Ocetotide prevents growth factor-induced proliferation of bovine retinal endothelial cells under hypoxia

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Abstract

Ocular diseases such as proliferative diabetic retinopathy are the major cause of blindness in industrialized countries. The main pathologic features of these diseases are hypoxia and overproduction of growth factors resulting in pathologic proliferation of endothelial cells and new vessel formation. Particularly, hypoxia and growth factors, such as VEGF, IGF-1, bFGF and TGFβ2, show a complex interaction in the onset and progression of the diseases. Therefore, to date, most therapeutic strategies for proliferative retinopathies have targeted proliferation of endothelial cells evoked by growth factors. Recently, a synthetic analog of somatostatin, octreotide, has been shown to inhibit the proliferation of various cells in vitro, including endothelial cells. In this study, we have investigated the proliferative response of bovine retinal endothelial cells (BREC) to growth factors under hypoxic conditions and the modulation by octreotide. We found a dose-dependent increase of cell proliferation with VEGF, IGF-1 and bFGF, and inhibition of hypoxia-induced cell proliferation with TGFβ2. Moreover, growth factor-induced, but not hypoxia-induced, cell proliferation was attenuated in the presence of octreotide. In contrast, TGFβ2 abolished hypoxia-induced BREC proliferation. Similar to octreotide BIM23027, a somatostatin receptor subtype 2 (SSTR2) receptor agonist was able to attenuate the growth factor-induced proliferation of BREC expressing mRNA and protein for SSTR2. Therefore, we postulate that octreotide exerts its effects mainly through binding to the SSTR2. Taken together, our findings point to octreotide as a promising candidate for the treatment of eye disorders involving growth factor-dependent proliferation of endothelial cells.


Introduction

The eye is one of the best models of blood vessel formation due to the diversity of angiogenic processes taking place throughout development and in adults (Risau 1994, Campochiaro 2000). During development, the eye is vascularized by either vasculogenesis, which means assembling of vessels from newly differentiated endothelial cells, or angiogenesis, which means sprouting of endothelial cells from pre-existing vessels (Battegay 1995, Hughes et al. 2000). In adults, new blood vessel formation occurs predominantly through angiogenesis (Campochiaro 2000). Although angiogenesis plays a crucial role in vessel repair and wound healing, it also accounts for pathologic events, such as tumor growth, as well as retinal and choroidal neovascularization (Risau 1994, Battegay 1995). Regardless of the pathologic or physiological nature of vascularization, the establishment of capillary networks requires a complex series of cellular events which are initiated and modulated by a multitude of growth factors including vascular endothelial cell growth factor (VEGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGFβ2), and further affected by systemic conditions such as hyperoxia and hypoxia (Wiedemann 1992, Battegay 1995, D’Amore 1994, Campochiaro 2000).

Vascular endothelial cell growth factor (VEGF) exerts its action through two tyrosine kinase receptors, namely VEGF receptor 1 (VEGFR1) and VEGFR2 (Campochiaro 2000). Binding of VEGF to VEGFR2 results in differentiation of the angioblasts into endothelial cells, whereas assembly of endothelial cells into tubes is mediated via activation of VEGFR1 (Campochiaro 2000). Insulin-like growth factor-1 (IGF-1) binds to IGF receptors (types I and II), and this binding is modulated by a system of IGF-binding proteins (IGFBP) and IGFBP proteases (Jones & Clemmons 1995, Feldmann et al. 2000). The consequence of IGF stimulation is the activation of endothelial cells as manifested by cell proliferation and migration (King et al. 1983, Grant et al. 1986, 1987, 1993b, Wilson et al. 2001). Basic fibroblast growth factor (bFGF) is a ligand of FGF tyrosine kinase receptors coupled to various downstream signaling pathways affecting cell survival, mitosis, differentiation, adhesion and...
motility (Gospodarowicz et al. 1987, Bensaid et al. 1992). Transforming growth factor β2 (TGFβ2) with its serine/threonine kinase receptors can stimulate or inhibit endothelial cell proliferation and control synthesis and degradation of the extracellular matrix (ECM) (Madri et al. 1988, Rifkin et al. 1993, Katsura et al. 2000). Additional spin to the system of growth factors affecting endothelial cells proliferation is provided by systemic conditions such as hypoxia/ischemia (Das et al. 2001). Hypoxia is essentially a pathologic condition and is thought to be the main driving force for retinal neovascularization (Yan et al. 2001, Humar et al. 2002). There are several ways by which hypoxia affects retinal neovascularization. Most importantly, hypoxia affects cell proliferation by induction of G1 cell cycle arrest (Gardner et al. 2001). The mechanism of this arrest is thought to be related to the mammalian target of rapamycin (mTOR) signaling pathway, which plays a central role in regulating cellular growth and induction of genes such as cyclin D (Schmelzle & Hall 2000, Humar et al. 2002). Additionally, hypoxia-inducible factor 1 alpha (HIF-1α) regulates metabolic genes and thus the availability of nutrients, which in turn affects mTOR (Humar et al. 2002). In pericytes, hypoxia causes decreased expression of TGFβ2, a major inhibitor of endothelial cell proliferation (Antonelli-Orlidge et al. 1989). It has been also reported that hypoxia positively influences the expression of growth factor receptors such as flt-1 (Gerber et al. 1997). Moreover, recent work by Hackett et al. (2002) points to hypoxia as a possible factor influencing angiopoietin-2 (Ang2) expression within areas of neovascularization in the retina. Hypothetically, Ang2 could increase the response of endothelial cells to VEGF as a result of the disruption of cell–cell contacts and extracellular matrix interaction. In general, growth factors seem to exert complex interactions under hypoxia to induce cell proliferation in eye disorders. Consequently, therapeutic strategies for diabetic retinopathy are currently focusing on the identification of pathways and receptors which would be able to antagonize VEGF-, IGF-1-, bFGF- or hypoxia-dependent action on endothelial cell proliferation. Promising candidates for such an action are somatostatin and its receptors (Boehm et al. 2001). The family of seven transmembrane domain G protein–coupled somatostatin receptors comprises five members termed SSTR1–5, which bind somatostatin 14 and somatostatin 28 as physiological ligands (van Hagen et al. 2000, Davis et al. 2001). The activation of the receptors leads to pronounced inhibition of adenylate cyclase and activation of tyrosine phosphatases. Additionally, binding of somatostatin to SSTR5 decreases the activity of the MAPK pathway, as opposed to the stimulatory action of SSTR1 and –4. Those signaling profiles mainly point to SSTR2, –3 and –5 as isoforms linked to inhibition of cell proliferation (Buscail et al. 1995, Danesi et al. 1997). The recently produced synthetic somatostatin receptor ligand, octreotide, shows high affinity binding to SSTR2 and –5, moderate binding affinity to SSTR3 and low affinity to both SSTR1 and –4. Accordingly, octreotide has been shown to exert antiproliferative effects on somatostatin receptor-expressing cells (Bruns et al. 2000). To date, octreotide has been found to inhibit the proliferation of human endothelial cells HUVEC–C (Danesi et al. 1997).

In our work, we have investigated the proliferative response of bovine retinal endothelial cells (BREC) to octreotide or the growth factors including VEGF, IGF-1, bFGF and TGFβ2 under hypoxia, systemic conditions associated with the development of various eye disorders. We provide evidence that octreotide is a potent inhibitor of growth factor-induced cell proliferation.

Materials and Methods

Preparation of retinal endothelial cell cultures

Bovine eyes were obtained from a local slaughterhouse. The eyes were freed from muscles, conjunctiva and connective tissues, and briefly immersed in 70% ethanol followed by 30 min soaking in a 5% penicillin/streptomycin solution (Gibco) and rinsing with sterile water. The vascular retina was dissected, freed from the underlying pigmented retina and homogenized in Hanks’ balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA), supplemented with 1% penicillin/streptomycin. The fragments of vascular retina were collected on 70 µm nylon sieves and incubated in PBS containing 20 U/ml type 2 DNAase I (Sigma) and 0.1% collagenase type I (Roche) at 37 °C for 90 min with agitation. The cell suspension was washed three times with HBSS–BSA and incubated with Dynabeads coated with antibody against CD31 (Dynal, Hamburg, Germany) in PBS/0.1% BSA for 15 min at 4 °C with gentle tilting and rotation. The endothelial cells bound to the beads were collected with a magnetic particle concentrator (MPC; Dynal), thoroughly washed and resuspended in Endothelial Microvascular Cell Growth Medium (ECGM) (PromoCell, Heidelberg, Germany). The cells were seeded on six–well plates coated with human fibronectin (10 µg/ml; Becton Dickinson, Heidelberg, Germany) and maintained in a humidified atmosphere of 5% CO2 at 37 °C. The medium was renewed every third day. After 7 days, the cells at passage number 0 were subcultured in 1:3 ratio into 35 cm2 culture flasks and than regularly passaged every 3 days. For hypoxia, cultures were incubated in a hypoxia incubator (Heraeus, Hanau, Germany) in PBS/0.1% BSA for 15 min at 4 °C with gentle tilting and rotation. The endothelial cells bound to the beads were collected with a magnetic particle concentrator (MPC; Dynal), thoroughly washed and resuspended in Endothelial Microvascular Cell Growth Medium (ECGM) (PromoCell, Heidelberg, Germany). The cells were seeded on six–well plates coated with human fibronectin (10 µg/ml; Becton Dickinson, Heidelberg, Germany) and maintained in a humidified atmosphere of 5% CO2 at 37 °C. The medium was renewed every third day. After 7 days, the cells at passage number 0 were subcultured in 1:3 ratio into 35 cm2 culture flasks and than regularly passaged every 3 days. For hypoxia, cultures were incubated in a hypoxia incubator (Heräus, Hanau, Germany) with a gas mixture containing 5% O2 and 5% CO2 balanced with nitrogen.

Proliferation assay

For DNA synthesis assays, cells were seeded on 96–well, flat–bottom plates (Nunc, Roskilde, Denmark) at a density
of 5 × 10⁴ cells/ml in ECGM medium and allowed to accommodate for 1 day. To assess the role of oxygen conditions on BREC proliferation, the ECGM medium was changed to serum-free medium, and the cells were cultured for 48 h under normoxia (21% O₂, 5% CO₂) or hypoxia (5% O₂, 5% CO₂). During the last 24 h, the culture medium was additionally supplemented with 1 µCi/ml [³H]thymidine. Subsequently, the cells were washed, dissociated in 0·25% trypsin-EDTA and harvested on glass-fiber filters. The filters were dried and the incorporated radioactive material was measured in 10 ml scintillation fluid in a beta-counter (Fuji, Rodgan, Germany). All experiments with growth factors, octreotide (Novartis) and BIM23027 (Sigma) were carried out under hypoxic conditions. After maintenance of cells for 24 h in the serum-free medium, cultures were simultaneously treated with 1 µCi/ml [³H]thymidine and the culture medium was additionally supplemented with 1 µCi/ml [³H]thymidine. Subsequently, the cells were washed, dissociated in 0·25% trypsin-EDTA and harvested on glass-fiber filters. The filters were dried and the incorporated radioactive material was measured in 10 ml scintillation fluid in a beta-counter (Fuji, Rodgan, Germany). All experiments with growth factors, octreotide (Novartis) and BIM23027 (Sigma) were carried out under hypoxic conditions. After maintenance of cells for 24 h in the serum-free medium, cultures were simultaneously treated with 1 µCi/ml [³H]thymidine and one or the combination of the following factors: VEGF (20 ng/ml), IGF-1 (10 ng/ml), bFGF (10 ng/ml), octreotide (10⁻⁵ to 10⁻¹⁰ M) and BIM23027 (10⁻⁷ M). All growth factors were obtained from R&D Systems (Wiesbaden, Germany). Each experiment was performed with cultures from at least three independent experimental set-ups. All cultures were used after the third replating.

Total RNA isolation and RT-PCR
Total RNA was isolated from 35 mm culture dishes of BREC using PeqGold RNAPure (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. The RNA concentration was calculated from UV absorbance of RNA solution measured at 260 nm. A total of 10 µg of RNA was reverse transcribed at 37 °C for 2 h using 200 U of M-MLV (Promega) and 2 µg random hexamer primers (Interactivaq, Ulm, Germany). Similarly, a sister reaction containing 10 µg of RNA was set up in which M-MLV reverse transcriptase was omitted. PCR was performed with 2 µl of the obtained templates in a total volume of 50 µl in the presence of 20 pmoles of the respective primers using recombinant Taq polymerase (Gibco). PCR amplification included 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. The quality of the RT reactions was proved by amplification of the GAPDH housekeeping gene with GAPDH-sense primer 5'-GCACAGTCAGGCAGAAGACG-3' and GAPDH-antisense primer: 5'-GGTCATAAGTCCCTCCACAGT-3' (338 bp product). The SSTR-2 cDNA was detected using the sequences of the bovine somatostatin receptor primers provided by Novartis (sense primer: 5'-TGAAGTCCTCAGGCATCCCGAAGAG-3', antisense primer: 5'-CCGGCTCACCTTGACCAAGCAG-3', product size: 298 bp). Reaction products were separated on 1·5% agarose gel in TAE buffer and stained with ethidium bromide.

Western blot analysis
For immunoblot analysis, cultured BREC were lysed by ultrasonification in 60 mM Tris–HCl, containing 2% SDS and 10% sucrose. Protein content of cell lysates was determined using the BCA protein estimation kit (Pierce,
Rockford, IL, USA) and BSA as a standard. Cell lysates were diluted 1:1 in sample buffer (250 mM Tris–HCl, pH 6.8, containing 4% SDS, 10% glycerol and 2% β-mercaptoethanol) and denatured at 95 °C for 5 min. Proteins (15 µg/lane) were separated by SDS– (10%) polyacrylamide gel electrophoresis and transferred to nitrocellulose by semidry electroblotting. Nonspecific binding sites were blocked with 5% non-fat milk for 30 min, and then the blots were incubated overnight at 4 °C with rabbit affinity purified anti-SSTR2 (1:500; Acris, Hiddenhausen, Germany). The immunoreaction was detected by incubating blots for 2 h at room temperature with anti-rabbit HRP-labeled secondary antibodies (Dianova, Hamburg, Germany) and was visualized with the enhanced chemiluminescence kit (Amersham).

Statistics

Statistical analysis was performed using the two-sample t-test for the means. A value of $P < 0.05$ was considered to be statistically significant.

Results

To measure the effects of growth factors on proliferation of BREC under hypoxia, confluent BREC cultures were treated with bFGF, VEGF, IGF-1 and TGFβ2 at concentrations of 1, 5, 10, 20 and 50 ng/ml in serum-free medium and the additional presence of 1 µCi/well of $[^3H]$thymidine for 24 h. Liquid scintillation counting of the cell lysates revealed dose-dependent effects of the

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Figure 2 Expression of somatostatin/octreotide receptor 2 in bovine retinal endothelial cells. (A) RT-PCR analyses revealed a single 298 bp product of SSTR2 mRNA. Additionally, a 338 bp product of GAPDH mRNA was amplified as an internal control using the same RT reaction as a template. (B) Western blotting using SSTR2-specific antibodies revealed a single immunoreactive protein band with an approximate molecular mass of 60 kDa.

Figure 3 BREC proliferation in response to octreotide in hypoxic conditions. Confluent BREC cultures were incubated with octreotide for 24 h, and the cell proliferation was determined by $[^3H]$thymidine incorporation. Maximal effective concentration of $10^{-6}$ M octreotide inhibited basal proliferation of BREC by 20% ($n = 3$; *$P < 0.0001$).
various growth factors on BREC proliferation. bFGF at a concentration of 10 ng/ml resulted in a maximal 2.7 ± 0.6-fold (n = 3; P < 0.0001) increase of endothelial cell proliferation as compared with the control (Fig. 1A). With increasing bFGF concentration (50 ng/ml), cell proliferation declined but was still significantly increased as compared with control (n = 3; P < 0.0001). For VEGF, BREC proliferation was maximal with 20 ng/ml (2.2 ± 0.6-fold; n = 3; P < 0.0001) and declined in the presence of 50 ng/ml (1.6 ± 0.6 fold; n = 3; P < 0.0001) of the growth factor (Fig. 1B). IGF-1 resulted in a maximal cell proliferation with 20 ng/ml (3.8 ± 1.8 fold; n = 3; P < 0.0001) and a slight drop of proliferation with 50 ng/ml (Fig. 1C). In contrast, BREC proliferation was potently decreased upon TGFβ2 treatment at all concentrations tested (n = 3; P < 0.0001) (Fig. 1D).

In a subsequent set of experiments, we sought to determine the proliferative response of BREC to co-stimulation with octreotide and growth factors under hypoxia. The expression of SSTR2, the target receptor for octreotide in BREC, was confirmed by RT-PCR amplification with specific primers and by Western blotting using specific antibodies (Fig. 2). The antibody detected a single protein band with an approximate weight of 60 kDa (He et al. 1989, Thermos et al. 1989). Additional PCR reactions using both SSTR2- and GAPDH-specific primers were performed on templates resulting from control reverse transcription reaction where reverse transcriptase was omitted. Both reactions yielded no PCR products, indicating that RNA preparations were free of contaminating DNA (Fig. 2). To determine maximal effective concentrations of octreotide, confluent endothelial cell cultures were maintained with octreotide (10⁻⁵ to 10⁻¹⁰ M), and cell proliferation was assessed by [³H]thymidine incorporation. Octreotide at a concentration of 10⁻⁶ M inhibited the basal proliferation of BREC by 20% (n = 3; P < 0.0001) (Fig. 3). Co-treatment experiments further revealed that octreotide massively inhibited the proliferation induced with 10 ng/ml of bFGF by 50% (n = 3; P < 0.0001), 20 ng/ml of VEGF by 60% (n = 3; P < 0.0001) and 20 ng/ml of IGF-1 by 60% (n = 3; P < 0.0001) (Fig. 4A–C). In contrast, co-treatment with octreotide (10⁻⁶ M) and TGFβ2 (50 ng/ml) did not further reduce proliferation of BREC as compared with single treatment with TGFβ2 (Fig. 4D).

The comparison to BREC cultures maintained under normoxia further revealed that TGFβ2 (50 ng/ml) basically abolished hypoxia-induced BREC proliferation (n = 3; P < 0.0001), whereas octreotide (10⁻⁶ M) only slightly interfered with BREC proliferation induced by hypoxia (Fig. 5). In addition our studies revealed a statistically significant (n = 3; P < 0.01) difference between BREC proliferation under normoxia and hypoxia/TGFβ2 (Fig. 5).

To substantiate the role of SSTR2 in octreotide effects, we have additionally determined the growth factor–induced proliferation of BREC in the presence of the SSTR2 receptor agonist BIM23027. Comparably to...
TGFβ, an analog of somatostatin, modulates the proliferative conditions. We have further determined how octreotide, BIM23027 (10⁻⁷ M) strongly inhibited the proliferation induced with 10 ng/ml of bFGF by 59% (n=3; P<0.001), 20 ng/ml of VEGF by 60% (n=3; P<0.002) and 20 ng/ml of IGF-1 by 38% (n=3; P<0.0001) (Fig. 6A–C). BIM23027 at this concentration was shown maximally to induce contractile action in rat isolated distal colon (McKeen et al. 1994).

Discussion

In our present study, we have investigated the influence of growth factors on proliferation of BREC under hypoxic conditions. We have further determined how octreotide, an analog of somatostatin, modulates the proliferative response of endothelial cells to VEGF, bFGF, IGF-1 and TGFβ2 under hypoxia. Hypoxia is the major cause of many proliferative ocular diseases. Hypoxia affects proliferation of endothelial cells independently of growth factor action through mTOR signaling pathways and induction of genes such as cyclin D1, which results in G1 cell cycle arrest (Gardner et al. 2001). According to the recently proposed model, intracellular signals induced by growth factors converge on hypoxic pathways, resulting in additional effects on cell proliferation.

We have observed a massive change of BREC proliferation under hypoxia. We further observed that under hypoxic conditions VEGF, bFGF and IGF-1 are able, dose dependently, to increase BREC proliferation as compared with hypoxic control. Relatively high concentrations of each growth factor were needed to evoke changes in cell proliferation. Therefore, it seems rather unlikely that the cells produce sufficient growth factor amounts over 1 day under hypoxic conditions to alter significantly their proliferation as compared with normoxia-incubated cells. Consequently, we suggest that the proliferation of untreated BREC cultures was stimulated by hypoxic pathways, rather than indirectly through growth factor upregulation.

Intriguingly, octreotide rather specifically interferes with growth factor-induced BREC proliferation. This conclusion is essentially based on the finding that under hypoxic conditions octreotide prevented growth factor-induced cell proliferation, but exerted only marginal effects on hypoxia-induced increases in BREC numbers. Ongoing studies further demonstrated that in addition to hypoxia, octreotide can also abolish growth factor-induced proliferation of BREC under normoxia (unpublished observations). This rather selective action of octreotide on growth factor–controlled endothelial cell proliferation could also explain why octreotide is ineffective in treating non-proliferative retinopathies which are not evoked by growth factor overproduction (van Hagen et al. 2000). In further support of this view, other recent studies found that inhibition of neovascularization by octreotide results from a direct inhibition of bFGF or IGF-1 activity on endothelial cells (Grant et al. 1993a, Danesi et al. 1997, Spraul et al. 2002).

Unlike octreotide, TGFβ2, attenuated hypoxia-induced BREC proliferation, thus pointing to a hypothetical mechanism in which TGFβ2 directly affects hypoxia-induced genes controlling cell proliferation. In fact, it has been shown that TGFβ2 can decrease hypoxia-induced cell proliferation by downregulating tumor promoter-induced tubule formation in collagen matrices (Mueller et al. 1987). However, since Emmanuel et al. (2002) have demonstrated that TGFβ2 evokes apoptosis in endothelial cells, we cannot completely rule out that the low proliferation rate seen in TGFβ2-treated BREC cultures is due to increased cell death. Hogg et al. (1999) have shown that TGFβ1, serum deprivation and hypoxia as individual conditions induce apoptosis in vascular endothelial cells to some extent, and they have also reported a pronounced increase in apoptotic cell death upon combination of the various conditions, such as treatment with TGFβ2 under hypoxia.

Octreotide shows high affinity binding for SSTR2, a somatostatin receptor subtype expressed by BREC, but also binds with lower affinity to SSTR5 (Bruns et al. 2000). A major role of SSTR2 in the observed inhibitory effects of octreotide on growth factor–induced BREC proliferation is confirmed by the additional finding that the proliferative response is equally sensitive to the SSTR2 receptor agonist BIM23027.
The observation that octreotide potently inhibits growth factor-induced BREC proliferation under hypoxic conditions suggests that octreotide could be a powerful drug against eye disorders where proliferation of retinal endothelial cells is growth factor-dependent. The effectiveness of this treatment can be further increased by also targeting TGF-β receptors, which would further prevent hypoxia-induced proliferation of endothelial cells.

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References

Antonelli-Orlidge A, Saunders KB, Smith SR & D’Amore PA 1989 An activated form of TGF-β is produced by co-cultures of endothelial cells and pericytes. PNAS 86 4544–4548.


Buscáil L, Esteve JP, Saint-Laurent N, Bertrand V, Reisine T, O’Carroll AM, Bell GI, Schilly AV, Vayssse N & Susini C 1995 Inhibition of cell proliferation by the somatostatin analogue RC-160 is mediated by somatostatin receptor subtypes SSTR2 and SSTR5 through different mechanisms. PNAS 92 1580–1584.


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