The migration and proliferation of endothelial cells play a pivotal role in various vascular diseases. We have previously reported that atrial natriuretic polypeptide (ANP) exerts an antigrowth effect on vascular smooth muscle cells via the guanylate cyclase–coupled mechanism. Because the endothelial cells are known to possess a large number of guanylate cyclase–coupled ANP receptors, we examined the action of ANP on the growth of endothelial cells. ANP (10^{-7} and 10^{-6} M) significantly attenuated serum-stimulated DNA synthesis of cultured bovine aortic endothelial cells with concomitant reduction of the increase in cell number. A ring-deleted analogue of ANP exerted less prominent antiproliferative action, and 8-bromo cyclic GMP (cGMP) mimicked the action of ANP, suggesting the involvement of cGMP cascade in the endothelial growth. Moreover, the proliferative action of exogenously administered basic fibroblast growth factor on endothelial cells was significantly attenuated by the simultaneously administered 8-bromo cGMP. Taken together, the present results demonstrate a potential novel role of ANP in the regulation of endothelial cell growth, which is implicated in angiogenesis or reendothelialization.

**Keywords:** atrial natriuretic peptides • endothelium • cell division • endothelial growth factors • cyclic GMP • fibroblast growth factor • atherosclerosis • neovascularization

Endothelial cell proliferation is one of the central events involved in angiogenesis, restenosis after angioplasty, tumor progression, or embryogenesis. Recent evidence has revealed that vasoactive peptides can regulate vascular smooth muscle cell (VSMC) growth. Vasoconstrictive substances, including angiotensin II, can act as growth promoters of VSMCs, whereas we and others have demonstrated that vasorelaxing factors, such as atrial natriuretic polypeptide (ANP) or endothelium-derived relaxing factor, can exert antiproliferative and antihypertrophic effects on VSMC growth.

Although the endothelial cells are known to possess a large number of guanylate cyclase–coupled ANP receptors, the role of ANP on endothelial functions has not been described. In the present study, therefore, under the hypothesis that ANP exerts growth regulation, we examined the action of ANP on basal and basic fibroblast growth factor (bFGF)–stimulated endothelial cell growth.

**Methods**

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. In preparation for experiments, the cells at 80–90% confluency were made quiescent by placing them for 3 days in DMEM with 0.5% calf serum. Cell cultures from early passages (three to eight passages) were used for the experiment.

**Determination of DNA Synthesis**

The relative rate of DNA synthesis was assessed by determination of tritiated thymidine incorporation into trichloroacetic acid, a precipitable material, as previously reported. Quiescent BAECs grown in 24-well Costar culture dishes were pulsed for 8 hours with tritiated thymidine (10 μCi/ml) (20–28 hours after the stimulation).

**Growth Curves**

For the determination of cell numbers, BAECs were placed into 24-well culture dishes at 1 x 10^4 cells per well and grown in DMEM with 5% calf serum with media changes every 24 hours. Cells were harvested with trypsin-EDTA (0.05% trypsin, 0.02% EDTA, GIBCO, Grand Island, N.Y.) solution. Counts were performed by hemocytometer measurement immediately after the cell harvest.

**Materials**

α-Rat ANP-(1–28), porcine brain natriuretic peptide 26, and des-[Gln³⁶,Ser⁴⁹,Gly⁵⁰,Leu⁵¹,Gly⁵²]ANP-(4–23)-NH₃ (C-ANP-[4–23]) were obtained from Peninsula Laboratories, Inc., Belmont, Calif. 8-Bromo cyclic GMP (cGMP) and bFGF were obtained from Sigma Chemical Co., St. Louis, Mo., and Genzyme Corp., Cambridge, Mass., respectively.
Statistics

Values are expressed as mean±SEM. Analysis of variance with subsequent Duncan's test was used to determine significant difference in multiple comparisons. A value of p<0.05 was considered significant.

Results

The confluent cultures were exposed to 10^{-7} and 10^{-6} M ANP or 10^{-6} M C-ANP-(4-23) for 15 minutes in the presence of 10^{-4} M isobutylmethylxanthine. The intracellular cGMP level was determined by radioimmunoassay as previously reported. The intracellular cGMP levels (pmol/mg protein) were 1.2±0.2 (vehicle), 95.7±32.2 (ANP 10^{-7} M), 145±35 (ANP 10^{-6} M), and 14.6±7.7 (C-ANP-(4-23) 10^{-6} M) (n=3-5). Therefore, ANP caused a 100-fold increase in cGMP production, whereas C-ANP-(4-23) exerted much less stimulatory effect.

ANP at 10^{-7} and 10^{-6} M significantly attenuated DNA synthesis of BAECs. [3H]Thymidine incorporation of BAECs was (counts per minute per well) 647,000±40,000 (vehicle group), 502,000±15,600 (ANP 10^{-7} M group, p<0.05 versus vehicle), and 500,000±26,000 (ANP 10^{-6} M group, p<0.05 versus vehicle) (n=6). The lower doses of ANP had no significant effect on [3H]thymidine incorporation. Porcine brain natriuretic peptide at the concentration of 10^{-7} M exhibited similar inhibitory effect as that of ANP (498,000±19,800 counts per minute per well), whereas C-ANP-(4-23) (10^{-7} M) showed significantly less inhibition (539,000±32,300 counts per minute per well, p<0.05 versus ANP 10^{-7} M). The analogues of cGMP and cyclic AMP (8-bromo cGMP and 8-bromo cAMP) showed dose-dependent inhibition on DNA synthesis of BAECs, as depicted in Table 1.

and 8-bromo cGMP significantly blunted the proliferation of BAECs.

To elucidate the possible mechanism of the antiproliferative action of ANP on endothelial cells, we hypothesize that ANP with subsequent activation of cGMP exerts its growth inhibitory action through the modulation of autocrine growth factors that are produced within the endothelial cells. We focus on bFGF, because it has been demonstrated to be one of the major autocrine growth factors regulating endothelial cell proliferation. The right panel of Figure 1 shows the interaction of bFGF and cGMP on DNA synthesis of BAECs. 8-Bromo cGMP (10^{-3} M) significantly reduced not only serum but also bFGF-stimulated DNA synthesis. This result suggests a functional antagonism between bFGF and cGMP for endothelial cell proliferation.

Discussion

The present study demonstrates that ANP inhibits endothelial cell proliferation. The effective dose of ANP...
for the antiproliferative action on endothelial cells is comparable to that for VSMCs, as reported previously.5,8

It has been well acknowledged that endothelial cells are responsive to ANP for the intracellular production of cGMP.9 In our BAECs, ANP markedly stimulated cGMP accumulation, whereas C-ANP-(4–23) had much less stimulatory effect. Recently, molecular cloning has defined three types of natriuretic peptide receptors: the ANP-C receptor, which is not coupled to cGMP production and may function in the clearance of ANP,10 and the ANP-A and ANP-B receptors, which are membrane forms of guanylate cyclase.12 It is generally accepted that the guanylate cyclase–linked receptors mediate the cellular effect of ANP. Because C-ANP-(4–23), which shows much higher affinity to the ANP-C receptor than the guanylate cyclase–coupled receptors,13 exerted significantly less effect on endothelial cell proliferation and because 8-bromo cGMP mimicked the ANP effect, the antiproliferative effect of ANP is considered to be mainly mediated by guanylate cyclase–coupled receptors.

Brain natriuretic peptide is a new member of the natriuretic peptide family, first isolated from the porcine brain.14 In the present study, porcine brain natriuretic peptide exerted almost equipotent action as compared with ANP. This result is compatible with the recent report of Suga et al14 that BAECs express predominantly the ANP-A receptor, which shows equal affinity to ANP and porcine brain natriuretic peptide. Both natriuretic peptides are reported to show almost the same stimulation of cGMP generation in BAECs.14 Therefore, natriuretic peptides are considered to exert the antiproliferative action on endothelial cells, mainly through the ANP-A receptor.

Because C-ANP-(4–23) showed weak but significant suppression on DNA synthesis of endothelial cells, the involvement of the ANP-C receptor cannot be completely ruled out. Although the coupling of the ANP-C receptor to the signal transduction system is not clearly demonstrated, Anand-Srivastava et al15 reported the possible coupling of the ANP-C receptor to the adenylate cyclase/cAMP system. They claimed that ANP as well as C-ANP-(4–23) inhibits cAMP production in VSMCs. In our BAECs, however, 8-bromo cAMP dose-dependently inhibited endothelial cell proliferation. The attenuation of cAMP production by ANP is, therefore, unlikely for the mechanism of the antiproliferation action of ANP, although we did not measure the intracellular cAMP level in the present study.

To explore the mechanism of the antiproliferative action of ANP on endothelial cells, we examined our hypothesis that vasoactive substances influence vascular wall growth by modulating growth factors produced within the vessel. Using the antisense DNA methodology to block the translation of mRNA of the target gene, we have already proved that angiotensin II exerts a part of its hypertrophic effect on VSMCs through the stimulation of platelet-derived growth factor production within VSMCs.16 In the present study, we focus on bFGF, because the neutralization experiments using anti-bFGF antibody demonstrate that bFGF modulates endothelial cell proliferation, migration, and plasminogen activator production,7 thus acting as a powerful autocrine/paracrine growth regulator. The present results indicate that ANP with subsequent cGMP activation can inhibit the proliferative action of bFGF within endothelial cells. Furthermore, we have the preliminary data that show that ANP suppresses the gene expression of bFGF. Therefore, the antigrowth properties of ANP on endothelial cells can be ascribed, at least in part, to the modulation of ANP on bFGF function. There are, however, other possible mechanisms of the antigrowth action of ANP. For example, ANP can potentiate the actions of endothelial growth inhibitors, including transforming growth factor-β or prostacyclin.

In summary, results of the present study indicate the novel role of ANP in the regulation of endothelial cell growth, which is implicated in various vasculopathy, such as in atherosclerosis and hypertension, mainly through cGMP-dependent cascade. The finding also suggests that other mediators that activate cGMP, such as endothelin-derived relaxing factor, may act as an autocrine growth regulator of the endothelium.

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