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Nerve Growth Factor-Induced Migration of Endothelial Cells

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ABSTRACT

Nerve growth factor (NGF) is a well known neurotropic and neurotrophic agonist in the nervous system, which recently was shown to also induce angiogenic effects in endothelial cells (ECs). To measure NGF effects on the migration of cultured ECs, an important step in neoangiogenesis, we optimized an omnidirectional migration assay using human aortic endothelial cells (HAECs) and validated the assay with human recombinant basic fibroblast growth factor (rhbFGF) and human recombinant vascular endothelial growth factor (rhVEGF). The potencies of nerve growth factor purified from various species (viper, mouse, and recombinant human) to stimulate HAEC migration was similar to that of VEGF and basic fibroblast growth factor (bFGF) (EC_{50} of ~0.5 ng/ml). Recombinant human bFGF was significantly more efficacious than either viper NGF or rhVEGF, both of which stimulated HAEC migration by ~30% over basal spontaneous migration. NGF-mediated stimulation of HAEC migration was completely blocked by the NGF/TrkA receptor antagonist K252a [(8*R**,9*S**,11*S**)-(ı)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)cycloocta(*c*,*d*,*e*)trindene-1-one] (30 nM) but not by the VEGF/Flk receptor antagonist SU-5416 [3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-indolin-2-one] (250 nM), indicating a direct effect of NGF via TrkA receptor activation on HAEC migration. Viper NGF stimulation of HAEC migration was additively increased by either rhVEGF or rhbFGF, suggesting a potentiating interaction between their tyrosine kinase receptor signaling pathways. Viper NGF represents a novel pharmacological tool to investigate possible TrkA receptor subtypes in endothelial cells. The ability of NGF to stimulate migration of HAEC cells in vitro implies that this factor may play an important role in the cardiovascular system besides its well known effects in the nervous system.

Angiogenesis is one of the major processes leading to the formation of new blood vessels. Angiogenesis is an essential part of embryonic development and also contributes to normal physiological events in adults. In addition, angiogenesis is an important hallmark of and contributor to many pathological states, including diabetes and cancer (Folkman, 2003). In response to a stimulus by an angiogenic growth factor, endothelial cells migrate into the interstitial space by first degrading the underlying basement membrane. Behind the front of migrating cells, other endothelial cells continuously proliferate to provide the necessary number of cells to generate the new vessel (neoangiogenesis) (Ausprunk et al., 1974).

Both migration and proliferation of endothelial cells (ECs) are pharmacological targets for drug discovery (Cai et al., 2000). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are known to induce migration and proliferation of endothelial cells in vitro and in vivo (Rousseau et al., 2000; Poole et al., 2001). These, like most other effects of VEGF and FGF, are mediated by activation of tyrosine kinase receptors Flk-1/KDR (VEGF) and FGF receptors (FGF), respectively, which leads to the phosphorylation of a variety of downstream targets, including cytoskeletal proteins, which in turn regulate EC migration (Kanda et al., 2004).

NGF is an evolutionary conserved polypeptide of the neu-

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ABBREVIATIONS: EC, endothelial cell; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; HAEC, human aortic endothelial cell; RAOEC, rat aortic endothelial cell; RAMEC, rat adrenal medullary endothelial cell; OM, omnidirectional migration; FGF, fibroblast growth factor; rhVEGF, human recombinant vascular endothelial growth factor; rhbFGF, recombinant human basic fibroblast growth factor; Flk-1/KDR, subtype of VEGF receptors (VEGFR2); SU-5416, 3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-indolin-2-one; K252a, (8*R**,9*S**,11*S**)-(ı)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)cycloocta(*c*,*d*,*e*)trindene-1-one; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; rhNGF, recombinant human nerve growth factor; mNGF, mouse nerve growth factor; vNGF, viper nerve growth factor; GM, growth medium; FBS, fetal bovine serum; EM, experimental medium; ANOVA, analysis of variance.

rotrophin family that plays a crucial role in the life of the sympathetic and sensory nervous systems (Levi-Montalcini, 1987). The majority of research on NGF has been performed using NGF isolated from the male mouse submaxillary gland. More recently, human recombinant NGF (Rask, 1999) and snake venom NGFs (Hayashi et al., 1996; Katzir et al., 2003) have become important additional NGF agonist tools.

Several recent reports indicate that NGF exerts a variety of effects on peripheral tissues, including the vasculature, suggesting that NGF may be a novel angiogenic factor (Cantarella et al., 2002; Lazarovici et al., 2005). In studying the effects of NGFs on EC migration, we used two different aortic ECs isolated from humans (human aortic endothelial cells, HAECs) and rats (rat aortic endothelial cells, RAOECs). Both these cell lines had been previously characterized in our laboratory for adhesion molecules and expression of various adenylate cyclase isoforms (Manolopoulos et al., 1995; Kanda et al., 1998). For comparison, we also used rat adrenal medullary endothelial cells (RAMECs), which respond to thrombin stimulation by secretion of different extracellular matrix proteins (Papadimitriou et al., 1997).

To investigate the effects of NGF on EC migration, we modified and optimized a previously described omnidirectional migration (OM) assay (Cai et al., 2000). Using this assay, we demonstrated that NGF from various species induced migration of EC cells, albeit with various efficacies. The effects of viper-NGF (vNGF) were comparable with those induced by human recombinant VEGF (rhVEGF) but were less efficacious than the strong migratory effect elicited by human recombinant bFGF (rhbFGF). NGF-induced EC migration was selectively blocked by K252a (NGF receptor inhibitor) but not by SU-5416 (VEGF receptor inhibitor). These results strongly support the concept that NGF may represent a novel angiogenic factor, which among other angiogenic effects induces migration of cultured aortic endothelial cells.

Materials and Methods

Materials. Reagents were purchased from Sigma-Aldrich (St. Louis, MO), with the exception of L-alanyl-L-glutamine and phosphate-buffered saline (PBS), which were purchased from Mediatech (Herndon, VA), and fetal bovine serum (FBS), which was from Hyclone Laboratories (Logan, UT).

Growth Factors. Human recombinant epidermal growth factor and vascular endothelial growth factor (rhVEGF 165) were purchased from Sigma-Aldrich. Human recombinant basic fibroblast growth factor (rhbFGF) was kindly provided by Cytolab Co. (Rehovot, Israel). Human recombinant nerve growth factor (rhNGF) and mouse β-nerve growth factor (2.5S-mNGF) were kindly supplied by Alomone Labs (Jerusalem, Israel). vNGF was purified as described previously (Hayashi et al., 1996; Katzir et al., 2003). Stock solutions of the different NGFs were routinely analyzed for activity in the PC12 bioassay (Katzir et al., 2003). Stock solutions of all growth factors (0.2–2.2 mg/ml) in PBS were aliquoted and stored at −20°C.

Drugs. The high-affinity NGF-receptor (trk) antagonist K252a was a gift from Kyowa Hakko Kogyo Co. (Tokyo, Japan). The selective VEGF receptor (Flk-1/KDR) antagonist SU-5416 was kindly provided by Dr. Aviv Gazit (Department of Organic Chemistry, The Hebrew University of Jerusalem, Israel). The drugs were dissolved in DMSO at concentrations of 1 mM (K252a) and 10 mM (SU-5416), aliquoted, and kept in the dark at −20°C.

Cell Culture. Several previously described EC lines were used in this study: RAMECs (Papadimitriou et al., 1997), RAOECs (Manolopoulos et al., 1995), and HAECs (Kanda et al., 1998). All cell lines were adapted to grow in a common growth medium (GM) composed of MCDB-131 and M-199 at a ratio of 1:1 supplemented with 7.5% (w/v) sodium bicarbonate, 10% FBS, 50 IU of penicillin, 50 μg/ml streptomycin, 25 μg/ml amphotericin B, 2 mM L-alanyl-L-glutamine, 2.3 μM hydrocortisone, 10 U/ml heparin, 10 ng/ml human recombinant epidermal growth factor, and 3 ng/ml rhbFGF at pH 7.4. The cells were grown in a humidified tissue culture incubator in 5% CO_2 at 37°C. For the migration experiments, we used EC cultures between passages 10 and 24. In terms of their migratory capabilities, the cells did not display any significant differences between early and late passages.

OM Assay. For this work, we modified and optimized the OM assay recently described by Dixit et al. (2001). In brief, in the first stage (Fig. 1A, step 1), we marked the outside bottom surface of six-well tissue culture plates (BD Biosciences, San Jose, CA) denoting the outer circumference of a cloning ring (4 mm in diameter; Fisher Scientific Co., Pittsburgh, PA). The center of the ring was also marked to allow for precise positioning of the ring inside the well before cell application and later on for accurate photography of cell migration (Fig. 1A). In the second stage (step 2), a ring was placed onto the marked circumference inside each well. Based on their size differences, the different EC lines were seeded inside the rings at densities of 60,000 (~4725 cells/mm^2), 50,000 (~3940 cells/mm^2), and 20,000 (~1575 cells/mm^2) for RAOECs, RAMECs, and HAECs, respectively. These densities were optimal for the formation of an instantaneous circular monolayer inside the rings. The EC suspensions were carefully applied into the center in a drop of 40 μl of GM and incubated at 37°C and 5% CO_2 for ~1 h (RAMECs) or ~2.5 h (RAOECs and HAECs). A 5-mm glass bead (Fisher Scientific Co.) was placed on top of the ring (Fig. 1A) to provide stability and firm contact to the bottom of the well. In the next stage (step 3), the rings were lifted, the cell monolayers were washed twice with prewarmed PBS (with calcium and magnesium), and the experiment was initiated by the addition of 2 ml of experimental medium (EM). EM is identical in composition to GM without rhbFGF supplementation. By excluding this particular growth factor, we were able to generate accurate growth factor dose-response effects and also to use rhbFGF as a positive control.

All growth factors were prepared aseptically in EM 1 day before the experiment and applied at the desired concentrations to the circular monolayers in a volume of 2 ml for a period of 3 days. In the

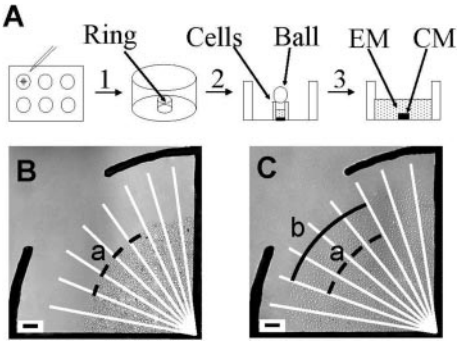

Fig. 1. OM assay schematics and migration measurements. A, schematic representation of the OM protocol setup. Marking the ring in the six-well plate was followed by precise placement of the ring (step 1) followed by addition of the cells in growth media and stabilization of the ring position with a glass ball (step 2) and removal of the ring, washing with PBS, and addition of EM to cover the ring formed cell monolayer (CM) (step 3). B and C, a quadrant of the circular monolayer is presented. Before plating, the bottom side of the well was etched (dark lines) to generate a reference line with the radius of 2 mm, representing the distance from the center of the ring to the periphery. The front of the monolayer is marked with a dotted line. To measure the migration of the cells, we compared the front of the cell migration at 0 time (a) and 3 days (b). To estimate precisely the distance of migration, 10 white lines (software generated radial grid) were superimposed on the images, and measurements of the radii (microns) from the center of the ring to the front were made.

control experiment, the cell monolayers were treated with EM only. In each experiment, EM supplemented with rhbFGF (50 ng/ml) and/or rhVEGF (10 ng/ml) served as positive controls. The growth factor receptor antagonists K252a and SU-5416 were dissolved in 0.01% DMSO, which in preliminary experiments was found to not affect migration. The cells were treated with the antagonists and incubated for 1 h at 37°C before the addition of the growth factors, both of which continued to be present for the entire experimental period. Every 2 days, the EM medium was changed with fresh medium that included the growth factors and/or drugs, according to the experimental protocols.

Data Acquisition and Evaluation. Unless otherwise stated, all experiments were terminated after 3 days. The monolayers were photographed at time 0 (Fig. 1B) before incubation with the growth factors and drugs and after 1 and 3 days (Fig. 1C). Monolayers were inspected on an inverted Nikon contrast microscope (Eclipse TE-2000-U; Nikon, Melville, NY) using a 2× long-working distance objective. All images were acquired digitally using a Hamamatsu black-and-white high-resolution camera and analyzed using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). In each circular monolayer, we separately photographed the four quadrants of the circle (Fig. 1, B and C). The quadrants were marked in the center of the tissue culture plate carefully preserving the 0, 90, 180, and 270° direction. The photographs were analyzed by superimposing a software generated radial grid (F. Dietrich and P. I. Lelkes, manuscript in preparation) along the circular cell monolayer (Fig. 1, B and C) to determine the migration of the cells at the front of the circular monolayer. Migration distance was defined as the difference between the cell monolayer fronts from the center of the ring measured at 3 days (Fig. 1Cb) compared with the cell monolayer front at 0 time (Fig. 1Ca). The mean ± S.D. of the migration of the cell monolayer is presented in micrometer or as percentage of migration with respect to control (untreated cells).

Statistical Analyses. In general, 10 radii were measured in each quadrant ($n_1 = 10$), taking into account at least three (the most regular cell front monolayers quadrant) of the four quadrants ($n_2 = 3$) to produce in each ring experiment, 30 measurements. A duplicate set of rings was used for a given experimental condition ($n_3 = 2$), generating 60 total measurements of each condition. Each OM experiment was repeated at least three times. The OM experiments described in Fig. 4 represent mean ± S.D. data from up to 10 different experiments ($n_4 = 3$–10). Statistical significance was determined using Student's t test and/or one-way analysis of variance (ANOVA) followed by Bonferroni's post-tests or Newman-Keuls multiple comparison tests. In general, we considered the difference between groups to be significant for $p < 0.01$, with certain exceptions listed in the text, where we accepted $p < 0.05$ as statistically significant.

Results

Optimization of the OM Assay. In our optimized assay, the cell monolayer front at time 0 was characterized by a smooth sharp border (Fig. 1Ba); after 3 days, the monolayer front was less well defined (Fig. 1Cb). As seen in Fig. 1C, the cells migrated radially outward to form a larger circle characterized by front b. To assess migration (change of circular cell monolayer front as a function of time), we measured the radial migration distance (Fig. 1, b to a). As seen in Fig. 2, RAMEC seeded at an initial density of 50,000 cells/ring migrated in 3 days, 2.5-fold farther than after day 1.

To further validate the assay, we investigated the effect of reducing the experimental medium serum concentration on RAMEC migration (Table 1). Using regular cell culture medium (supplemented with 10% FBS), we observed significant "spontaneous" migration, which might be attributed to angiogenic factors normally present in serum.

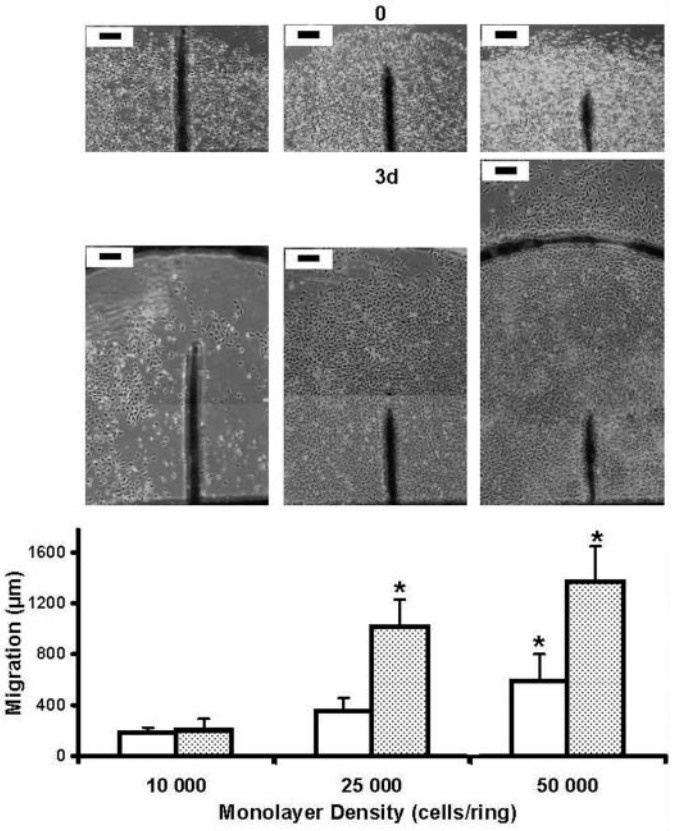

Fig. 2. Effect of cell monolayer density and time dependence on endothelial cell migration. RAMECs at different densities were plated, and the migration experiment was performed in GM. The ring monolayers were photographed (top of light micrographs; scale bar, 250 μm) immediately upon plating (0) and after 3 days at the end of the experiment (3d). The bottom represents histograms of the monolayer front cell migration (measured in micrometers) after 1 day (open columns) and 3 days (dotted columns). *, $p < 0.01$ compared with the group of 10,000 cells/ring using Student's t test.

TABLE 1
Effect of serum concentration on RAMEC migration

Serum Concentration	Migration[a]
%	$\mu m/3$ day
0.1	407 ± 27
1	579 ± 43[b]
2	586 ± 35[b]
5	930 ± 46[b]
10	814 ± 57[b]

[a] These values (mean ± S.D.) represent the difference between the radius of migration at time 0 subtracted from the radius of migration after 3 days.
[b] Statistical significance (*, $p < 0.01$) for all experimental groups was calculated by Student's t test comparing each experimental group to 0.1% FBS concentration ($n = 40$–50).

To test the effect of growth factors on HAEC migration with minimal contribution of proliferation and because NGF effect on migration was maximal at 2% FBS (Fig. 4, inset), all further migration experiments were performed in 2% FBS. As seen in Fig. 3, spontaneous migration varied between the three endothelial cell lines, with RAMEC > HAEC > RAOEC. One possible reason for these differences in the migration of diverse ECs may be the distinct levels of endogenous growth factors released into the medium (Hannan et al., 1988). Given the importance of HAECs as a model system for the human vasculature, most of the subsequent experiments were carried out with HAECs.

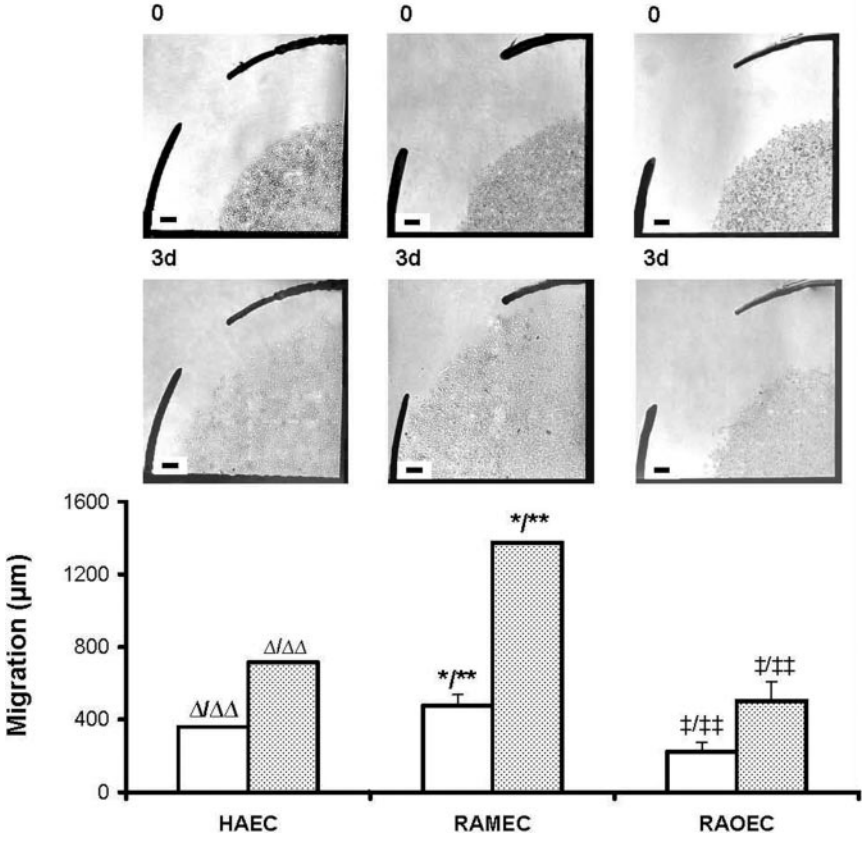

Fig. 3. Comparison of spontaneous migration among different endothelial cells. HAECs (20,000 cells/ring), RAMECs (50,000 cells/ring), and RAOECs (60,000 cells/ring) were plated, and the migration experiment was performed in EM. The ring monolayers were photographed (top of light micrographs; scale bar, 250 μm) immediately upon plating (0) and after 3 days (3d). The bottom represents histograms of the monolayer front cell migration (measured in micrometers) after 1 day (open column) and 3 days (dotted columns). $*$, Δ, $\ddagger$, $p < 0.01$, by comparison between the different endothelial cells, one to the other at each individual time point using Student's t test. Single symbols represent comparisons between the various species; double symbols represent comparison of the migration distances of the same species measure at days 1 and 3, respectively.

NGF-Induced Endothelial Cell Migration. To further validate the OM assay, we characterized the effects of two known angiogenic factors, rhVEGF and rhbFGF. As seen in Fig. 4, both these angiogenic growth factors significantly stimulated migration of HAEC cells by 1.4- (1 day) and 1.6-fold (3 day) for rhbFGF and 1.2- (1 day) and 1.3-fold (3 day) for rhVEGF, respectively. Furthermore, the data in Fig. 4 also indicate that, among the different NGFs, vNGF had the strongest effect by stimulating HAEC migration by 1.1- and 1.3-fold on days 1 and 3, respectively. By contrast, identical doses of mNGF and rhNGF had a weak (3–5%) stimulatory effect, which became statistically significant after 3 days (Fig. 4). Viper NGF stimulated migration of RAMECs in a serum-dependent manner (Fig. 4, inset). Upon 3 days of treatment with growth factors, migration of RAOECs was moderately enhanced by vNGF (13 ± 4%), whereas exposure to rhbFGF and rhVEGF resulted in an 80 ± 4 and 20 ± 3% enhancement of migration, respectively (data not shown). Together, these data provide evidence that NGFs enhances migration in all of the three endothelial cells investigated.

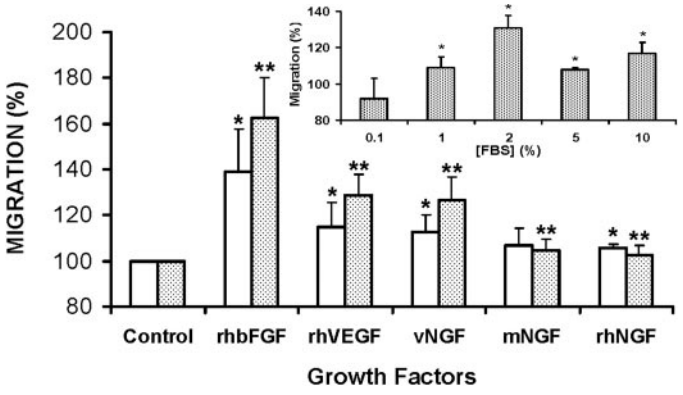

Fig. 4. NGF-induced migration of endothelial cells. A comparison with rhbFGF and rhVEGF effect on HAEC cells. Inset, effect of serum concentration on vNGF-induced migration of RAMEC cells. HAECs and RAMECs were plated at a density of 20,000 and 50,000 cells/ring, respectively, and were treated with different growth factors for 3 days in EM or at different FBS concentrations (inset): rhbFGF, 50 ng/ml; rhVEGF, 10 ng/ml; vNGF, 50 ng/ml; mNGF, 50 ng/ml; and rhNGF, 50 ng/ml. Cell migration in percentage was calculated after 1 day (open columns) and 3 days (dotted columns). $*$, $p < 0.01$ compared with control. $**$, $p < 0.05$ compared with control using Student's t test and ANOVA (with Bonferroni's post-test). Migration distance of control was at 1 day, 332 ± 30 μm (100%), and at 3 days, 503 ± 42 μm (100%).

To further quantitatively characterize growth factor stimulation of HAEC migration, we established dose-response curves for rhVEGF, rhbFGF, and vNGF (Fig. 5). Apparent EC_{50} values were calculated from the log linear part of the dose-response curves generating values of 0.4, 0.5, and 0.6 ng/ml for rhVEGF, vNGF, and rhbFGF, respectively. These EC_{50} values indicate a similar high potency among these three growth factors in stimulating HAEC migration. However, a comparison of the maximal effects (Fig. 5) suggests that vNGF similar to rhVEGF was less efficacious than rhbFGF.

Selectivity of NGF-Induced HAEC Migration. To demonstrate that NGF induced-migration of HAECs is a specific receptor-mediated event, we compared the inhibitory effects of two selective antagonists, K252a, a well known antagonist of TrkA, which is the high-affinity NGF receptor (Berg et al., 1992), and SU-5416, which is a selective inhibitor of Flk-1/KDR, one of the VEGF receptors (Mendel et al., 2000a). As seen in Fig. 6, K252a at a nontoxic, selective concentration of 30 nM completely blocked vNGF-induced stimulation of

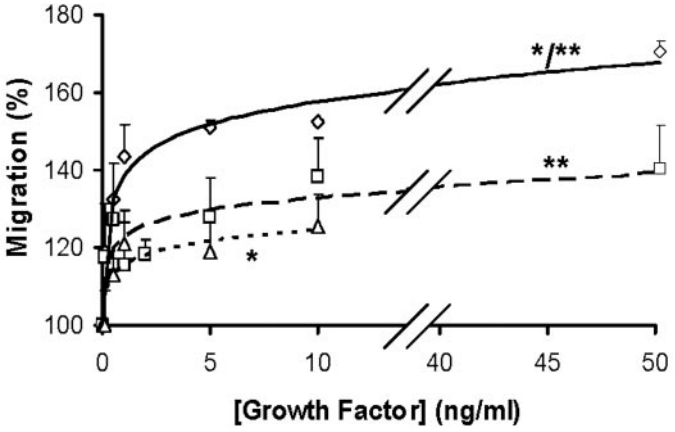

Fig. 5. Dose-response curves of the effect of growth factors on induction of migration of HAECs. HAECs were plated at a density of 20,000 cells/ring and were treated for 3 days with the different growth factors at concentrations up to 50 ng/ml in EM. The migration (percentage of control) is presented as mean ± S.D. At higher concentrations than 50 ng/ml, the migration of the cells was not further affected. *, $p < 0.05$ rhbFGF compared with rhVEGF (one-way ANOVA with Bonferroni's post-test); **, $p < 0.05$ vNGF compared with rhbFGF (one-way ANOVA with Newman-Keuls post-test). Δ, rhVEGF; □, vNGF; ◇, rhbFGF. Control migration was 335 ± 19 μm (100%).

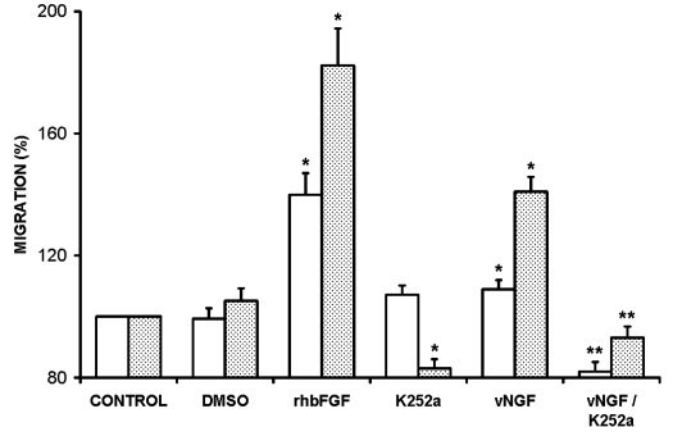

Fig. 6. K252a blocks NGF-induced migration of HAECs. HAECs were plated at a density of 20,000 cells/ring and were treated with vNGF (50 ng/ml) for 1 (open columns) and 3 days (dotted columns) in the absence or presence of a nontoxic concentration of 30 nM K252a. Cell migration was measured in microns and is presented as mean ± S.D. *, $p < 0.01$ compared with control; **, $p < 0.01$ the effect of K252a compared with vNGF treatment alone (one-way ANOVA with Bonferroni's post-test). Control migration was at 1 day, 379 ± 10 μm (100%), and at 3 days, 578 ± 18 μm (100%).

HAEC migration at both 1 and 3 days of treatment, indicating that this effect of vNGF is TrkA-mediated. We also noticed that K252a inhibited the spontaneous migration of HAEC cells by 20 ± 1%, suggesting either a constitutive release of NGF by the cells or a nonspecific effect of K252a on other cellular protein kinases involved in regulation of migration. When HAECs were treated with a concentration of 1 μM SU-5416, which is clinically relevant (Mendel et al., 2000b), the drug almost completely inhibited rhVEGF-induced cell migration (Fig. 7A). From dose-response experiments in which HAECs were exposed for 3 days to 10 ng/ml rhVEGF in the presence and absence of various concentrations of SU-5416, the apparent IC_{50} of SU-5416 was calculated to be ~0.9 μM (Fig. 7B). At this concentration, SU-5416 exerted a significant nonspecific inhibitory effect on vNGF-induced HAEC migration (data not shown). However, at a nontoxic concentration of 0.25 μM ($IC_{5-10\%}$), we observed a significant inhibition of SU-5416 on rhVEGF-induced but not vNGF-induced HAEC migration (Table 2).

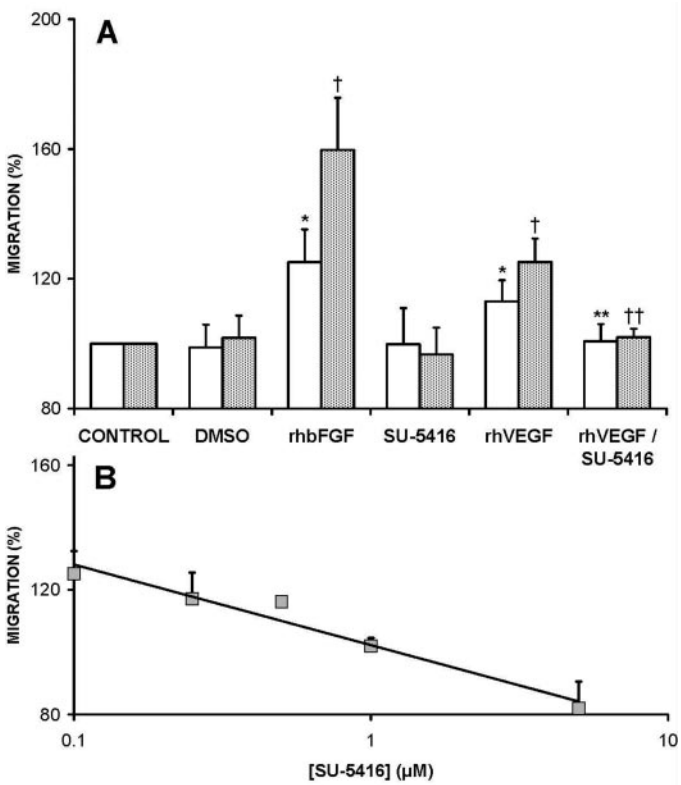

Fig. 7. SU-5416 dose-response inhibitory effects on VEGF induced migration of HAECs. HAECs were plated at a density of 20,000 cells/ring and were treated with rhbFGF (50 ng/ml), vNGF (50 ng/ml), and rhVEGF (10 ng/ml) for 1 day (A) or 3 days (A and B) in the absence or presence of SU-5416 at 1 μM (A) or at different concentrations (B). Cell migration is measured in percentage of control and is presented as mean ± S.D. after 1 day (open columns, A) and 3 days (dotted columns, A). Panel B shows the dose response of migration by SU-5416 measure at day 3. The regression coefficient was −0.95 (B). After the first day, *, $p < 0.05$ compared with control; **, $p < 0.05$, the effect of SU-5416 compared with VEGF treatment alone (one-way ANOVA with Newman-Keuls post-test). After the third day, †, $p < 0.01$ compared with control; ††, $p < 0.01$, the effect of SU-5416 compared with VEGF treatment alone (one-way ANOVA with Bonferroni's post-test). Control migration was at 1 day, 347 ± 22 μm (100%), and at 3 days, 618 ± 59 μm (100%).

TABLE 2

Stimulatory effect of vNGF on migration of HAECs is not blocked by SU-5416

The OM assay was performed with 20,000 cells/ring. After 1 h, the ring was removed, the cells were gently washed with PBS, and the growth factors in EM were added in the presence or absence of drugs: 0.01% DMSO, 10 ng/ml VEGF, 50 ng/ml vNGF, and 0.25 μM SU-5416. After 3 days, the experiment was stopped, and the cell monolayers were photographed using a 20× magnification 2× objective. The migration was measured and analyzed according to *Materials and Methods*.

Growth Factor	Migration	
	DMSO	SU-5416
	% over control	
Control	100	106 ± 9
rhVEGF	121 ± 6	99 ± 5*
vNGF	117 ± 7	118 ± 5

* $p < 0.01$; statistical significance was calculated by either Student's t test comparing each experimental group to the respective control DMSO value or by one-way ANOVA (with post-Bonferroni's test) ($n = 60$).

Potentiation of NGF-Induced HAEC Migration by rhVEGF and rhbFGF. Our data so far described independent stimulatory activities of three different angiogenic factors: vNGF, rhVEGF, and rhbFGF. We investigated possible physiologically relevant interactions among different combinations of these growth factors at concentrations that, according to the dose response curves presented in Fig. 5, individually generated up to ~10% stimulation of total HAEC migration. By comparison with the stimulatory effects of each individual growth factor alone, exposure to either the three pairs of growth factors or to a combination of all three angiogenic factors yielded significant additive enhancement of HAEC migration (Table 3).

Discussion

As the most significant outcome of this study, we report for the first time that several nerve growth factor agonists, in particular viper NGF, stimulated the migration of cultured human aortic endothelial cells. The potency of vNGF was similar to that of the well known angiogenic growth factors VEGF and bFGF; however, the efficacy of vNGF was lower than bFGF but similar to VEGF. Importantly, K252a, a potent relatively specific Trk-A NGF-receptor antagonist, blocked HAEC migration stimulated by NGF but not by VEGF. Conversely, SU-5416, a selective antagonist of the Flk-1/KDR VEGF receptor, blocked VEGF-induced but not vNGF-induced HAEC migration. These findings support the notion that viper nerve growth factor is a potent angiogenic NGF agonist, which acts via TrkA receptor-mediated signaling pathways.

For this study, we adapted and optimized a recently described omnidirectional migration assay (Dixit et al., 2001). An important validation of our OM assay is the reproducible stimulation of the migratory response of diverse ECs upon treatment with bFGF and VEGF, which has previously been documented in a variety of two- and three-dimensional migration assays (Yoshida et al., 1996; Vernon and Sage, 1999). In our hands, the EC_{50} values for all growth factors were in the range of 0.4 to 0.6 ng/ml, indicating that our OM assay is very sensitive, probably because we used a radial migration measurement approach and not a surface area approach (Dixit et al., 2001). These values are in line with the K_d values obtained for bFGF and VEGF binding to their respective receptors (Neufeld and Gospodarowicz, 1985; Soker et al., 1996). By comparison, in previous studies using these growth factors, concentrations between 1 and 10 ng/ml were used to measure the effects of VEGF and bFGF on the migration of cultured ECs (Yoshida et al., 1996; Ghosh et al., 2002).

TABLE 3

Potentiation of NGF induced HAEC migration by rhVEGF and rhbFGF

The OM assay was performed with 20,000 cells/ring. After 1 h, the ring was removed, the cells were gently washed with PBS, and the growth factors were applied in EM: 0.25 ng/ml rhVEGF, 0.1 ng/ml rhbFGF, and 0.1 ng/ml vNGF. After 3 days, the experiment was stopped, the cell monolayers were photographed using a 2× objective, and the migration was measured and analyzed according to *Materials and Methods*.

Growth Factor	Migration
	% over control
vNGF	109 ± 3*
rhbFGF	110 ± 3*
rhVEGF	109 ± 4*
vNGF/rhbFGF	119 ± 4*,**
vNGF/rhVEGF	123 ± 5*,**
rhbFGF/rhVEGF	120 ± 4*,**
vNGF/rhbFGF/rhVEGF	128 ± 6*,**

* $p < 0.01$ compared with control, Student's t test.

** $p < 0.05$ compared with each single growth factor, ANOVA (with Bonferroni's post-test).

A comparison between the maximal effects of these growth factors on HAEC migration indicates that rhbFGF is more efficacious than rhVEGF (Fig. 5). This finding is consistent with other migration studies performed with bovine aortic endothelial cells in which bFGF and VEGF induced ~155 and ~135% stimulation, respectively, of EC migration in a "wound model"-type migration assay (Ghosh et al., 2002).

A known difficulty in assessing cell migration in most in vitro assays is that cell proliferation may contribute in part to the measured migration (Cai et al., 2000). Preliminary data indicate that at day 3 of vNGF-stimulated migration, only 6 ± 1% HAECs were proliferating at the front of the circular monolayer (unpublished results). Thus, the contribution of proliferation to HAEC migration in the present OM assay may be relatively small and requires further investigation.

In this study, we compared the effects of several NGF agonists on HAEC cell migration. Mouse and human recombinant NGFs generated a weak yet statistically significant stimulatory signal. By contrast, viper NGF stimulated the migration of HAECs with a potency and an efficacy similar to that of rhVEGF. The higher potency of vNGF by comparison to the other NGFs may be attributed either to the Asn-21 glycosylation found only in vNGF (Katzir et al., 2003) but not in mouse and human recombinant NGF (Hayashi et al., 1996) or to an increased affinity toward TrkA receptors due to primary sequence changes compared with the other NGFs. It is well known that glycosylation of NGF and FGF results in an increased stability, probably causing more efficient and/or prolonged receptor stimulation (Delli-Bovi et al., 1988; Murphy et al., 1989).

Interestingly, the order of potency by which the various NGF analogs stimulated HAEC migration (vNGF $\gg$ mNGF $>$ rhNGF) is opposite to the order by which these agonists induce neurite outgrowth (rhNGF $>$ mNGF $>$ vNGF) in PC12 cells overexpressing neuronal human recombinant TrkA receptors (Katzir et al., 2003). This observation suggests that the TrkA receptor in HAECs may be of another subtype than human neuronal TrkA. Together, we propose that vNGF may serve as an important tool to study angiogenic effects of NGF in ECs in vitro and in vivo (Lazarovici et al., 2005).

Two receptor antagonists, K252a and SU-5416, are important tools for probing the specificity and selectivity of NGF- and VEGF-induced HAEC migration, respectively. At the low concentrations used in our studies, both K252a and SU-5416 are highly specific antagonists of the cognizant high-affinity receptors for NGF and VEGF, TrkA, and Flk-1/KDR, respectively. Under these conditions, we demonstrated that NGF-induced HAEC migration was not blocked by SU-5416, excluding the possibility that NGF effect is mediated directly by autocrine release by VEGF. This latter possibility has been favored in a recent in vivo study (Manni et al., 2005).

NGF-induced migration of aortic ECs confirms and extends recent similar observations in porcine aortic ECs (Rahbek. et al., 2005) and human choroidal ECs, but interestingly,

not in human retinal ECs (Steinle and Granger, 2003). NGF-induced migration of ECs is in line with previous reports on the angiogenic effects of mouse and human recombinant NGF in vitro and in vivo. NGF has been shown to stimulate proliferation of HUVECs (Cantarella et al., 2002), human choroidal endothelial cells (Steinle and Granger, 2003), human dermal microvascular endothelial cells (Raychaudhuri et al., 2001), and rat brain endothelial cells (Moser et al., 2004). NGF also promoted survival of mice aortic endothelial cells (Tanaka et al., 2004) and increased neoangiogenesis in the chick chorioallantoic membrane (Cantarella et al., 2002). In view of the well known phenomenon of EC heterogeneity (Lelkes et al., 1996), future studies will focus on the similarities and differences in angiogenic response among ECs of different tissue origins in response to NGF.

Generally, in the cardiovascular system, a number of different angiogenic factors are operative concomitantly. Hence, we studied the possible relationship among VEGF, bFGF, and NGF. As described in Table 3, the three growth factors additively stimulated EC migration, suggesting similarities in their mechanism of action. Previous studies indicated the presence of Flk-1/KDR (Endo et al., 2003), FGF receptor (Motamed et al., 2003), and TrkA (Rahbek et al., 2005) in aortic endothelial cells. As soon as one of the above-mentioned receptors is stimulated, the tyrosine phosphorylation of its cellular substrates initiates the signaling pathways that result in cell migration. Hence, stimulation of yet another tyrosine kinase-activating receptor may induce only a weakly additive effect. A synergistic effect would have required mobilization of a completely different signal transduction pathway. We propose that the potentiation of two-dimensional HAEC migration by the above-mentioned growth factors is analogous to similar effects seen in vitro in three-dimensional models (Pepper et al., 1992) as well as in in vivo experiments (Asahara et al., 1995).

The signaling pathways of NGF-induced migration or chemotaxis of endothelial and nonendothelial cells involve phosphatidylinositol 3-kinase, extracellular signal-regulated kinase 1 and 2, Src, Rho GTPase Rac 1, cdc42 kinase, and paxillin (Escalante et al., 2000; Steinle and Granger, 2003; Ho et al., 2005; Rahbek et al., 2005). An important issue to be elucidated is the role of each of the two NGF receptors, viz., TrkA and p75NTR in NGF-induced HAEC migration. p75 NTR modulates the migration of primary melanoma (Shonukan et al., 2003) and Schwann cells (Yamauchi et al., 2004). Our preliminary DNA array data indicate that HAECs express p75 NTR; inhibition of NGF-induced HAEC migration by K252 implicates the presence of trkA, in line with the findings by Rahbek et al. (2005). The precise role and signaling pathways of both NGF receptors are currently under investigation.

In conclusion, we propose that NGF-induced migration of ECs may be paradigmatic for the widespread cross-talk between the nervous and cardiovascular systems (Lazarovici et al., 2005). For example, sympathetic denervation results in significant blood vessel growth (Torry et al., 1991) that may be related to an increased NGF production by aortic tissue (Ueyama et al., 1991). It is tempting to speculate that NGF is necessary to induce proliferation and/or migration of ECs, which in turn will lead to the repair of cardiovascular tissue. Our observation of NGF-induced migration of cultured aortic cells may be relevant also for angiogenic processes in vivo.

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