Permissive Role of Nitric Oxide in Endothelin-induced Migration of Endothelial Cells

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Endothelin (ET) synthesis is enhanced at sites of ischemia or in injured vessels. The purpose of this study was to explore the possibility of autocrine stimulation of endothelial cell migration by members of the endothelin family. Experiments with microvascular endothelial cell transmigration in a Boyden chemotactic apparatus showed that endothelins 1 and 3, as well as a selective agonist of ETB receptor IRL-1620, equipotently stimulated migration. Endothelial cell migration was unaffected by the blockade of ETA receptor, but it was inhibited by ETB receptor antagonism. Based on our previous demonstration of signaling from the occupied ETB receptor to constitutive nitric oxide (NO) synthase (Tsukahara, H., Ende, H., Magazine, H. I., Bahou, W. F., and Goligorsky, M. S. (1994) J. Biol. Chem. 269, 21778–21785), we next examined the contribution of ET-stimulated NO production to endothelial cell migration. In three independent cellular systems, 1) migration and wound healing by microvascular endothelial cells, 2) wound healing by Chinese hamster ovary cells stably expressing ETB receptor with or without endothelial NO synthase, and 3) application of antisense oligodeoxynucleotides targeting endothelial NO synthase in human umbilical vein endothelial cells, an absolute requirement for the functional NO synthase in cell migration has been demonstrated. These findings establish the permissive role of NO synthesis in endothelin-stimulated migration of endothelial cells.

Since we and others have previously demonstrated that occupancy of ETB receptors in endothelial cells is accompanied by the activation of constitutive endothelial NO synthase (9, 10), we hypothesized that the observed motogenic effects of members of the ET family may in fact be mediated by the release of NO. Indeed, there is emerging evidence that NO release serves as a prerequisite for epithelial and endothelial cell motility (11–13). Leiboovich et al. (13) have shown that production of angiogenic activity by activated monocytes (assayed by chemotaxis of endothelial cells and corneal angiogenesis) is absolutely dependent on L-arginine and NO synthase. These observations are in concert with findings reported by Ziche et al. (12) who detected the potentiation by sodium nitroprusside of the angiogenic effect of substance P in the rabbit cornea. Our own observations expand this function to the classical angiogenic signal, vascular endothelial growth factor. We demonstrated that endogenous NO production by the endothelial cells is a prerequisite for the motogenic and angiogenic effects of this factor. In the present study, we used three different approaches (migration and wound healing by endothelial cells, wound healing by Chinese hamster ovary cells expressing ETB receptor with or without eNOS, and application of antisense oligodeoxynucleotides targeting eNOS) to provide evidence that the effect of ET-1 on cell migration is mediated via ETB receptor and requires functional enzymatic machinery for NO generation.

MATERIALS AND METHODS

Cell Culture and Reagents—Renal microvascular endothelial cells (RMVEC) were previously established and characterized (15). This cell line shows expression of receptors for acetylated low density lipoprotein, staining with an antibody to von Willebrand antigen, and capillary tube formation. Human umbilical vein endothelial cells (HUVEC) were isolated according to the previously established technique (9, 16). RMVEC were grown in gelatin-coated dishes in M199 culture medium (Mediatech, Washington, D. C.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) HUVEC were cultured in M199 supplemented with 100 μg/ml heparin, 30 μg/ml endothelial cell growth factor, and 10% fetal bovine serum. Endothelin-1 (ET-1) and endothelin-3 (ET-3) were purchased from Calbiochem. IRL-1620, BQ123, and BQ788 were generously provided by Dr. Magazine (Queens College, Flushing, NY). N⁵-Nitro-L-arginine methyl ester (l-NAM) was obtained from Bachem California (Torrance, CA), and N-nitroso-N-acetyl-DL-penicillamine was purchased from Molecular Probes, Inc. (Eugene, OR).

Functional Expression of ETₐR and eNOS in CHO Cells—The generation and characterization of individual cell lines used in this study have been described previously (9). Briefly, CHO-ETₐR cell cultures stably integrated copies of the ETₐR cDNA cloned, and the amplified insert encompassing the ETₐR open reading frame was digested with PstI, gel-purified, and ligated into the eukaryotic expression plasmid pMT2. This plasmid is driven by the adenovirus major late promoter and
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contains the sequences encoding murine dihydrofolate reductase imme-
mediately downstream of the cDNA insert, thereby allowing for the estab-
lishment of stably integrated cell lines with greatly amplified copy
numbers when propagated in the presence of methotrexate (17). Dihy-
drofolate reductase-deficient CHO cells (18) were propagated in nucleo-
somes of the modified Dulbecco's medium (Sigma), supplemented
with 10% dialyzed fetal calf serum, and subsequently plated at a den-
sity of 5 × 10^5/100 mm^2 dish. Cells were transfected with 30 μg of
pMT2-ETB or pMT2-wild type using the calcium phosphate transfection
method of Chen and Okayama (19). After 5 days, selection and ampli-
ification of a miniature gold electrode, have been reported previously
tric cell-substrate impedance sensor, a device that detects with high
ultra-high sensitivity and frequency selectivity for the probe (25
maximized by supplementation of the medium with 50 μg/ml penicillin and 100 μg/ml streptomycin. After
wounding, the images were recorded immediately and 3 and 6 h later and
stored for analysis (Universal Imaging Corp., West Chester, PA). The
surface area of wounds in the consecutive images was measured, the
size of the initial wound was expressed as 100%, and the timed pro-
gression of wound healing was expressed as fractions of the initial size.

**Antisense Oligodeoxynucleotide Treatments—** The antisense phosphi-
rhiothide derivatives of oligodeoxynucleotides (SODN) to the human
endothelial constitutive NO synthase (eNOS) 5'-AGT TGC CCA TGT
TAC TGT GCG TCC CTG-3', nucleotides 56–30 of human eNOS cDNA
(20), as well as the sense 5'-GAG GGA CCC ATA OTA ACA TGA GCA
AGT ACC-3' and scrambled 5'-TCG GTA CCT GCT GAT GCT-3'
TTCC-3' phosphorothioate sequences were synthesized using an auto-
lated solid-phase DNA synthesizer (Applied Biosystems, Foster City,
CA). These sequences included the 5'-untranslated region of eNOS
cDNA and initiation codon. Thus-designed sequences showed no homol-
gy with other known mammalian sequences deposited in the GenBank
dbase, as screened using a Blast program (21). All the S-ODNs were
purified using oligonucleotide purification cartridges, dried down,
and resuspended in Tris-EDTA (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0),
and quantified spectrophotometrically.

Prior to migration assays, HUVEC were incubated with 10 μM S-
ODNs for 12 h under a serum-free condition. During typical experi-
ments in a Boyden chemotactic chamber (see above), S-ODNs were
deposited in the medium containing 0.1% bovine serum albumin
in a dose-dependent manner, with 2 mM l-NAME resulting in
complete abrogation of the ET-1-induced cell migration. Under the
conditions of NO inhibition, endothelial cell migration could be
restored in the presence of 8-bromo-cyclic GMP (100 μM), as shown
in Fig. 2. To elucidate the type of ET receptor(s) involved in the
observed stimulation of RMVEC migration, transwell migration
experiments were performed with different endothelins and in the
presence of selective inhibitors of their receptors. As sum-
marily in Fig. 3, ET-1 and ET-3 were equipotent in stimulat-
ing endothelial cell migration. A selective ET_{A}R agonist, IRL-
1620 exhibited a similar stimulatory effect, suggesting that the
observed phenomena were mediated via ET_{A}R. This conclusion is
further supported by the observation that a selective ET_{A}R

**RESULTS**

**Motogenic Effect of Endothelin Is Mediated via ET_{B} Receptor and
Is NO-dependent—** With regard to NO release and locomotory
responses, RMVEC displayed sensitivity to ET-1 within the
physiologic concentration range (Fig. 1). ET-1 stimulated
SO production by RMVEC, and l-NAME blunted NO release in
a dose-dependent manner, with 2 mM l-NAME resulting in
complete inhibition of NO release (Fig. 1A). Transwell migrat-
on RMVEC, studied in a modified Boyden chamber, showed the
similar exquisite sensitivity to ET-1. A statistically signif-
ificant almost 50% increase in the number of migrated cells was
detected with ET-1 concentrations of 10 pM, and it almost
doubled with further elevation of ET-1 concentration to 1 nM
(Fig. 1B). Inhibition of NO synthesis with 2 mM l-NAME com-
pletely abrogated the ET-1-induced cell migration. Under the
conditions of NO inhibition, endothelial cell migration could be
restored in the presence of 8-bromo-cyclic GMP (100 μM), as shown
in Fig. 2.
receptor antagonist BQ123 did not affect ET-1-induced endothelial cell transmigration, whereas a selective ETBR antagonist BQ788 completely abrogated the stimulatory action of ET-1. These findings were in concert with ET-1-induced NO release. While BQ123 did not interfere with NO production by endothelial cells stimulated with 1 nM ET-1, pretreatment with BQ788 abrogated NO release in response to ET-1 but not to bradykinin (Fig. 4). It is therefore concluded that ET-1 induces endothelial cell migration via the ETαR and that the previously demonstrated coupling of this receptor to eNOS (9) is critical for NO production and cell migration.

Effects of Endothelin on the Rate of Endothelial Wound Healing—The rate of endothelial wound healing was examined by electroporation-induced RMVEC denudation from the surface of a gold miniature electrode, as previously detailed (25). ET-1 accelerated wound healing at 10 h by 66.0 ± 3.2% (n = 5) compared with the control medium (40.4 ± 2.0% (n = 5), p < 0.05) (Fig. 5). The rate of restitution of endothelial integrity was equally enhanced by IRL-1620 and by ET-3, and this effect was abolished by co-application of ETB receptor antagonist BQ788, further implicating ETB receptor in the observed phenomena (Fig. 5, A and B). The addition of l-NAME to ET-1- or ET-3-stimulated endothelial cells virtually abrogated this response (Fig. 5, C and D).

Effects of Endothelin on Wound Healing in CHO Cells—To further test the above premise that NO production is required for ET-1-induced RMVEC migration, experiments were performed in a model system of CHO cells expressing ETBR and eNOS, as verified and detailed previously (9). ET-1 was ineffective in stimulating wound healing in CHO cells transfected with ETβR alone (Fig. 6). However, co-expression of both ETαR
and eNOS in CHO cells imparted on them the migratory response to ET-1 and resulted in acceleration of wound healing. The data further confirm the permissive role of NO production in ET-1-induced migration.

Effects of Endothelin in the Presence of Antisense Oligodeoxynucleotides Targeting eNOS—In the next series of experiments, a selective knockdown of eNOS was performed using antisense S-ODNs. To avoid any possible species-specific variations in the sequence, experiments were performed in HUVECs using an antisense construct directed against the initiation codon of human eNOS cDNA. Cells were treated with either antisense, sense, or scrambled S-ODNs, as detailed under “Materials and Methods.” The expression of immunodetectable eNOS in endothelial cells subjected to S-ODNs is presented in Fig. 7, confirming the adequacy of treatments. Functional analysis of endothelial cells subjected to S-ODNs was accomplished by monitoring NO release. Fig. 8A depicts typical tracings of ET-1-induced NO release. Both sense and scrambled S-ODN-treated cells responded to ET-1 with increased NO release, similar to that observed in intact control cells. In contrast, antisense S-ODN-treated HUVECs failed to produce NO in response to ET-1. These data confirm the validity of the utilized S-ODN constructs. To study the effects of ET-1 on motility of S-ODN-treated HUVECs, experiments were performed in a modified Boyden apparatus. As summarized in Fig. 8B, pretreatment of HUVECs with antisense S-ODN resulted in a dramatic deceleration of transmigration, as compared with intact control cells and cells exposed to sense or scrambled constructs.

DISCUSSION
In this study, two different endothelial cell preparations, derived from rats and humans, showed a consistent acceleration of motility in response to members of the endothelin family. Through application of specific agonists and antagonists of known endothelin receptors, ET<sub>A</sub> and ET<sub>B</sub>, it was possible to implicate ET<sub>B</sub> receptor in the observed responses. However, these effects of ET were mediated by endogenous NO production, as confirmed by the results of three independent experimental approaches. In the first series of experiments, l-NAME prevented ET-1-induced endothelial cell transwell migration and wound healing. In the second series, CHO-ET<sub>B</sub>NOS cells, a model cell system devoid of complexity inherent to endothelial cells, showed NO dependence of migration induced by ET-1. Finally, the application of eNOS isoform-selective antisense S-ODNs, but not sense or scrambled constructs, to endothelial cells suppressed their migratory responsiveness to ET-1. From these observations, we derive a conclusion that stimulated NO production serves a permissive role in ET-induced acceleration of endothelial cell motility and wound healing.

ET<sub>B</sub> receptor in endothelial cells serves a unique purpose for autocrine feedback regulation of several physiologic functions by the produced ET-1. The application of anti-ET γ-globulin to cultured endothelial cells inhibits the rate of proliferation (26, 27). Moreover, this receptor is implicated in ET-induced vaso-dilation (10). We have recently demonstrated that this non-isopeptide-selective receptor is functionally coupled to eNOS and coordinates the release of NO from endothelial cells (9).
Stably transfected CHO-ETbNOS cells were successfully employed in those studies to reveal, in a simplified model system, that receptor-enzyme coupling involves the tyrosine kinase- and calcium/calmodulin-dependent pathways (9). Hence, the studies established a physiologically meaningful communication and coordination between endothelium-derived vasoconstrictor and vasodilator systems that control the vascular tone. Other investigators have also viewed ET-1 generated by endothelial cells as an autocrine motility factor (7, 8). However, results presented herein implicate for the first time the integral complex system of endothelium-derived vasoactive agonists, endothelin and NO, in endothelial cell motility and implicate this system in vascular remodeling.

We have previously demonstrated that NO production is a prerequisite for epithelial cell migration guided by several motogens, including hepatocyte growth factor and epidermal growth factor (11). The establishment of head-to-tail gradients of NO synthase in locomoting, but not in stationary, epithelial cells was construed as an important regulator of locomotion driven by these guidance cues (11). More recently, we have established that NO interferes with processes of endothelial cell adhesion to matrix proteins and participates in locomotion initiated by vascular endothelium growth factor. These findings provided an experimental basis for a hypothesis ascribing to NO the function of a modulator of cell-matrix adhesion. In this vein, ET-induced NO production by endothelial cell can be considered as a particular example of a more generalized phenomenon of NO-regulated cell motility.

The described NO production serving as a prerequisite for endothelial cell locomotion in response to activation of ETb receptor may explain a host of pathophysiologic observations on inadequate angiogenesis despite enhanced generation of ET-1. In hypercholesterolemic pigs and in humans with atherosclerotic lesions, ET-1 generation is augmented (28–30).

![Fig. 6. Effect of ET-1 on the rate of wound healing by genetically engineered CHO cells.](image)

Confluent cultures of wild type (WT) CHO cells as well as the cells stably expressing ETb receptor alone (CHO-ETbR) or together with eNOS (CHO-ETbR/NOS) were wounded as detailed under “Materials and Methods.” The initial size of each wound was expressed as 100%, and the rate of wound healing was assessed as a decrease in wound size (panel A depicts representative images of wound healing). Note that ET-1 (1 nM) accelerated wound healing (* denotes p < 0.05 versus control, n = 5–7 separate experiments) only in CHO-ETbR/NOS cells, and this effect was inhibited by L-NAME (panel B). Wild type CHO cells did not respond to ET-1 (not shown).

![Fig. 7. Immunohistochemical detection of eNOS in HUVEC subjected to different phosphorothioate oligodeoxynucleotide constructs.](image)

Cells were pretreated with oligodeoxynucleotides (10 μM each) for 12 h prior to experiments. A, control cells, B–D, cells pretreated with the antisense, sense, or scrambled S-ODNs, respectively, E, control cells stained with the secondary antibody. Scale bar, 50 μm.

![Fig. 8. Effects of antisense oligodeoxynucleotides targeting the initiation codon of human eNOS cDNA on nitric oxide release (A) and transwell migration (B) of human umbilical vein endothelial cells.](image)

Phosphorothioated antisense (AS), sense (S), and scrambled (Scr) constructs were designed and prepared as detailed “Materials and Methods.” Cells were pretreated with oligodeoxynucleotides (10 μM each) for 12 h prior to experiments. As shown in A, 1 nM Et-1 did not stimulate NO release from cells pretreated with antisense oligodeoxynucleotides, whereas control (C) cells or cells pretreated with sense and scrambled constructs responded to ET-1 with increased release of NO. (Inset to A shows the time and amplitude scale.) Tracings are representative of 3 separate experiments. In B, companion cells were utilized for ET-1-induced transwell migration experiments. Transmigration was halted only in cells pretreated with antisense oligodeoxynucleotides (* denotes p < 0.005 versus control).
The reason for the insufficient angiogenesis toward ischemic sites could be conceptually explained by the fact that NO production by the endothelium is suppressed under these conditions (31–33). Similarly, endothelial wound healing after balloon angioplasty is retarded, neointimal formation by proliferating smooth muscle cells is enhanced, and endothelin receptor antagonist SB 209670 protects angioplastic vessels against neointimal formation (14). Interpretation of these observations may rest on the above presented findings on the permissive role of NO in endothelial cell migration and wound healing.

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