Nerve Growth Factor, Brain-Derived Neurotrophic Factor, Neurotrophin-3 and Glial-Derived Neurotrophic Factor Enhance Angiogenesis in a Tissue-Engineered *In Vitro* Model

Mathieu Blais, MSc, Philippe Lévesque, Eng., MSc, Sabrina Bellenfant, BSc, and François Berthod, PhD

Skin is a major source of secretion of the neurotrophic factors nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial-derived neurotrophic factor (GDNF) controlling cutaneous sensory innervation. Beside their neuronal contribution, we hypothesized that neurotrophic factors also modulate the cutaneous microvascular network. First, we showed that NGF, BDNF, NT-3, and GDNF were all expressed in the epidermis, while only NGF and NT-3 were expressed by cultured fibroblasts, and BDNF by human endothelial cells. We demonstrated that these peptides are highly potent angiogenic factors using a human tissue-engineered angiogenesis model. A 40% to 80% increase in the number of capillary-like tubes was observed after the addition of 10 ng/mL of NGF, 0.1 ng/mL of BDNF, 15 ng/mL of NT-3, and 50 ng/mL of GDNF. This is the first characterization of the direct angiogenic effect of NT-3 and GDNF. This angiogenic effect was mediated directly through binding with the neurotrophic factor receptors tropomyosin-receptor kinase A (TrkA), TrkB, GFR α -1 and c-ret that were all expressed by human endothelial cells, while this effect was blocked by addition of the Trk inhibitor K252a. Thus, if NGF, BDNF, NT-3, and GDNF may only moderately regulate the microvascular network in normal skin, they might have the potential to greatly increase angiogenesis in pathological situations.

Introduction

THE NERVE GROWTH FACTOR (NGF), the brain-derived neurotrophic factor (BDNF) and the neurotrophin-3 (NT-3) are members of the neurotrophin family of growth factors. Neurotrophins are well known to regulate growth, survival, differentiation, function, and plasticity of neuronal cells.¹ NGF is the preferred ligand for the tyrosine kinase receptor tropomyosin-receptor kinase A (TrkA), BDNF for TrkB, and NT-3 for TrkC. NT-3 has also been shown to activate TrkA and TrkB.² Trk activity leads to activation of Ras, phosphatidylinositol 3-kinase, phospholipase C- γ 1, and signaling pathways downstream of these proteins, including the mitogen-activated protein kinases.³ All neurotrophins can also bind with smaller affinity the p75 receptor.¹ The neurotrophin family belongs to a larger collection of secreted factors called neurotrophic factors. They include, among others, the glial-derived neurotrophic factor (GDNF) with its multicomponent receptor system GFR α -1 and c-Ret.⁴

The epidermis is known to be a major source of neurotrophic factors, including NGF, BDNF, NT-3, and GDNF, to

support skin innervation.^{5,6} Moreover, NGF has been demonstrated to promote keratinocyte proliferation, which express TrkA and p75 in the basal layer of the epidermis.⁷ Thus, neurotrophic factors do not only control the cutaneous innervation, but could also modulate epidermal homeostasis. In addition, NGF and BDNF have been shown to promote angiogenesis in other tissues and context.⁸⁻¹² Angiogenesis is defined as the formation of new blood vessels from pre-existing ones. The link between NGF and angiogenesis is notably intriguing in the context of skin repair, remodeling, and pathogenesis. NGF is known to be upregulated in psoriatic skin, while increased angiogenesis contribute to this disease. Thus NGF, as well as other neurotrophic factors could participate at least in part to the modulation of the dermal microvascular network in normal skin as well as in pathological situation like wound healing. However, if NGF expression in the epidermis has been well described, the specific secretion of BDNF, NT-3 and GDNF in skin was not known. Most importantly, the secretion of each factor from the epidermis and from the dermal fibroblasts, endothelial cells, and Schwann cells has never been quantified.

We hypothesized that NGF, BDNF, NT-3, and GDNF were all expressed in the skin in significant amounts and that these neurotrophic factors are highly potent angiogenic factors.

Materials and Methods

Cell isolation

Fibroblasts were isolated from human skin biopsies after breast reductive surgeries as previously described,¹³ using 0.2 IU/mL collagenase H (Roche Diagnostics). The study was approved by the CHA research ethical committee DR-002-951. Cells were grown in Dulbecco-Vogt modification of Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics: 100 U/mL penicillin G (Sigma-Aldrich) and 25 µg/mL gentamicin (Shering) in 8% CO₂ at 37°C. Human umbilical vein endothelial cells (HUVEC) were obtained from healthy newborns by enzymatic digestion with 0.25 µg/mL thermolysin (Sigma-Aldrich) as previously described.¹⁴ Human dermal microvascular endothelial cells (HDMEC) were purified from human foreskin using Dynabeads coupled with anti-CD31 antibodies (DynaL Biotech).¹⁵ HUVEC and HDMEC were grown on collagen-coated surface in Endothelial Growth Medium-2 (EGM-2; Cambrex Bioscience Baltimore, Inc.) and characterized as previously described.¹⁶ Sensory neurons and Schwann cells were extracted from the dorsal root ganglia of mouse embryos (E13) and were cultured as previously described.¹⁷

Indirect immunofluorescence

Indirect immunofluorescence assays were performed on paraformaldehyde (4%) or formaldehyde (3.7%) followed by methanol (100%) fixed culture cells or frozen tissue sections embedded in optimal cutting temperature compound (Sukura, Finetek U.S.A., Inc.). For tissues fixed with paraformaldehyde, a further step of permeabilization was performed with phosphate buffered saline containing 0.5% (v/v) Triton X-100 (Bio-Rad). The primary antibodies used were, goat polyclonal anti-human TrkA, TrkB, TrkC, GFR α -1 and c-Ret (R&D Systems), mouse monoclonal anti-human NGF R/TNFRSF16 (R&D Systems), sheep polyclonal anti-human platelet-endothelial cellular adhesion molecule-1 (PECAM-1; R&D Systems), rabbit polyclonal anti-human von Willebrand Factor (vWF; Dako Diagnostics Canada, Inc.), sheep polyclonal anti-mouse NGF, BDNF, NT-3, and GDNF (Millipore). The secondary antibodies used were, goat anti-rabbit IgG Alexa Fluor[®]488, chicken anti-goat IgG Alexa Fluor[®]488, donkey anti-sheep IgG Alexa Fluor[®]594, chicken anti-rabbit IgG Alexa Fluor[®]594 (all from Invitrogen), goat anti-mouse IgG fluorescein isothiocyanate (FITC), goat anti-rabbit IgG FITC, rabbit anti-goat IgG Biotin (all from Upstate), Alexa488-conjugated Streptavidin (Invitrogen). Cell nuclei were labeled with Hoechst reagent 33258 (Sigma-Aldrich). As a control, the primary antibody was omitted.

ELISA analysis

Supernatants of cultured cells were harvested, clarified by centrifugation, and frozen at -80°C. Lysate of epidermis were obtained after digestion with dispase II (Sigma-Aldrich) for 3 h at 37°C and crushing of the epidermis in liquid nitrogen. The lysis buffer contained 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl

fluoride (PMSF; Sigma-Aldrich), 10 µg/mL aprotinin (Sigma-Aldrich), 1 µg/mL leupeptin (Sigma-Aldrich), 0.5 mM sodium vanadate (Sigma-Aldrich), and Complete Protease Inhibitor Cocktail (Roche Diagnostics). NGF, BDNF, NT-3, and GDNF contents from culture supernatants or epidermis were assayed at 450 nm using the E-Max ImmunoAssay system (Promega) according to the manufacturer's instructions.

Preparation of the tissue-engineered model of angiogenesis

Collagen sponges were prepared as described previously.^{17,18} The model was prepared by the addition of a 1:1 ratio suspension of human dermal fibroblasts and HUVEC or HDMEC on the top of the collagen sponge. Each cell type was seeded at a concentration of 2.1×10^5 cells/cm² and culture medium was added 2 h later. The sponges were cultured for 10 days in immersion and fed with a 1:1 ratio of EGM-2: DMEM, supplemented with 75 µg/mL ascorbic acid. Subsequently, they were cultured for 7 days in a 3:1 ratio of DMEM-Ham's F-12 medium supplemented with 10% FBS, 0.4 µg/mL hydrocortisone, 5 µg/mL bovine insulin, 100 µg/mL ascorbic acid and antibiotics. The constructs were then elevated to the air-liquid interface for the remaining 14 days.

Treatment with neurotrophic factors and inhibitors

NGF 2.5S (Invitrogen), BDNF, NT-3, and GDNF (Cell Sciences) were added in the culture medium at three successive doses on days 17, 19, and 21 at the following concentrations: NGF (1, 10, 50, and 100 ng/mL), BDNF (0.01, 0.1, 1, and 10 ng/mL), NT-3 (1, 15, 60 and 120 ng/mL), and GDNF (1, 10, 50, 100 ng/mL). For the stimulation of the construct containing HDMEC and all other experiments using neurotrophic factors, 10 ng/mL of NGF, 0.1 ng/mL of BDNF, 15 ng/mL of NT-3, or 50 ng/mL of GDNF were tested. For the Trk receptors inhibition studies, constructs were cultured with the above mentioned neurotrophins, and with 100 nM of the inhibitor of TrkA, B, C (K252a; Calbiochem) dissolved in 1 mM dimethyl sulfoxide (DMSO), or 1 mM DMSO alone in the controls.

Quantification of capillary-like tubes and evaluation of their maximal depth of migration in the angiogenesis model

Six microns transversal histological sections covering the whole construct were stained with Masson's trichrome and visualized with a Nikon Eclipse E600 microscope (Nikon). Capillary-like tubes (CLT) were counted by a single observer in a blinded fashion. To evaluate the maximal depth of migration, immunofluorescent staining of human PECAM-1 was performed on whole construct transversal sections. Individual high-resolution images were assembled in Adobe[®] Photoshop[®] 10.0 (San Jose, CA) to reconstitute the total section of the construct. The maximum depth of migration of the CLT was determined in an automated fashion using MATLAB[®] version 6.5 (Math Works) with the resolution of 1 pixel (0.5 µm).

Western immunoblotting

Cells were lysed in 50 mM Tris-HCl, pH 7.4, 1% Nonidet p40, 150 mM NaCl, 5 mM EDTA, 10 mM sodium orthovanadate, Complete Protease Inhibitor Cocktail, 1 mM PMSF. The

primary antibodies were, goat polyclonal anti-human TrkA (R&D Systems), rabbit polyclonal anti-human phospho-TrkA (Tyr490 and Tyr674/675; Cell Signaling Technologies), rabbit polyclonal antibody anti-mouse TrkB (Santa Cruz), mouse monoclonal anti-actin (Upstate). Immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus Western blotting kit, Amersham Biosciences) using rabbit anti-goat IgG horseradish peroxidase-conjugated (Upstate), goat anti-rabbit IgG horseradish peroxidase-conjugated (Sigma-Aldrich), or goat anti-mouse IgG horseradish peroxidase-conjugated (Upstate). For the endothelial cells stimulation with NGF for western blot analysis of TrkA phosphorylation, confluent HUVEC were starved overnight in EGM-2 containing 0.1% serum and incubated with EGM-2 containing 0.1% serum and 100 ng/mL NGF (Invitrogen) for 5 or 15 min.

Intracellular phosphorylation analysis after treatment with neurotrophic factors

For the HDMEC stimulation, cells were starved 15 h in EGM-2 containing only 0.1% serum and incubated with EGM-2 containing only 10 ng NGF (Invitrogen), 0.1 ng/mL BDNF, 15 ng/mL NT-3, or 50 ng/mL GDNF (Cell Science). Then, Proteome Profiler human phospho-kinase antibody arrays (R&D Systems) were used according to the manufacturer's instructions. Significant results are presented as the mean of at least three different experiments as the relative pixel densities in respect to the control, more or less the standard deviation.

Statistical analysis

Values were expressed as the means more or less the standard deviation. Statistical analysis was performed using the Student's *t*-test with a bilateral distribution and equal variances, with, $p < 0.05$ regarded as significantly different. Each experiment was reproduced at least twice to ensure reproducibility.

Results

Expression of NGF, BDNF, NT-3 and GDNF in normal human skin

NGF immunostaining of human skin was homogeneously distributed in the dermis and the epidermis (Fig. 1A). Although NGF and all other neurotrophic factors tested were not detected in supernatants of cultured keratinocytes, they were measured by ELISA in native lysed epidermis, which expressed 5.6 ± 1.8 pg/mg of NGF (Table 1). Cultured fibroblasts released 53.65 ± 25.8 pg/ 10^6 cells/mL NGF, and Schwann cells 35.6 ± 10.8 pg/ 10^6 cells/mL. BDNF immunoreactivity was present in the epidermis and in the dermis (probably corresponding to capillaries) (Fig. 1B). Epidermis contained 12.0 ± 8.9 pg/mg BDNF, while Schwann cells secreted 37.3 pg/ 10^6 cells/mL BDNF (Table 1). HUVEC secreted 68.3 pg/ 10^6 cells/mL BDNF, but HDMEC did not express detectable BDNF. NT-3 staining was found in all layers of the epidermis (Fig. 1C). Fibroblasts expressed 45.0 ± 14.4 pg/ 10^6 cells/mL of NT-3 while 7.9 ± 6.6 pg/mg

FIG. 1. Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), and Glial-derived neurotrophic factor (GDNF) expression in human skin. The expression of neurotrophic factors in human skin was assessed by immunofluorescent staining of normal human skin cross sections. The four neurotrophic factors NGF (A), BDNF (B), NT-3 (C) and GDNF (D) were expressed in epidermis, while NGF and BDNF were also detected in the dermis. Scale Bar in D = 40 μ m. Color images available online at www.liebertpub.com/tea

TABLE 1. RELEASE OF NGF, BDNF, NT3 AND GDNF BY EPIDERMIS AND FIBROBLASTS, ENDOTHELIAL CELLS, SCHWANN CELLS AND NEURONS

	NGF	BDNF	NT3	GDNF
<i>Tissue lysate (pg/mg)</i>				
Human epidermis	5.6±1.8	12±8.9	7.9±6.6	39.8±27.2
<i>Culture supernatant (pg/million of cells/mL)</i>				
Dermal fibroblasts	53.6±25.8	-	45±14.4	-
HUVEC	-	68.3±29.4	-	-
HMVEC	-	-	-	-
Schwann cells	35.6±10.8	37.3±19.2	-	42±25.6
Sensory neurons	-	-	-	-

Neurotrophic factors were quantified by ELISA from culture supernatant of human fibroblasts and endothelial cells, rat Schwann cells and mouse sensory neurons, and from fresh human epidermis. A minimum of three different samples from three different donors were used for each assay. The results are presented as the mean ± standard deviation (-: no detection).

NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3, neurotrophin 3; GDNF, glial-derived neurotrophic factor; HUVEC, human umbilical vein endothelial cells; HMVEC, human microvascular endothelial cells.

NT-3 were measured in lysed epidermis. Immunostaining for GDNF was detected in the suprabasal layers of the epidermis (Fig. 1D) that expressed 39.8±27.2 pg/mg GDNF in native lysed epidermis and 42.0±25.6 pg/mg for Schwann cells culture supernatant (Table 1).

NGF, BDNF, NT-3 and GDNF promote angiogenesis

To assess whether neurotrophic factors could promote angiogenesis and to compare their specific angiogenic potential, we used a tridimensional tissue-engineered model of angiogenesis made of human dermal fibroblasts and endothelial cells cultured in a collagen sponge. The number of CLT in the model cultured in presence of each neurotrophic factors was significantly higher compared to controls (Fig. 2B). These differences were clearly visible on the Masson's trichrome staining (Fig. 2A). There was 40% more CLT in constructs treated with 10 ng/mL of NGF ($p=0.006$), 80% more CLT in constructs treated with 50 ng/mL GDNF ($p=0.00002$) or 0.1 ng/mL BDNF ($p=0.0001$), and 70% more CLT in constructs treated with 15 ng/mL NT-3 ($p=0.0004$), compared to control. These results were confirmed with HDMEC extracted from human skin and treated with 10 ng/mL of NGF, 0.1 ng/mL of BDNF, 15 ng/mL of NT-3 and 50 ng/mL of GDNF. The increase in the number of CLTs was limited to 20% in the constructs made of HDMEC (Fig. 2C).

The addition to the construct of K252a, a specific inhibitor of Trk, abolished the increase of CLT induced by NGF, BDNF, or NT-3 (Fig. 2D).

To further investigate the remodeling of the CLT network induced by neurotrophic factors in the model, the maximal depth of migration of the CLT was measured according to immunohistochemical staining of PECAM-1 in the constructs made of HUVEC (Fig. 3A–E) and HDMEC. The CLT were observed migrating significantly deeper in the models in respect to the controls for all the conditions with the neurotrophic factors (Fig. 3F, G). The average depth of migration with HUVEC was 244±28 µm in control, 359±83 µm in NGF ($p=0.03$), 361±131 µm in BDNF ($p=0.06$), 394±72 µm in

NT-3 ($p=0.006$) and 387±32 µm in GDNF ($p=0.0001$). The average depth of migration with HDMEC was 307±15 µm in control, 443±29 µm in NGF ($p=0.02$), 354±13 µm in BDNF ($p=0.05$), 380±15 µm in NT-3 ($p=0.008$), and 376±21 µm in GDNF ($p=0.05$).

To assess whether the neurotrophic factors influence the proliferation of endothelial cells, HDMEC, and HUVEC were cultured in presence of 10 ng/mL of NGF, 0.1 ng/mL of BDNF, 15 ng/mL of NT-3 and 50 ng/mL of GDNF, but no difference was observed in the number of cells in presence or absence of these neurotrophic factors for 24 h in monolayer cultures (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/tea). Moreover, this result was confirmed using a colorimetric method to assay the relative number of HDMEC over a 72 h period of subconfluent culture (Supplementary Fig. S1B). Finally, the proportion of HUVEC (over fibroblasts) cultured in the 3D construct with and without neurotrophic factors was quantified by flow cytometry after staining of endothelial cells with antibodies against vWF. NGF, BDNF, NT-3, and GDNF did not induce a significant change in the proportion of HUVEC present in the constructs (Supplementary Fig. S1C).

Human endothelial cells express the receptors for NGF, BDNF, NT-3 and GDNF

Given the increased number and depth of migration of CLT in presence of neurotrophic factors, we assessed whether the neurotrophic factors receptors were expressed by endothelial cells and/or fibroblasts. The expression of TrkA and TrkB, the preferred high affinity receptors for NGF and BDNF, respectively as well as the low affinity receptor for all neurotrophins, p75, were shown by indirect immunofluorescence both on HUVEC (Fig. 4A–C) and HDMEC (Fig. 4F–H). The preferred high affinity receptor for NT-3, TrkC, was not found to be expressed. However, NT-3 can bind TrkA and TrkB as well as p75. The expression of the GDNF multicomponent receptor complex GFRα-1 and c-Ret tyrosine kinase coreceptor was shown on HUVEC (Fig. 4D, E) and HDMEC (Fig. 4I, J). No staining for TrkB, TrkC, p75, c-Ret, and GFRα-1 was observed in fibroblasts (not shown).

Western blot analysis of TrkA showed that HUVEC and HDMEC express a variant of the 140 kDa full length receptors at 68 kDa (Fig. 4K; lane 1 for keratinocytes, lane 2 for HUVEC, lane 3 for HDMEC, lane 4 for fibroblasts). We showed that stimulation of HUVEC with NGF induces the phosphorylation of TrkA on both Tyr490 and Tyr674/675 (Fig. 4M). The phosphorylation on Tyr674/675 has been shown to increase the kinase activity of the receptor,¹⁹ while the phosphorylation on Tyr490 is required for the recruitment of the adaptator protein SHC and the subsequent initiation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (AKT) signaling pathways.^{20,21}

The main variant TrkB-T1 was also expressed by HUVEC and HDMEC is (Fig. 4L; lane 1 keratinocyte, lane 2 HUVEC and lane 3 HDMEC). TrkB-T1 was also found in fibroblasts (Fig. 4L; lane 4). There was also a strong expression of a band corresponding to the molecular weight of the variant TrkB-T-shc in HUVEC, HDMEC, and a light one in fibroblasts. The full length variant of TrkB was detected for HUVEC and HDMEC.²²

FIG. 2. Neurotrophic factors induced a significant dose-dependent increase in capillary-like tubes (CLT) formation by human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC). Neurotrophic factors were added in the culture medium of the construct between days 17 and 24. The analyses were performed on biopsies taken 1 week after the removal of the neurotrophic factors after 31 days of culture. **(A)** CLT formation was observed on Masson's trichrome staining of 4 μ m thick tissue cross sections. **(B)** In constructs made with HUVEC, the addition of each neurotrophic factor induced the formation of more CLT, with a maximum at 10 ng for NGF, 0.1 ng for BDNF, 15 ng for NT-3 and 50 ng for GDNF (* p < 0.05; ** p < 0.02; n = 5). **(C)** These optimal concentrations of neurotrophic factors were used in constructs made of HDMEC instead of HUVEC and also promoted an increase in the number of CLT (* p < 0.05; ** p < 0.02; n = 5). **(D)** The increase in CLT number induced by NGF, BDNF and NT-3 in our model of angiogenesis was abolished by the inhibition of the Trk receptors with 100 nM of K252a (* p < 0.05; n = 4). Scale Bar in A = 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 3. The maximum depth of migration of the CLT was increased by the addition of neurotrophic factors in the culture medium. (A–E) Since endothelial cells were seeded on the top of the construct, and the neurotrophic factors were added in the culture medium underneath the tissue, the CLT expansion from the top to the bottom shows the chemoattractant potential of these factors. The CLT were stained with an antibody against human platelet-endothelial cellular adhesion molecule-1 on 5 μ m thick cross-sections. The maximum depth of migration of the CLT was determined using Matlab software. (F, G) The addition of neurotrophic factors increased the maximum depth of migration in the construct compared with the control for both HUVEC (F) and HDMEC (G) (** $p < 0.02$, $n = 5$; * $p < 0.05$, $n = 4$). Scale Bar = 250 μ m.

Neurotrophic factors activate angiogenesis-related signaling pathways in endothelial cells

A 19% increase in the relative phosphorylation level (RPL) of focal adhesion kinase (FAK), a 12% increase in the RPL of Hsp27 and a 61% decrease in the RPL of phospholipase C gamma 1 (PLC γ -1) were observed with NGF (Table 2). For BDNF, a 23% increase in the RPL of Mek 1/2 was detected. NT-3 induced an increase in the RPL of 26% for p27, 21% for p70s6 kinase and for Lck, 20% for FAK and 18% for Fgr, along with an 86% decrease of the ribosomal S6 kinase of RSK 1/2/3. For GDNF, we detected a 12% increase in the RPL of STAT-Y701 and a 15% decrease of the RPL of Pyk2-

Y402. In addition, we observed no significant difference in the RPL of proteins implicated in a proapoptotic response like p53 (Table 2).

Discussion

Skin is known to be a sensory organ. It promotes sensory nerve fibers migration and maintenance at least in part through secretion of NGF by keratinocytes. We hypothesized that keratinocytes could secrete other neurotrophic factors, such as BDNF, NT-3 and GDNF, as well as fibroblasts and endothelial cells and that these factors could contribute to dermal angiogenesis as it was already known for NGF.

FIG. 4. Human endothelial cells express TrkA, TrkB, p75, GFR α -1 and c-ret. The expression of the receptors for NGF, BDNF, NT-3 and GDNF on HUVEC and HDMEC was assessed by immunofluorescent staining and western blot analysis. **(A–J)** Expression of TrkA, TrkB, p75, GFR α -1 and c-ret on HUVEC (in green, **A** to **E**, respectively) and HDMEC (**F** to **J**, respectively), stained in red with vonWillebrand Factor antibodies. **(K)** Western blot analysis of TrkA expression on total cell lysates. TrkA is expressed on keratinocytes, the positive control (lane 1), on HUVEC (lane 2) and HDMEC (lane 3), but not on fibroblasts (lane 4). The antibody, which can bind the extracellular portion of TrkA, revealed a TrkA variant at 68 kDa that was only present in endothelial cells. **(L)** Western blot analysis of TrkB expression on total cell lysates. We found a weak band at 140 kDa for keratinocyte (the positive control, lane 1), for HUVEC (lane 2) and for HDMEC (lane 3) that correspond to the Full length variant. For HUVEC and HDMEC (lane 2 and 3, respectively), the strongest staining correspond to the variant TrkB-T1 at 100 kDa. This variant could also be found in fibroblast lysate (lane 4). A band at 90 kDa corresponding to the variant TrkB-T-Shc was also strongly expressed in endothelial cells and weakly expressed by fibroblasts. **(M)** To assess whether TrkA cell receptors on endothelial cells could be activated, cells were stimulated for 5 min (lane 2) or 15 min (lane 3) with 100 ng/mL of NGF and the phosphorylation at Tyr490 and Tyr674/675 of the TrkA receptor was analyzed by western blot, showing phosphorylation of TrkA on both residues after 5 min. Scale bar in **J**: 15 μ m. Color images available online at www.liebertpub.com/tea

Our results confirm that NGF, BDNF, NT-3, and GDNF are expressed by the epidermis, as previously shown at the mRNA level.²³ In addition, we showed that BDNF and GDNF were released in greater amounts than NGF and NT-3 in the epidermis. Neurotrophins derived from keratinocytes were only detected in native lysed epidermis, in contrast with the other cell types for which they were easily detected in the conditioned medium. Since keratinocytes naturally express high level of proteases notably during differentiation and wound healing, these enzymes could have degraded neurotrophins in the conditioned medium.^{24,25} BDNF and GDNF were not detected by fibroblasts, in contrast with NGF and NT-3. BDNF was expressed by HUVEC, but not by HDMEC in monolayer culture. BDNF secretion by endothelial cells has already been shown in the brain as a major

guidance pathway for neural stem cell migration.²⁶ We assessed the secretion of neurotrophic factors by Schwann cells since they are known to be a major source of neurotrophic factors in the nerve regeneration context and since it was shown elsewhere that there are between 140 and 450 Schwann cells per mm² in the skin.^{27,28} However, because we found that Schwann cells secrete NGF, BDNF, and GDNF at levels similar to the others cell types that we tested in the conditioned medium, we suggest they should not be considered as the major source of neurotrophic factor in the skin. There was no secretion of NT-3 by Schwann cells, as reported elsewhere.²⁹ As expected from the literature, sensory neurons don't produce neurotrophic factors.

The next step of this project was to investigate the potential impact of these neurotrophic factors release on the

TABLE 2. RELATIVE PHOSPHORYLATION LEVEL OF INTRACELLULAR KINASE IN HDMEC STIMULATED WITH NEUROTROPHIC FACTORS

	NGF	BDNF	NT-3	GDNF
FAK-Y397	1,19±0,09*	Mek 1/2 S222, 1,23±0,06** S222/226	p27-T198 1,26±0,05**	STAT1-Y701 1,12±0,03**
HSP27-S78/S82	1,12±0,05**		p70s6kin-T421/S424 1,21±0,1*	Pyk2-Y402 0,85±0,06**
PLCγ1-Y783	0,39±0,09**		Lck-Y394 1,21±0,09**	
			FAK-Y397 1,2±0,04**	
			Fgr-Y412 1,18±0,00**	
			RSK 1/2/3-S380 0,14±0,06**	

HDMEC were stimulated or not for 15 min with 10 ng/mL NGF, 0,1 ng/mL BDNF, 15 ng/mL NT-3 or 50 ng/mL GDNF and screened with a human phospho-kinase array of antibodies. The significant results are presented as the percentage of the relative variation in respect to the control. (* $p < 0.05$; ** $p < 0.02$; $n = 3$).

skin microvasculature. Indeed, the skin capillary network is assumed to be modulated by conventional angiogenic growth factors, such as vascular endothelial growth factor (VEGF). However, neurotrophic factors may also participate to the control of skin angiogenesis, and may become in pathological situation the main source of angiogenic factors in the skin. To better quantify the specific angiogenic potential of each neurotrophic factor, we investigated their effect on CLT formation *in vitro* using our well-characterized tissue-engineered model of angiogenesis.^{30–32} We demonstrated a major increase in the number of CLT for all neurotrophic factors. Indeed, NGF, GDNF, and NT-3 induced a 40% to 80% increase in the number of CLT. In addition, BDNF induced an 80% increase of CLT at a 100 to 500-fold lower concentration compared to the other three neurotrophins (0.1 ng/mL). Therefore, BDNF was the most potent angiogenic neurotrophic factors we tested. An angiogenic effect was also obtained with the angiogenesis model made of HDMEC, whereas the increased number of CLT was limited to 20%. This much lower difference is not specific to neurotrophic factors, and is due to the much lower number of CLT formed by HDMEC, justifying the use of HUVEC in a first step to better discriminate the angiogenic potential of compounds.

Since neurotrophic factors were not supposed to be major players in the angiogenic process, it was surprising to see their huge angiogenic potential compared with VEGF that was limited to a 20% increase in our angiogenesis model.³¹ Our results suggest that neurotrophic factors induce tubulogenesis and migration of endothelial cells rather than their proliferation. Indeed, the proliferation was unchanged when assayed in classical proliferation assays and the ratio endothelial cells/fibroblasts was unchanged in the 3D model after treatment with neurotrophic factors. We measured the maximum depth of migration of CLT in the construct and we observed that all neurotrophic factors induced a deeper migration of CLT into the sponge. This result demonstrates that neurotrophic factors could probably modulate the organization of the capillary network through a chemoattractant effect.

Our results suggest a direct interaction between neurotrophic factors and endothelial cells. Indeed, the expression of TrkA, TrkB, p75, GFRα-1/c-Ret, the receptors for NGF, BDNF, NT-3, and GDNF was shown on both HUVEC and HDMEC. A competitive inhibitor for all Trk receptors, K252a, abolished the angiogenic effect induced by NGF,

BDNF, and NT-3. Meanwhile, western blot analysis showed a phosphorylation on some critical kinase effectors of HDMEC after treatment with NGF. These results suggest that the angiogenic effect is mediated specifically through the Trk receptors.

To better analyze the signaling pathways they activated, we investigated the phosphorylation status of the kinase effectors in HDMEC after treatment with neurotrophic factors. First, stimulation by NGF induced the phosphorylation of FAK and Hsp27. FAK is known to promote endothelial cell migration,³³ while hyperphosphorylation of Hsp27 might promote cell cycle arrest.³⁴ There was also a strong decrease of PLCγ-1 potentially linked to a mitogenic signal.^{35,36} For BDNF, an increase for Mek 1/2, expressed downstream the TrkB signaling pathway, was observed.² For NT-3, an increase for FAK was observed, as well as of Lck and Fgr, two members of the Src family required for the angiogenic process, possibly via the reorganization of the actin cytoskeleton affecting cell migration.³⁷ NT-3 induced an increase in phosphosrylation of p27, reported to modulate cell migration,³⁸ and p70-S6, reported to occur in mitogenic HUVEC.³⁹ Finally, GDNF promoted phosphorylation for Stat1, reported notably in proliferating endothelial cells.⁴⁰ Overall, the phosphorylation status of intracellular kinase after stimulation with each neurotrophic factors is coherent with a pro-angiogenic effect.

This study shows that neurotrophic factors might participate to the regulation of skin microvasculature through the release, mainly from epidermis, of NGF, BDNF, NT-3 and GDNF. These neurotrophic factors can promote angiogenesis by a direct effect on endothelial cells. In addition, this angiogenic potential may be highly potent in pathological situation where neurotrophic factors could be markedly overexpressed.

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Disclosure Statement

The authors declare no competing financial interests exist.

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Address correspondence to:

François Berthod, PhD

Département de Chirurgie

Faculté de Médecine

Centre LOEX de l'Université Laval

Centre de recherche du CHU de Québec

Hopital de l'Enfant-Jésus, Aile R

1401, 18e rue

Québec, Canada G1J 1Z4

E-mail: francois.berthod@fmed.ulaval.ca

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