly correlates with the number of metabolically active cells in the culture. After incubation of the cells for a period of 24, 48 and 72 h, 20 µl/well of the reagent WST-1 were added and incubated for 1 h at 37 °C. An increase in the number of viable cells resulted in an increase in the overall activity of mitochondrial dehydrogenases in the sample with an ensuing increase in formazan dye formation. The formazan dye was quantified by measuring the optical density of the dye solution at 450 nm with a scanning multiwell spectrophotometer (Spectramax 250, MWG-Biotech, Germany) using 630 nm as the internal reference. In pilot experiments the optical density correlated with the number of both cell types under two different medium conditions (Fig. 1). Cell numbers smaller than 1000 did not correlate (not shown). Therefore, cell numbers

of greater than 1000 were used throughout the study. All results in the study were based on at least five parallel measurements each time and repeated up to five independent experiments.

Data analysis and statistics

Effect is defined as the growth factor-induced increment in optical density exceeding controls expressed relative to the increase of the controls over the culture time. Data are presented as medians (± SEM) of the percentage of control. Statistical comparisons between groups were performed using the Wilcoxon rank sum test or one-way ANOVA followed by a post hoc test (Neuman-Keuls or Dunnett's), as appropriate. Differences among medians were considered significant when $P < 0.05$.

RESULTS

In pilot experiments cell proliferation was measured at various serum concentrations and seeding densities. Finally, serum concentration was adjusted so as to result in 40% and 50% confluency (visual inspection) of MIEC and HUVEC after 48 h in basal culture medium without growth factors at a seeding density of 6000 cells. In complete medium (containing supplements for the optimal growth of endothelial cells) after 72 h a confluency of 70% and 100% (visual inspection) was reached in MIEC and HUVEC, respectively.

At a seeding density of 6000 cells/well and in serum-containing medium the proliferation in the presence of the growth factors was similar to that in untreated cultures at 24 h (HUVEC, MIEC) and 48 h (HUVEC) and therefore likely due to a normal mitotic increase in cell number probably owing to serum effects. On MIEC the proliferative activity of the cytokines was observed already after 48 h (data not shown), but with maximal effects after 72 h. Untreated cultures exhibited a significant increase in cell number up to 72 h in the presence of serum except for only a small increase in MIEC at low seeding density. In the absence of serum only HUVEC proliferated (Fig. 2). In FCS absence no loss of cellular adherence to the gelatine coated plates was observed.

After 72 h a significant mitogenic effect of some growth factors was observed in both cell types in the presence of FCS at high seeding densities (Fig. 3). In HUVEC (Fig. 3a), maximum stimulatory effects of VEGF_{121}, VEGF_{165} and FGF-2 were obtained with 100 ng/ml (VEGF_{121} 37 ± 3%, FGF-2 34 ± 3%, $P < 0.001$, VEGF_{165} 16 ± 5%, $P < 0.05$). PlGF-1 caused no proliferative response and 100 ng/ml PlGF-2 inhibited cell proliferation (−4 ± 5%, $P < 0.01$). MIEC (Fig. 3b) were more sensitive to VEGF_{121} and VEGF_{165}, which achieved maximum effects at lower concentrations (10 ng/ml 46 ± 7% and 50 ng/ml 26 ± 3%, respectively, $P < 0.001$) than in HUVECs. FGF-2 increased the proliferation in a dose-dependent manner with maximum effects at 100 ng/ml (49 ± 9%, $P < 0.001$). PlGF-1 induced a weak proliferative response at various concentrations (1 ng/ml 13 ± 6%, 100 ng/ml 11 ± 5%, $P < 0.01$), but PlGF-2 was only mitogenic at 0.1 ng/ml (10 ± 5%, $P < 0.05$). The mitogenic potencies of VEGF_{121}, VEGF_{165} and FGF-2 (100 ng/ml) were similar in both cell types. Among the PlGFs only PlGF-1 was mitogenic on MIEC whereas in HUVEC PlGF-1 and -2 were not mitogenic (Fig. 3c).

The incubation of HUVEC and MIEC (6000 cells/well) in serum-free basal medium with 100 ng/ml of the respective growth factor for 24 h (Fig. 4a) and 48 h (Fig. 4b) resulted in more pronounced effects than in the presence of serum, and revealed differences in the kinetics of the cytokine effects. 1–50 ng/ml of the cytokines induced a similar mitogenic response on both cell types under these conditions (data not shown). In HUVEC pronounced proliferative responses

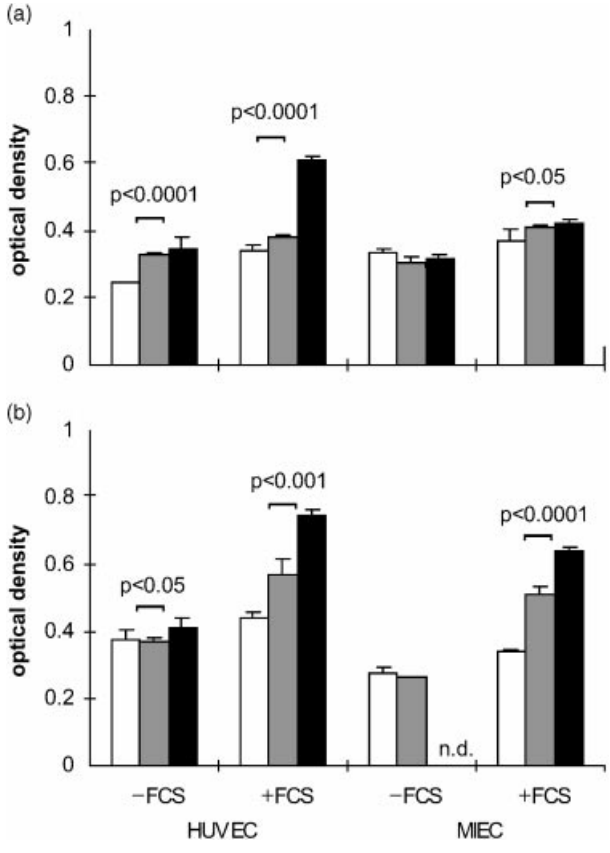

Figure 2. Influence of seeding density and FCS on the growth characteristics of HUVEC and MIEC in the absence of cytokines. (a) 3000 and (b) 6000 cells/well were seeded in the presence or absence of FCS and the optical density of the WST-1 reaction product was measured after 24 h (□), 48 h (■) and 72 h (■). In the presence of serum all control cultures exhibited a significant increase in cell density, whereas in the absence of serum only HUVEC proliferated. n.d. not determined.

were found after 24 h culture with VEGF_{121} (219 ± 21%, $P < 0.001$) and VEGF_{165} (148 ± 21%, $P < 0.01$), whereas the FGF-2 effect was weaker (76 ± 14%, $P < 0.01$). PlGF-1 and -2 did not stimulate proliferation under this condition. After 48 h only VEGF_{121} (176 ± 27%, $P < 0.001$) and VEGF_{165} (123 ± 23%, $P < 0.05$) stimulated HUVEC. MIEC responded to all tested cytokines after 24 h, except for FGF-2. VEGF_{121} and VEGF_{165} induced a proliferation of 105 ± 14% and of 86 ± 8% ($P < 0.01$), respectively. Under serum-free conditions PlGF-2 became mitogenic in MIEC (Fig. 3a,b) in contrast to serum presence (Fig. 3c). In HUVEC the cytokine-induced effects were greater ($P < 0.05$) after 24 h than after 48 h, whereas in MIEC the kinetics were reversed. Culture of MIEC for 72 h in serum-free medium caused a gradually loss of viability.

When the seeding density was reduced to 3000 cells/well the mitogenic effects of the cytokines (100 ng/ml) after 24 h without FCS were different from those with 6000 cells/well in both cell types (Fig. 5). At this low seeding density also the PlGFs became mitogenic in HUVECs with effects similar to those of the VEGFs. Except for VEGF_{165} the effects on MIEC were in general more pronounced than in HUVEC (Fig. 5). The addition of 2.5% FCS to basal medium

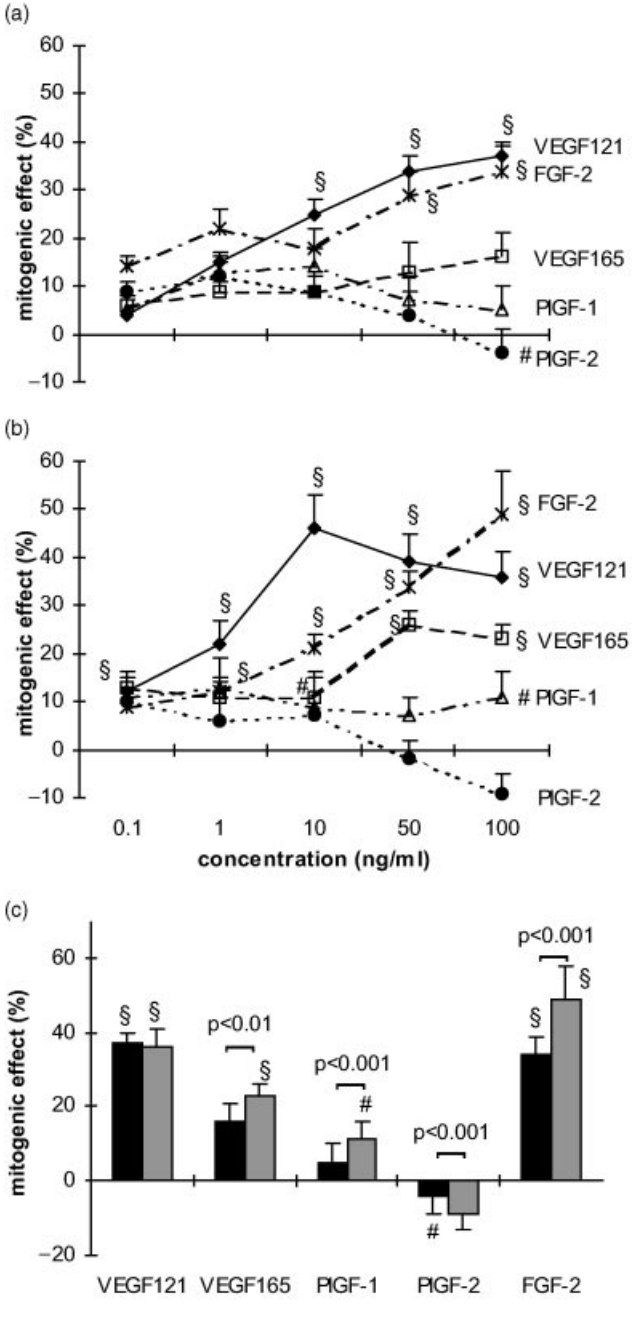

Figure 3. Dose-dependent effects of cytokines on HUVEC (a) and MIEC (b) expressed as percentage increase over untreated controls. Six-thousand cells/well were incubated for 72 h in basal medium with 2.5% FCS containing various amounts (0.1–100 ng/ml) of VEGF_{121}, VEGF_{165}, PlGF-1, PlGF-2 and FGF-2. (c) Comparison of the mitogenic effects of the cytokines at a concentration of 100 ng/ml between HUVEC (■) and MIEC (■). At this concentration VEGF_{121}, VEGF_{165} and FGF-2 are potent cell mitogens, PlGF-1 and PlGF-2 did not produce a significant effect under these conditions, except for a weak proliferative effect of PlGF-1 on MIEC. *$P < 0.05$, #$P < 0.01$, §$P < 0.001$ vs. untreated control.

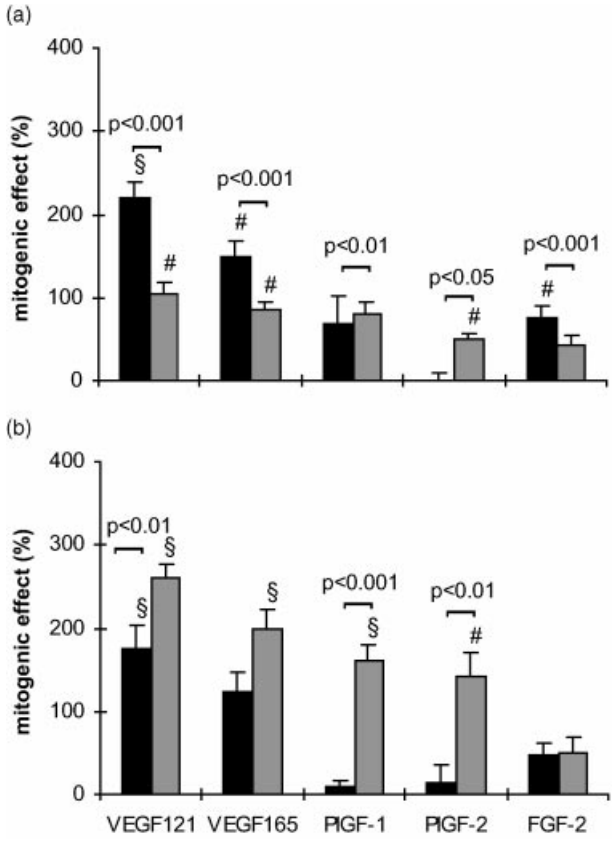

Figure 4. Kinetics of the cytokine effects on HUVEC (■) and MIEC (■) expressed as percentage increase over untreated controls. Six-thousand cells/well were incubated for 24 h (a) and 48 h (b) in serum-free basal medium in the presence or absence (control) of 100 ng/ml of the respective growth factor. In general, the response of MIEC increases, whereas that of HUVEC decreases from 24 to 48 h. $*P < 0.05$, $\#P < 0.01$, $\S P < 0.001$ vs. untreated control.

containing 100 ng/ml of the respective cytokine for 24 h caused a reduction in the mitogenic effect of PlGFs and FGF-2 in both cell types at a seeding density of 3000 cells/well (Fig. 5b).

DISCUSSION

Cell proliferation was quantified by the tetrazolium salt WST-1, which is reduced by mitochondrial dehydrogenases to a soluble and intensely coloured formazan. Its concentration, and hence optical density, depends on the number of metabolically active cells. These assays can be carried out in a microculture format and are therefore widely used to determine cellular growth (Marshall, Goodwin & Holt 1995).

The response of HUVEC to growth factors and inhibitors has been more widely characterized than that of any other endothelium (Bicknell 1993), nevertheless available information about the mitogenic effects of VEGFs and PlGFs is contradictory. The only agreement reached so far is the higher mitogenic potency of VEGFs as compared to PlGFs, probably due to the interaction of both growth factors with their type III receptor-tyrosine kinases.

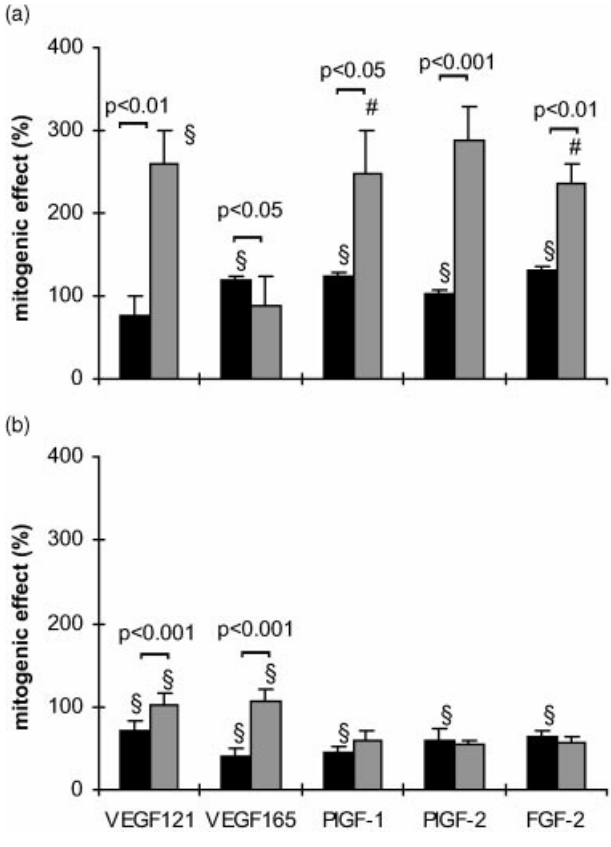

Figure 5. Serum-dependent effects of the cytokines on HUVEC (■) and MIEC (■) expressed as percentage increase over untreated controls. Three thousand cells/well were incubated for 24 h in serum-free basal medium (a) or in medium with FCS (b), in the presence or absence (control) of 100 ng/ml of the respective growth factor. The proliferative response of MIEC to growth factors is more dependent on serum presence than that of HUVEC. Note that at this seeding density PlGF-1 and PlGF-2 elicited a mitogenic effect also in HUVEC $*P < 0.05$, $\#P < 0.01$, $\S P < 0.001$ vs. untreated control.

HUVEC proliferated well in serum-containing medium regardless of the seeding density even in the absence of the endothelial growth factors and, thus, seem to be less demanding in their selective requirements than MIEC. Even serum-starvation for 72 h did not lead to a growth arrest. In contrast MIEC are less 'robust' and require the presence of some growth factors present either in serum or added to the culture medium. Whether this reflects distinct differences in the intracellular machinery regulating cell cycle progression is unknown but would deserve further investigation.

In the present study high doses of both VEGF_{121} and VEGF_{165} effectively stimulated proliferation under serum-free conditions, high cell seeding densities and during an incubation time of 24 h on both cell types. However, the reactivity of the PlGFs clearly differed between both cell lines. The MIEC responded to high doses of PlGF-1 and -2, whereas HUVEC did not respond, although the corresponding flt-1 receptor was localized on both cell types (Nomura *et al.* 1995; Detmar *et al.* 1997). Maximum effects of the growth factors related to the untreated control were achieved on HUVEC after 24 h and on MIEC after 48 h. This could indicate a lower efficiency

of the cytokine-induced stimulatory action on serum-starved HUVEC compared to MIEC. The presence of serum delayed the maximum growth factor response in HUVEC to 72 h, whereas it did not change the kinetics of response in MIEC. This difference in the kinetics may reflect that MIEC are more specific in their reaction to cytokines (Bicknell 1993).

After 72 h a significant mitogenic effect of some growth factors was observed on both cell types at high seeding densities. Increasing amounts of VEGF_{121} and VEGF_{165} lead to an increase of the mitogenic effect on HUVEC, whereas PlGF-1 and -2 failed to induce proliferation. On MIEC maximum effects of VEGF_{121} were achieved at lower concentrations and PlGF-1 induced a weak proliferative response. Apart from this both cell types similarly responded to the growth factors under this conditions.

The more uniform effects of PlGFs, if any, as compared to the VEGFs may reflect differential binding to and activation of their receptors. VEGFs bind with higher affinity to flt-1 than to the KDR receptor, resulting at first in effects not related to proliferation and became only active after binding to the obviously more essential Kdr receptor. Trophoblast growth is limited by VEGF mediated NO release via flt-1 receptor activation (Ahmed *et al.* 1997) indicating a growth suppressive function of the VEGF/flt-1 complex. PlGFs do not bind to KDR receptors, but PlGF/flt-1 interactions induce a weak proliferative response (Khalig *et al.* 1996; Sawano *et al.* 1996). A 10–20 fold molar excess of PlGF seemed to potentiate the mitogenic activity and vascular permeability action of low-dosed VEGF *in vitro* and *in vivo*, probably because of a competitive displacement of VEGF from flt-1 by PlGF, which may result in an increase in VEGF binding to the more relevant KDR receptor (Park *et al.* 1994; Sawano *et al.* 1996).

The most abundant isoforms VEGF_{121}, VEGF_{165}, PlGF-1, PlGF-2 and their receptors were shown to be colocalized in HUVEC and MIEC (Hauser & Weich 1993; Nomura *et al.* 1995; Detmar *et al.* 1997; Yonekura *et al.* 1999). It can be speculated that PlGFs modulate VEGF action by an autocrine mechanism. Alternatively, the formation of heterodimers between KDR and flt-1 might confer new properties or ligand specificities upon these receptors (Hauser & Weich 1993).

FGF-2, expressed widely during embryogenesis and in tissues of the human fetus, is a potent endothelial cell mitogen and has angiogenic activity *in vivo*. In accordance with our data, FGF-2 was reported to be more potent in stimulating HUVEC proliferation than VEGF_{165} at conditions of high cell numbers, serum concentration (10% FCS) and after 72 h of incubation (Yoshida, Anad-Apte & Zetter 1996). Under serum-free conditions FGF-2 was mitogenic predominantly at low seeding densities. At high seeding densities HUVEC responded to FGF-2 only after 24 h but MIEC did not respond at all. Interestingly, renal microvascular endothelial cells did not show any proliferative response to FGF-2 also under similar conditions (Khalig *et al.* 1999), suggesting that the absence of effect on microvascular endothelial cells is a general phenomenon.

The seeding density of the cells is one of the most important parameter when studying mitogenic effects of any growth factors on any cell type. The present study clearly confirmed this for both HUVEC and MIEC. Therefore, the seeding density is one parameter confounding studies into mitogenic effects of growth factors on endothelial cells, and may account for some of the opposing results published in the literature. Cells at low seeding densities are in the cell cycle and are more sensitive to growth factors, probably as a result of the lower self-stimulatory interactions between the cells.

After addition of serum, i.e. when the cells are in a proliferating state, the cell-specific differences of HUVEC and MIEC in their mitogenic response to growth factors are reduced. In general, cells in serum-free medium are more sensitive to growth factors compared to cells incubated in medium containing fetal calf serum. The smaller differences in cytokine sensitivity between the cell types in the presence of FCS could be the consequence of the reduced overall sensitivity under these

conditions. This, in turn, could be explained by other factors, such as the mitogenic effect of serum masking part of the cytokine effects, or the survival promoting effect of the cytokines.

In FCS absence no loss of cell adherence to the surface of the plates was observed. This differs from the apoptosis in HUVEC induced by serum deprivation (Gerber *et al.* 1998), but is likely the result of different matrices (Fukai *et al.* 1998) on which the cells had been cultured, i.e. gelatine (this study) and plastic (Gerber *et al.* 1998). VEGF and FGF-2 inhibit apoptosis and delay senescence in serum-free cultured HUVEC, whereas flt-1-specific ligands such as PlGF or an flt-1 selective VEGF mutant did not promote the survival of serum-starved primary human macrovascular endothelial cells (Gerber *et al.* 1998). This appears to be a cell-specific phenomenon, because in MIEC FGF-2 did not prevent apoptosis in contrast to VEGF (Watanabe & Dvorak 1997). In our study the mitogenic effects induced by the VEGFs were less serum-dependent compared with the PlGFs, which are effective mainly under serum-free conditions, i.e. when the cells are in G_0. Therefore, it can be speculated that PlGFs act as competence factors stimulating the G_0/G_1 transition, whereas the VEGFs with their cytokine sensitivity mainly in the presence of FCS may predominantly act as progression factors thereby stimulating the G_1/S transition. The latter notion is in line with recently published data demonstrating a VEGF-induced entry of endothelial cells in the S-phase of their cell cycle, an effect which was mediated by PI3-kinase (Thakker *et al.* 1999). The fact that in FCS absence HUVEC responded to the PlGFs only at low cell density, i.e. when the cells were in the cell cycle, further corroborates above hypothesis.

Throughout our study VEGF_{121} was more potent than VEGF_{165}. A similar result was found on MIEC using low serum conditions (2.5% FCS) (Birkenhäger *et al.* 1996). In two other studies, however, a higher activity of VEGF_{165} compared to VEGF_{121} was found, but under higher serum conditions than here (20% and 5% FCS) (Soker *et al.* 1997) or on different types of endothelial cells (Keyt *et al.* 1996). Therefore, the discrepancies in the literature about the relative potencies of VEGF_{121} vs. VEGF_{165} may be the result of different serum concentrations or cellular models used.

Collectively, all cytokines tested can act as endothelial cell mitogens under certain conditions, which vary among the cytokines and between the type of endothelial cell, i.e. whether they have been derived from the macro- or microvasculature. This differential reactivity of macro- and microvascular endothelial cells to VEGFs and PlGFs was noted particularly in the absence of serum. Addition of fetal calf serum to the culture medium reduces the cell-specific differences of HUVEC and MIEC in their proliferative response to growth factors. MIEC are more sensitive to changing cell culture conditions than HUVEC and generally stronger respond to the cytokines. This lower flexibility of the MIEC to changing ambient conditions may be a further indication of their highly specialized function. However, one has to keep in mind that some of the effects found here were very small though statistically significant. It remains to be demonstrated in further experiments with different endpoints whether these *in vitro* effects reflect *in vivo* effects of biological significance.

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It is a honour to dedicate this work to Prof. W. Burkl on the occasion of his 80th birthday.

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