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Modulation of Insulin-like Growth Factor-I Production of Cultured Retinal Vascular Endothelial Cells by Oxygen, Glucose and Growth Hormone

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Purpose: To evaluate the interaction of oxygen, glucose, and growth hormone (GH) on insulin-like growth factor-I (IGF-I) production of cultured bovine retinal vascular endothelial cells.

Methods: Confluent cultures of bovine retinal vascular endothelial cells were incubated under 3% or 20% oxygen in Dulbecco's modified Eagle's medium (containing 1 g/L or 4.5 g/L glucose), with or without the addition of 10 ng/mL GH. After incubation times of 0, 24, 48, and 72 hours, IGF-I was measured in the supernatant, and cells were counted.

Results: Highest levels of IGF-I were reached after 72 hours with 4.5 g/L glucose and 10 ng/mL GH under 3% O_2. All IGF-I levels found in 3% O_2 samples were significantly higher than those found in 20% O_2 samples. No statistical significance was found regarding glucose concentration or GH supplement.

Conclusions: Oxygen turned out to be the sole modulating factor for IGF-I production of cultured retinal vascular endothelial cells. **Jpn J Ophthalmol 2002;46:226–229** © 2002 Japanese Ophthalmological Society

Key Words: Glucose, growth hormone, insulin-like growth factor-I, oxygen, retinal vascular endothelial cells.

Introduction

Insulin-like growth factor-I (IGF-I) is known to play an important role in the promotion of angiogenesis. IGF-I levels are drastically elevated in the vitreous body of patients suffering from proliferative diabetic retinopathy.

IGF-I is thought to be the mediator of growth hormone in the peripheral tissues. However, it has also been shown that in poorly controlled diabetic patients, growth hormone levels are elevated even though normal or low levels of IGF-I are found, indicating that in a clinical situation there is no association between the two factors.

Ischemia is known as the major event in the onset of proliferative diabetic retinopathy. In vitro cultures of bovine retinal endothelial cells have shown a huge change in growth factor concentrations when exposed to different oxygen concentrations. It has also been demonstrated that the production of IGF-I by endothelial cells is altered by different oxygen levels.[1] In mammalian cells, glucose and oxygen consumption are inversely related. Under conditions in which oxygen is limited, a dramatic rise in the demand for glucose in order to maintain adenosine triphosphate turnover has been described.[2]

Because vascular endothelial cells are the first to be involved in the onset of proliferation, resulting in neovascularization, it has been the aim of this study to investigate whether the known effect of varying oxygen tensions on the IGF production of cultured retinal vascular endothelial cells[1] is influenced by

Received: May 1, 2001
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Published by Elsevier Science Inc.

0021-5155/02/$–see front matter
PII S0021-5155(01)00499-3

supplementing the culture medium with different concentrations of glucose, with or without the addition of growth hormone.

Materials and Methods

Isolation and Culture of Retinal Endothelial Cells

Bovine retinal vascular endothelial cells were isolated and cultured by standard methods. These cells were cultivated in a humidified atmosphere of 5% CO_2 and 20% O_2 at 37°C.

To examine these cells, cell smears were made on slides for each passage. Cells in passage 2 up to passage 5 were used for experiments. Passages were performed by weak digestion to eliminate and suppress pericytes.

Antibodies and Immunohistochemical Staining

A standard two-stage indirect immunohistochemical technique was applied using anti-von Willebrand factor (vWF)/Factor VIII antibody (clone F8/86; DAKO A/S, Glostrup, Denmark), and anti-smooth-muscle-actin antibody (smAc, clone 1A4; DAKO).

Assay of Experiments

Confluent cultures were incubated with either Dulbecco's modified Eagle's medium (DMEM; including 1 g/L glucose) alone or DMEM supplemented with 4.5 g/L glucose, with or without 10 ng/mL growth hormone (GH) under 3% or 20% oxygen. After incubation times of 0, 24, 48, and 72 hours, supernatant was collected and cells were trypsinized and counted in a counting chamber. All experiments were repeated six times using two wells per time point.

Radioimmunoassay for IGF-I

IGF-I was measured in the supernatant using a radioimmunoassay (Nichols Institute Diagnostics, Bad Nauheim, Germany). This radioimmunoassay was modified for lower IGF levels. IGF-binding proteins were removed by acid-ethanol extraction.

Statistical analysis was performed using an analysis of variance.

Results

The morphology of cultured bovine retinal vascular endothelial cells exhibited a cobblestone phenotype, especially in small colonies, and an elongated phenotype in confluent contact-inhibiting monolayers (Figure 1A). Immunohistochemical staining of the cultures with anti-von Willebrand factor antibody (vWF) revealed specific and strongly positive labeling of bovine retinal vascular endothelial cells with granular perinuclear staining (Figure 1B). With anti-smAc antibody, these bovine cells in suspension and in monolayers were not stained. Pericytes were positively stained with anti-smAc antibody, whereas contamination of cultures by pericytes was ≤5% (estimated by counting cells).

The values of the IGF-I production measured are shown in Figure 2. All IGF-I levels found in 3% O_2 samples were significantly higher than those found in 20% O_2 samples. No significance was found concerning glucose concentration or GH supplement. No cell proliferation was found over the 72-hour period. Moreover, a reduction in cell count was evident in all samples. The decrease in cell count was more severe in samples incubated with 3% O_2 than in samples incubated with 20% O_2.

Discussion

With the technique described, we were able to obtain almost pure cultures of bovine retinal vascular endothelial cells for in vitro experiments. Our results show that these cultured bovine cells produce IGF-I in a time-dependent manner. Highest IGF levels were found after 72 hours of incubation, the longest incubation time in our study. Among the exogenous stimuli tested (oxygen, glucose, GH), only oxygen levels turn out to have statistically significant influence on IGF production in cultured bovine retinal vascular endo-

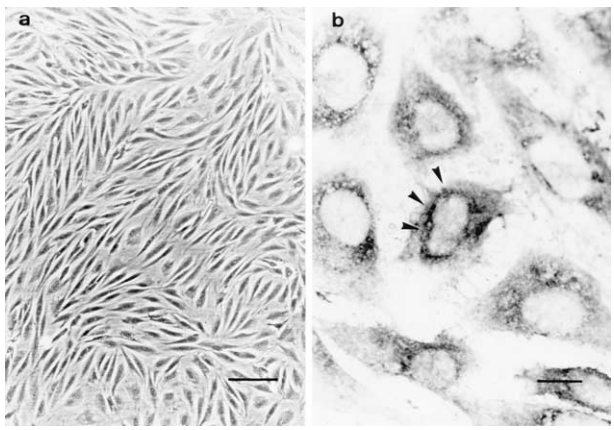

Figure 1. Bovine retinal vascular endothelial cells in culture. (**A**) Phase contrast micrograph of confluent culture of bovine retinal vascular endothelial cells of the third passage. Bar = 100 μm. (**B**) Micrograph of bovine retinal vascular endothelial cells of the first passage, immunostained with anti-von Willebrand factor. The reaction product of the immunostaining is mainly perinuclear (arrows). Bar = 10 μm.

Jpn J Ophthalmol
Vol 46: 226–229, 2002

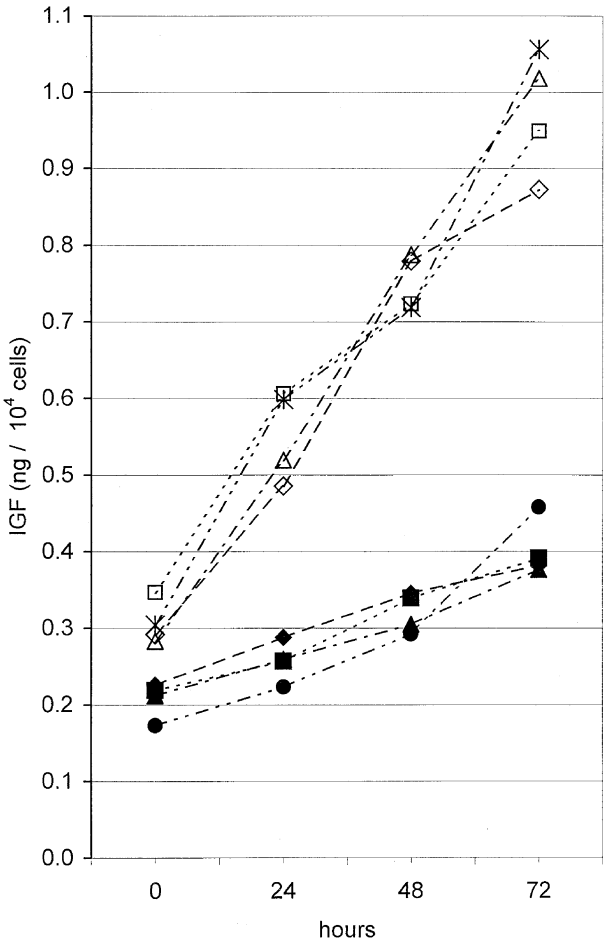

Figure 2. Insulin-growth factor-I (IGF-I) production of retinal vascular endothelial cells. IGF-I production per 10^4 cells after 0, 24, 48, and 72 hours. ◆, 20% O_2, 0 GH, 1 g glucose; ■, 20% O_2, 10 GH, 1 g glucose; ▲, 20% O_2, 0 GH, 4.5 g glucose; ●, 20% O_2, 10 GH, 4.5 g glucose; ◇, 3% O_2, 0 GH, 1 g glucose; □, 3% O_2, 10 GH, 1 g glucose; △, 3% O_2, 0 GH, 4.5 g glucose; ✻, 3% O_2, 10 GH, 4.5 g glucose.

thelial cells. All samples grown in a 3% O_2 environment were found to produce significantly more IGF-I than samples grown in a 20% O_2 environment, thus confirming the findings by Boulton et al.[1] Our observations were not affected in a statistically significant manner by other supplements, such as high-glucose concentrations or GH or both of them together.

Moriarty et al[3] also demonstrated that retinal endothelial cells produce IGF-I in a time-dependent manner using plasma-free and plasma-containing media. In plasma-containing media they could not find any indication of IGF-I production of retinal vascular endothelial cells but only in RPE cells and only at the 72-hour point of time, with much lower values than in plasma-free medium.[3] Other authors have also pointed out that the IGF amount measured is dependent on the media and the radioimmunoassay.[4] Brooks et al[4] observed IGF-I production of bovine microvascular cells in a serum-free medium, but not in serum-containing media. The idea of possible inhibition of IGF production by serum factors, and therefore a negative feedback, has been proposed.[5] Due to a loss in sensitivity it is also more difficult to measure IGF production in serum-containing media than in serum-free media. Our own preliminary experiments have shown that retinal endothelial cells cultivated in serum-containing media first utilized the exogenous IGF before starting to produce their own IGF. This observation led us to conduct all our experiments in a serum-free environment, taking into account a negative influence of this setting on endothelial cell proliferation. In fact, no cell proliferation could be found in our experiments. In contrast, we observed a decrease in cell count in both a 20% O_2 and a 3% O_2 environment, however, more severely in a 3% O_2 environment. We contributed this decrease in cell count to the lack of serum, because in serum-containing media we found cell proliferation in a time-dependent manner (unpublished data). This observation is partly in contrast to the report by Moriarty et al,[3] who reported that cell numbers of retinal endothelial cells, pericytes and RPE cells remained constant in both plasma-containing and plasma-free medium throughout the time course of the experiment. Only fibroblasts that had remained constant in plasma-containing medium showed a 50% reduction in cell number after 48-hour incubation in plasma-free medium.

In our experiments we found that IGF production is oxygen-dependent and decreases with increasing oxygen levels in bovine retinal vascular endothelial cells. Cultured cells are routinely grown in an environment of 95% air/5% CO_2, comparable to our 20% O_2 environment. This environment has misleadingly been regarded as normoxia. Measurements of oxygen tension within these media, however, showed a mean pO_2 of 135 mm Hg, thus being much higher than normal oxygen tension under physiological conditions in vivo (70–90 mm Hg).[1] pO_2 measurements in our media showed the 20% environment to be hyperoxic with a mean pO_2 of 150 mm Hg, whereas 3% O_2 environment resulted in a pO_2 of 90 mm Hg, thus approximating physiological conditions. Boulton et al[1,6] showed that the IGF production of retinal endothelial cells increased towards hypoxia. However, cell proliferation was greatest at physiological pO_2 levels and decreased with hypoxia and hyperoxia.

GH is known to play an important role in cell proliferation and neovascularization.[7] Many of its effects have been shown to be IGF-mediated; however, a direct effect of GH on peripheral receptors cannot be ruled out. The fact that GH supplementation in our 3% O_2 environment experiments only mildly and statistically not significantly increased IGF production might support the fact that GH effects on retinal endothelial cells are not IGF-mediated. Rymaszewski et al[7] showed that supplementation of physiological doses of GH to human retinal endothelial cell cultures resulted in a 100% greater cell number, however, only in the presence of serum. In the serum-free environment of our experiments we found no significant effect of GH supplementation on cell number or IGF production of bovine retinal vascular endothelial cells.

In vivo, temporary worsening of diabetic retinopathy has been shown to be related to a sudden correction of chronic hyperglycemia. Increasing IGF-I levels have also been found with hypoglycemia. Glucose and oxygen consumption of mammalian cells has been shown to be inversely related (Pasteur effect), with a dramatic rise and a demand for glucose during oxygen limitation.[2] In our experiments, high glucose levels led to an insignificant increase in IGF-I production; acute glucose deprivation, however, was not tested.

Taking IGF-I as an indicator of the proliferative activity of endothelial cells our results helped us to identify oxygen as the dominant stimulator. However, from these in vitro experiments no firm conclusions can be drawn concerning the bioactivity of IGF, which is also dependent on other factors such as IGF receptors or IGF-binding proteins.[3]

Conclusion

In conclusion, cultured bovine retinal vascular endothelial cells produce IGF-I in a time-dependent manner. The highest IGF-I levels were reached after 72 hours in a 3% O_2 environment. Incubation in 3% O_2 led to a significantly higher IGF-I production than 20% O_2 incubation. Modulation of glucose levels or GH supplementation had no significant effect on IGF-I production.

This study was in part supported by *Aktion Kampf der Erblindung*. The authors are grateful to Dr. R. Fimmers of the Institute of Medical Biometrics, Computer Science and Epidemiology, for his help with statistical analysis. The authors also thank Ursula Rau-Schneider, whose technical expertise made this work possible.

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