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Short communication

Recombinant human erythropoietin stimulates vascular endothelial growth factor release by glomerular endothelial cells

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Abstract

We designed the present study to address the question of whether recombinant human erythropoietin stimulates DNA synthesis and vascular endothelial growth factor (VEGF) secretion in vitro using cultured bovine glomerular endothelial cells (GENs). Recombinant human erythropoietin dose-dependently stimulated the proliferation of GENs in culture, and this proliferative effect was inhibited by 1 μg/ml anti-VEGF antiserum. The increase in VEGF concentrations in the supernatants containing 10 U/ml rHuEpo was abolished by incubation with 10 μg/ml of anti-human rHuEPO antiserum, 0.2 μg/ml actinomycin D or 10 μg/ml cycloheximide. Taken together, rHuEpo stimulates GEN proliferation in vitro and VEGF release from these cells is associated with stimulation of RNA-dependent DNA and protein synthesis.

Keywords: Glomerular endothelial cell; Erythropoietin; Vascular endothelial growth factor; Angiogenesis

1. Introduction

The angiogenic process involves the degradation of the vascular basal membrane by a protease, proliferation and migration of endothelial cells through the extracellular matrix, and pericyte activation and alignment of the migrating cells to form a tubular structure (Form et al., 1986). The rationale for the potential effect of recombinant human erythropoietin on endothelial cells derives from the fact that endothelial cells descend from angioblasts, which in turn derive from mesenchymal cells. Endothelial cells possess an EPO receptor (Anagnostou et al., 1990). It has recently been reported that rHuEpo stimulates migration and proliferation, as well as endothelin-1 release by endothelial cells (Carlini et al., 1993) and stimulates angiogenesis in vitro (Carlini et al., 1995). We also reported that endothelin-1 mediates rHuEPO-stimulated glomerular endothelial cell (GEN)-dependent proliferation of mesangial cells (Nitta et al., 1995a).

Vascular endothelial growth factor (VEGF) is a specific growth factor acting on the vascular endothelium and is assumed to be important for angiogenesis (Leung et al., 1989). VEGF is thought to be important factor in the regulation of angiogenesis during fetal development, in wound healing, and during the growth of benign and malignant tumors (Ferrara et al., 1992). We previously reported that GENs in culture express and secrete VEGF in vitro (Uchida et al., 1994). The present study was designed to test the hypothesis that rHuEPO modulates VEGF secretion and promotes GEN proliferation in culture.

2. Materials and methods

2.1. Culture of bovine GEN

Bovine GENs were cultured as previously described (Uchida et al., 1994; Nitta et al., 1995b). RPMI 1640 medium was supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 nM selenium, together with 10 ng/ml acidic fibroblast growth factor and 0.1 mg/ml heparin. To characterize the cloned endothelial cells, the uptake of acetylated-low density lipoprotein and the expression of factor-VIII-related antigen were tested as described previously (Uchida et al., 1994).

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2.2. Cell proliferation assays

Thymidine incorporation was measured as previously described (Nitta et al., 1995a). GENs were transferred to 24-well plates, 10^4 cells per well, and allowed to rest for 24 h in RPMI medium with 2% serum-deprived serum. Then they were stimulated for 22 h with 0.1 to 10 U/ml rHuEPO in the presence or absence of 1 μg/ml anti-human VEGF antiserum (Uchida et al., 1994) or 10 μg/ml anti-human rHuEPO dissolved in the medium. The stimulated cells were then pulsed with 0.5 mCi/ml [^{3}H]thymidine for 2 h, harvested on fiberglass paper, and counted for radioactivity. In some experiments, cells were trypsinized and counted with a hemocytometer.

2.3. Measurement of VEGF in the culture supernatant

Since the amino acid sequence of VEGF is almost compatible in cattle and humans, a sandwich enzyme-linked immunoadsorbent assay (ELISA) was used to determine the VEGF concentration in the culture supernatant following standard protocols. Briefly, microtiter plates were coated with a first monoclonal antibody against human $VEGF_{165}$. After addition of 100 μl recombinant human VEGF or duplicate samples and incubation for 2 h at 37°C, the wells were washed and incubated with goat anti-human $VEGF_{121}$ IgG conjugated with horseradish peroxidase. After 2 h at 37°C, the wells were washed and a substrate solution composed of *O*-phenylenediamine was applied. The reaction was stopped with 2 N sulfuric acid, and absorbance was measured at 492 nm with an ELISA reader. A standard curve was prepared from the standards and the sample values were determined with a computer program (ELISA-Microsoft, Dynatech, Chantilly, VA, USA). Values are expressed as pg/ml. A measure of total human VEGF load per ml of supernatant was obtained by subtracting the VEGF concentration found in the supernatant. The detection limit of this assay was 0.5 pg/ml. The antibody does not crossreact with human platelet-derived growth factor, human epidermal growth factor, or human fibroblast growth factor.

2.4. Chemicals

rHuEPO and anti-human rHuEPO antiserum were kindly provided by Chugai Pharmaceutical, Tokyo, Japan. [^{3}H]thymidine was purchased from Amersham Japan, Tokyo, Japan. A human VEGF ELISA kit was from Immuno Biological Laboratories, Fujioka, Gunma, Japan. Standard recombinant human VEGF was from R&D System, Minneapolis, MN, USA. Culture flasks and 24-well culture plates were from Falcon, Lincoln, NJ, USA. Other culture reagents were purchased from Sigma, St. Louis, MO, USA.

2.5. Statistical analysis

Results are expressed as means ± S.E. Comparisons between two groups were made using Student's *t*-test. Comparisons among three or more groups were performed by using One-way analysis of variance (ANOVA) followed by Bonferroni's test to evaluate statistical significance between any two groups. Data were considered statistically significant if $P < 0.05$.

3. Results

Fig. 1a shows the effect of rHuEPO on [^{3}H]thymidine incorporation into GENs in culture. After a 24-h incubation, rHuEPO promoted [^{3}H]thymidine incorporation in a dose-dependent manner. This effect induced by 10 U/ml rHuEPO was inhibited by 42% in the presence of 1 μg/ml rabbit anti-human VEGF antiserum, which has been previ-

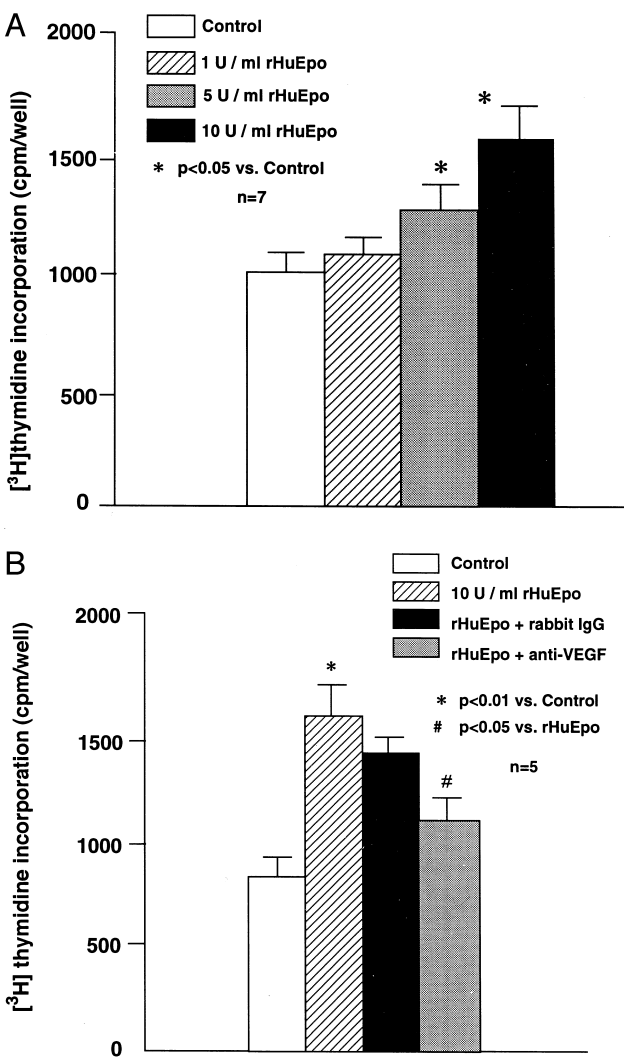

Fig. 1. (a) Effect of various concentrations of rHuEPO on DNA synthesis in glomerular endothelial cells in culture ($n = 7$). (b) Effect of anti-human VEGF antiserum on DNA synthesis in cells stimulated with 10 U/ml rHuEPO for 24 h ($n = 5$). DNA synthesis was assessed by [^{3}H]thymidine incorporation. Data represent means ± S.E. $^*P < 0.05$ vs. control.

ously characterized (Uchida et al., 1994), whereas control non-immune rabbit IgG had no effect (Fig. 1b). Anti-human VEGF rabbit antiserum had no effect on GEN proliferation at basal levels. rHuEPO did not alter the morphologic appearance of the cells, or lactate dehydrogenase (LDH) release by the glomerular endothelial cells (data not shown), suggesting that cellular toxicity of rHuEPO could be ruled out as a cause of the changes in [^{3}H]thymidine incorporation. We also assessed the change in cell numbers in a proliferation assay. Cell numbers significantly increased when GENs were incubated for 24-h with 10 U/ml rHuEPO (control; $1.15 \pm 0.21 \times 10^4$ cells vs. rHuEPO; $2.45 \pm 0.63 \times 10^4$ cells).

To investigate the mechanism of the stimulatory effect of rHuEPO on DNA synthesis in cultured GENs, we examined whether these cells secrete VEGF, an endothelial growth stimulator, in an autocrine fashion. When the cells were incubated with various concentrations of rHuEPO for 24 h, the VEGF concentration in the supernatant significantly increased in response to rHuEPO (Fig. 2a). This effect induced by 10 U/ml rHuEPO was significantly inhibited by 36% in the presence of 10 μg/ml anti-human rHuEPO antiserum. However, control human IgG had no effect on the VEGF concentration. No significant toxicity of these doses of anti-human VEGF was detected on the basis of cell counts, phase-contrast morphology, or measurements of LDH released by GENs (data not shown). Moreover, we examined whether recombinant human VEGF stimulated DNA synthesis in GENs in culture. After 24-h incubation, VEGF promoted [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 2b).

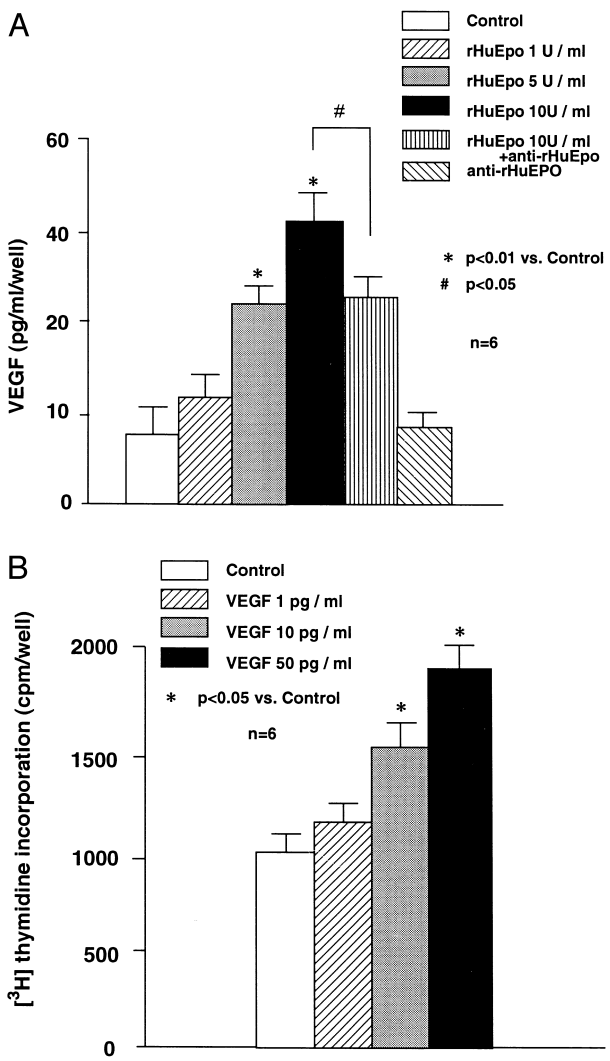

Fig. 2. Effect of rHuEPO on VEGF production by cultured glomerular endothelial cells in the presence or absence of 10 μg/ml anti-human rHuEPO antiserum ($n = 6$). (b) Effect of recombinant human VEGF on DNA synthesis in glomerular endothelial cells in culture ($n = 6$). VEGF in the culture supernatant was measured by a sandwich ELISA and DNA synthesis was assessed by [^{3}H]thymidine incorporation. Data represent means ± S.E. $P < 0.05$ vs. control.

It has been reported that 0.2 μg/ml actinomycin D or 10 μg/ml cycloheximide inhibits protein and DNA-dependent RNA synthesis in endothelial cells, respectively (Thomas and Rita, 1986). To assess the relationship between VEGF production and protein or DNA-dependent inhibition of RNA synthesis in rHuEPO-treated GENs, a subsequent study was carried out in which 2×10^4 cells were grown to confluence on 6 well-plates. After being quiescent, the cells were incubated with or without 0.2 μg/ml actinomycin D or 10 μg/ml cycloheximide for 0.5 h. After the cells were washed, the medium was exchanged for medium with or without 10 U/ml rHuEPO for an additional 22 h incubation. At the end of incubation, VEGF concentrations in the supernatants were determined. The VEGF concentrations in the supernatants of media containing 10 U/ml rHuEPO were significantly inhibited by 0.5-h incubation with actinomycin D (42.8 ± 2.2 vs. 20.8 ± 2.5 pg/ml) or cycloheximide (42.8 ± 2.1 vs. 18.9 ± 1.6 pg/ml).

4. Discussion

rHuEPO is widely used to treat anemia in patients with end-stage renal failure and does not alter residual glomerular function in these patients (Lim et al., 1990). However, the direct effect of rHuEPO on the residual glomeruli has not been elucidated. Hypoxia is reported to stimulate VEGF mRNA induction, suggesting that VEGF may be overproduced in the nephron under conditions of hypoperfusion. Since glomerulosclerosis is thought to be similar to arteriosclerosis, VEGF may act on GENs in end-stage renal failure patients who are under treatment with rHuEPO.

We previously demonstrated that bovine GENs in culture express VEGF mRNA and secrete VEGF in response to protein kinase C activator (Uchida et al., 1994) and mesangial cell proliferation is promoted by endothelin-1 secreted from cultured GENs stimulated with rHuEPO (Nitta et al., 1995a). We therefore focused on VEGF as a modulator on rHuEPO-stimulated GEN proliferation.

The present study demonstrates that rHuEPO dose-dependently stimulates DNA synthesis in GENs in vitro. The underlying mechanism of this effect is unknown, but we favor the hypothesis that the interaction between the hormone and other growth factors such as those contained in the supernatants of these cells may have a significant effect on GEN proliferation. We herein demonstrated that the stimulatory effect of rHuEPO on DNA synthesis is in part mediated by VEGF secreted by GENs in association with an increase in the synthesis of RNA-dependent DNA and protein. A recent report has demonstrated that rHuEPO stimulates VEGF secretion and increases VEGF receptor expression in bovine aortic endothelial cells (Victoria et al., 1998). These data suggest that rHuEPO and VEGF synergistically stimulate DNA synthesis in cultured GENs and act as a co-factor for glomerular remodeling.

A possible link between angiogenesis and atherosclerosis is suggested by the observation that neovascularization of the vasa vasorum is commonly observed in the atherosclerotic area (Barger et al., 1984). Whether proliferation of the vasa vasorum precedes or follows plaque formation is not known. It has recently been reported that T-lymphocytes surrounding the atherosclerotic plaque produce VEGF (Couffinhal et al., 1997), suggesting that VEGF modulates the migration of monocytes and macrophages in the atherosclerotic lesions.

Immunoperoxidase staining and in situ hybridization studies have shown that, in normal human kidney, VEGF protein and mRNA are localized predominantly in visceral glomerular epithelial cells, podocytes (Brown et al., 1992). In many glomerular diseases, VEGF-expressing cells are decreased in number or absent in areas of focal or global glomerular sclerosis (Shulman et al., 1996). However, the role of VEGF in the pathogenesis of glomerulonephritis is still unknown. We have recently found an increased serum VEGF level that is significantly correlated with the crescent frequency or the grade of interstitial injury and the rate of glomerular infiltration by macrophages in rapidly progressive glomerulonephritis, suggesting that hypoxia may cause severe inflammatory changes in kidney, resulting in an increased serum VEGF level.

In conclusion, rHuEPO promoted DNA synthesis in cultured GENs. This effect is mediated, at least in part, by the autocrine release of VEGF. The clinical implications of our findings remain to be elucidated.

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