p75 Neurotrophin Receptor Participates in the Choroidal Antiangiogenic and Apoptotic Effects of T-Lymphocyte–Derived Microparticles

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Purpose. Choroidal neovascularization (CNV) is a major cause of vision loss in which choroidal vessels penetrate the RPE—an important source of growth factors, including nerve growth factor (NGF), whose activation via the p75NTR receptor promotes apoptosis and inhibits angiogenesis. We demonstrated previously that human T-lymphocyte–derived microparticles (LMPs) significantly inhibit angiogenesis in several models of ocular neovascularization. We investigated how LMPs modulate pro- and antiangiogenic microenvironments during choroidal angiogenesis.

Methods. Antiangiogenic effects of LMPs were investigated using a rat model of choroidal angiogenesis. The impact of LMPs on expression of major angiogenic factors was assessed by real-time quantitative PCR (qPCR). To determine whether p75NTR signalling was implicated in LMPs-induced activities, we used a specific antibody and short hairpin RNA (shRNA) targeting p75NTR. Cellular apoptosis was determined via evaluation of activated caspase-3 and annexin V binding.

Results. The LMPs time-dependently inhibited choroidal angiogenesis by more than 64% after 48 hours of treatment. Removal of the RPE from choroidal explants abolished the antiangiogenic effects of LMPs. The mRNA levels of pigment epithelium-derived factor (PEDF) and NGF were increased significantly following LMPs treatment of intact, but not RPE-removed choroids. Downregulation of PEDF and p75NTR significantly blocked the antiangiogenic effects of LMPs. Finally, induction of choroidal endothelial cell apoptosis by LMPs was dependent on p75NTR.

Conclusions. We demonstrate for the first time to our knowledge that LMPs markedly inhibit choroidal angiogenesis via mechanisms that are dependent on the integrity of the RPE, and that are mediated largely by the PEDF and proapoptotic activities of p75NTR.

Keywords: choroidal neovascularization, lymphocytic microparticles, neurotrophins, PEDF, p75NTR

Choroidal neovascularization (CNV) triggered by multietiologic factors is a major cause of vision loss. It represents the growth of new blood vessels from the choriocapillaris into the RPE, a specialized monolayer of cells that secretes antiangiogenic factors, such as pigment epithelium-derived factor (PEDF), and proangiogenic factors, such as VEGF.[1,2] The balance of antiangiogenic and proangiogenic factors is crucial for the physiological stability of the choroid.[3,4]

Whereas anti-VEGF therapies are the current standard treatment for CNV,[5,6] they do not address the underlying cause of this pathology and are associated increasingly with drug resistance. Promising therapeutic approaches include treatments directed against the vascular and extravascular components of CNV, such as glia and RPE cells[7,8]; therapies that target inflammatory and apoptotic mediators also may prove beneficial and warrant further investigation.[9–14]

Human T-lymphocyte–derived microparticles (LMPs) possess strong antiangiogenic properties in vivo,[15–17] and exert numerous actions on a variety of cells and tissues.[18–20] We have established that LMPs strongly suppress in vivo corneal neovascularization and tumor angiogenesis,[15,16] and limit neovascularization during the vasoproliferative phase of ischemic retinopathy.[17] The antiangiogenic effects of LMPs are linked to their anti-VEGF properties and to their ability to increase expression of the CD36 antiangiogenic factor. However, to date, the regulation of cellular apoptosis by LMPs largely is unknown.

The neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) comprise a family of secreted growth factors that have been studied extensively for their actions on the nervous system. Neurotrophins are known to regulate angiogenesis through autocrine and paracrine mechanisms, which are mediated by two structurally unrelated

cell surface receptors: the common neurotrophin receptor p75NTR and the tropomyosin kinase receptors (Trks).[21–23] Studies have shown that p75NTR has a critical role in triggering apoptosis of endothelial cells and vascular progenitor cells, and in suppressing neovascularization.[24,25] Notably, a role of LMPs in mediating intercellular cross-talk and apoptosis has been suggested.[19,26] Thus, p75NTR may contribute to the inhibitory actions of LMPs during choroidal angiogenesis.

In our study, we investigated whether LMPs modulate pro- and antiangiogenic microenvironments during choroidal angiogenesis. We demonstrated that LMPs inhibit choroidal angiogenesis significantly through mechanisms that are dependent on the structural integrity of the RPE, and that are mediated largely by the antiangiogenic factor PEDF and proapoptotic activities of p75NTR.

Materials and Methods

Animals

Newborn and adult Sprague-Dawley rats were purchased from Charles River Laboratories (St. Constant, QC, Canada). All animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Animal Care Committee of CHU Sainte-Justine (Montreal, QC, Canada).

LMPs Production

The LMPs were generated as described previously.[15] Human CEM T cells were purchased from ATCC (Manassas, VA) and cultured with X-VIVO medium (Cambrex, Walkersville, MD). Briefly, CEM T cells were treated with 0.5 μg/mL actinomycin D for 24 hours. A supernatant was obtained by centrifugation at 750*g* for 15 minutes, then 1500*g* for 5 minutes to remove cells and large debris. The LMPs from the supernatant were washed after 3 centrifugation steps (50 minutes at 12,000*g*) and recovered in PBS. To investigate whether heat-denatured LMPs are good control for LMPs, we performed cell growth assay and found that heat-denatured LMPs still possess 40% effectiveness in inhibition of endothelial cell growth (Yang C, Tahiri H, Qiu Q, Hardy P, unpublished data, 2012). As washing medium from the last supernatant has been used widely as control vehicle for each individual microparticles,[18,27–29] this particular medium was used as a control in following experiments unless otherwise noted.

Cell Culture

Brain microvascular endothelial cells (RBMVEC) were obtained from Cell Applications, Inc. (San Diego, CA), and cultured in rat brain endothelial cell growth medium (Cell Applications, Inc.), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Primary RPE Culture

Primary RPE cultures were obtained as described previously from 9-day-old rat pups.[30,31] Animals were euthanized, and eyes enucleated and maintained overnight at room temperature in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), and then incubated for 45 minutes with 2 mg/mL trypsin/collagenase I at 37°C. After trypsin inhibition with DMEM containing 10% fetal calf serum (FCS), the RPE layer was harvested. RPE cells were plated in 8-well LabTek (Nunc; Thermo Fisher Scientific, Inc., New York, NY) at a rate of RPE from 1 eye per well in DMEM containing 10% FCS and 1% penicillin/streptomycin. To evaluate whether mediators secreted by RPE cells inhibit capillary formation, conditioned medium from confluent RPE cells in the presence or absence of LMPs (30 μg/mL, 48 hours) was collected and used to treat choroidal explants.

RNA Isolation and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from choroidal tissue and RPE cells using an RNA extraction kit (Qiagen, Mississauga, ON, Canada). DNase-treated RNA then was converted into cDNA using M-MLV reverse transcriptase (Invitrogen). Quantitative analysis of gene expression was performed using an ABI Prism 7700 sequence detection system and the SYBR Green Master Mix Kit (BioRad Laboratories, Inc., Hercules, CA). For each sample, reactions were performed in duplicate, and threshold cycle numbers were averaged. Gene expression was normalized to 18S, and the percentage of reduction was calculated according to a previously described formula.[32] PCR primers targeting rat VEGF-A, VEGFR-1, VEGFR-2, PEDF, NGF, p75NTR, and tropomyosin-receptor–kinase (TrkA) were synthesized by Alpha DNA (Montreal, Quebec, Canada) based on the following sequences: VEGF-A, forward 5′-ACGAAAGCGCAAGAAATCCC-3′ and reverse 5′-TTAACTCAAGCTGCCTCGCC-3′; VEGFR-1, forward 5′-GACTGCTGACCTGCGAAGCC-3′ and reverse 5′-AGGAATCTCACGGGGCTCGG-3′; VEGFR-2, forward 5′-TGGAT GAGCGCTGTGAACGC-3′ and reverse 5′-ACTTGGCCAAAG GCACCACG-3′; PEDF, forward 5′-AAGAGTGCTTCCA GAATTGTG-3′ and reverse5′-CCCAGTTGTTAATCTCCT GAAGG-3′; NGF, forward 5′-GGACTCACAGGAGCAAGCGC-3′ and reverse 5′-GCCCTTGATGTCCGTGGCTG-3′; p75NTR, forward 5′-TGCACAGCGACAGTGGCATC-3′ and reverse 5′-TCCT CACGCTTGGTCAGGGG-3′; TrkA, forward 5′-GAATGATGTG GGCCGGGCTG-3′ and reverse 5′ –GAAGAACCA GCGCAGG GACG-3′; and 18S, forward 5′-CCTGCGGCTTTAAATTTGACT CA-3′ and reverse 5′-GCTATCAATCTGTCAATCCTGTC-3.′

Preparation of Choroidal Explants and RPE-Removed Choroidal Explants

Choroidal explants were prepared from adult Sprague-Dawley rats as described previously.[33] Eyes were isolated rapidly under aseptic conditions. Preparation of choroidal explants was performed under a stereomicroscope with an optic fiber light source in a sterile vertical laminar flow hood. Blood vessels, and connective and fatty tissue were removed from the exterior of the eyeballs in a Petri dish containing DMEM. After removing the cornea, lens, corpus vitreum, and retina, the posterior segment containing the sclera and choroid was sectioned into 1- to 2-mm sections and placed in growth factor-reduced basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA).

The RPE-removed choroidal explants were prepared according to a previously described procedure.[34] In a Petri dish containing 1× Hank's balanced salt solution (HBSS; Invitrogen), the blood vessels, and connective and fatty tissue were removed from the exterior of the eyeballs. Eyes then were incubated at 37°C for 45 minutes with 2% Dispase II (neutral protease; Roche Applied Science, Indianapolis, IN) in HBSS. After removing the cornea, lens, corpus vitreum, and retina, the RPE layer was peeled off from the choroid with fine forceps. The posterior segment containing the sclera and choroid without the RPE was sliced into 1- to 2-mm sections and placed in growth factor–reduced basement membrane matrix. The effectiveness of the RPE removal was assessed by histologically.

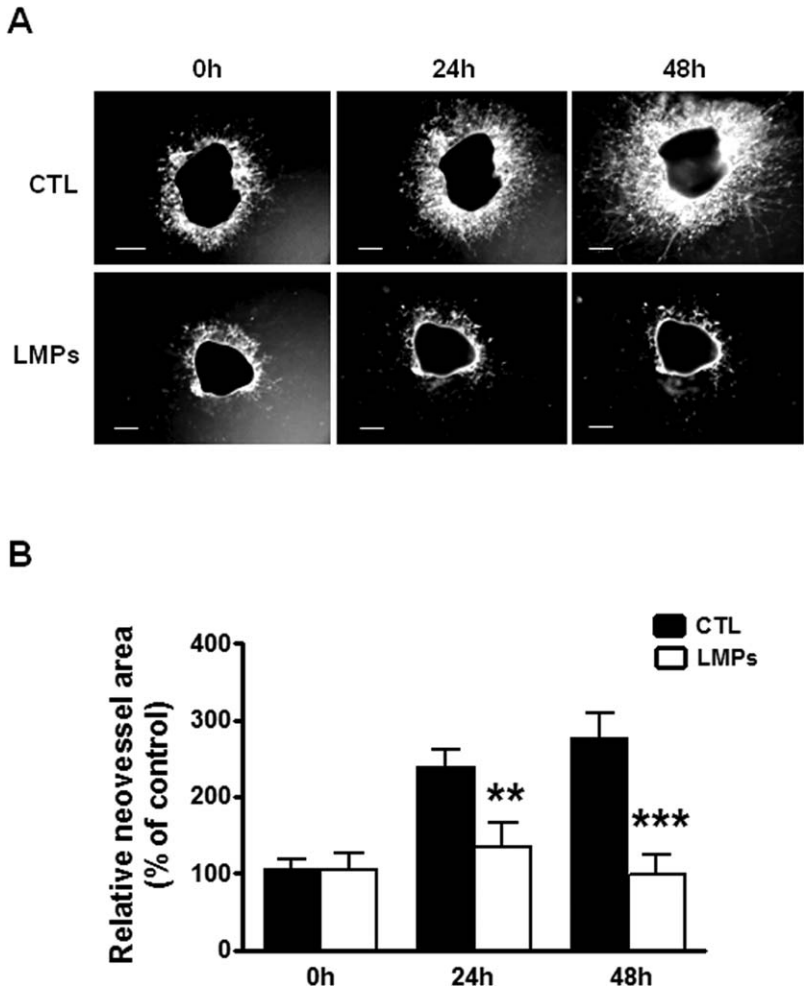

FIGURE 1. (**A**) Representative images of neovessels arising from choroidal explants incubated after 24- and 48-hour treatment with 50 μg/mL of LMPs. *Bar*: 400 μm. (**B**) The area of neovessel formation in choroidal explants was quantified using Image-Pro Plus software, and normalized to control ($n = 7$ per group). Values are depicted as means ± SEM of 5 separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

Treatment of Choroidal Explants and Measurement of Angiogenesis

Choroidal explants were cultured at 37°C in 5% CO_2 for 3 days. The culture medium was changed on day 4 and explants were treated with saline or 50 μg/mL of LMPs (or indicate concentrations) until day 6. In some experiments, choroidal explants were preincubated with the following antibodies for 3 hours before LMPs treatment: 45 μg/mL anti-p75NTR (Millipore Corporation, Billerica, MA), 45 μg/mL anti-PEDF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and 45 μg/mL anti-rat IgG (Sigma-Aldrich, St. Louis, MO). Photographs were taken daily from days 4 to 6 using an Axiovert 200M inverted microscope (Carl Zeiss International, Jena, Germany). The angiogenic response was determined by measuring the area covered by vessels (neovessel formation) using Image-Pro Plus software (MediaCybernetics, Rockville, MD), and presented as a percentage of that in control groups (set as 100%).

Immunohistochemical Staining of Choroidal Explants

The choroidal explants were fixed in cold 4% paraformaldehyde for 1 hour followed by rinsing with PBS (pH 7.4). Explants then were permeabilized in 1.0% Triton X-100 and blocked in 10% normal goat serum. The rabbit anti-activated caspase-3 (Asp175, 1:200 in PBS; Cell Signaling Technology, Beverly, MA) with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen) or annexin V conjugated to green-fluorescent FITC dye (FITC annexin V, 1:200; Invitrogen) was used to detect apoptotic cells. Phycoerythrin-conjugated anti-rat CD31 (PECAM-1, 1:200; BD Pharmingen; BD Biosciences) and mouse monoclonal to RPE65 (ab13826, 1:100; Abcam, Cambridge, UK) with secondary antibody 594 Fab goat anti-mouse (1:500; Invitrogen) were used to identify specifically endothelial and RPE cells, respectively. Alexa-488–conjugated phalloidin (1:200; Invitrogen) was used to determine the cell morphology. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:5000; Invitrogen). Explants then were mounted onto glass slides with the epithelium facing upwards using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were taken using fluorescence microscopy (Eclipse E800; Nikon Corp., Tokyo, Japan).

Preparation of Short Hairpin RNAs (shRNAs) and Lentivirus Production

Lentiviral pLKO.1 vector expressing shRNA against rat p75NTR and control pLKO.1 vector containing scramble shRNA were obtained from OpenBiosystems (Huntsville, AL). Infectious lentiviruses (LV.shRNA-p75NTR) were generated by transiently cotransfecting plasmids encoding proteins essential for viral

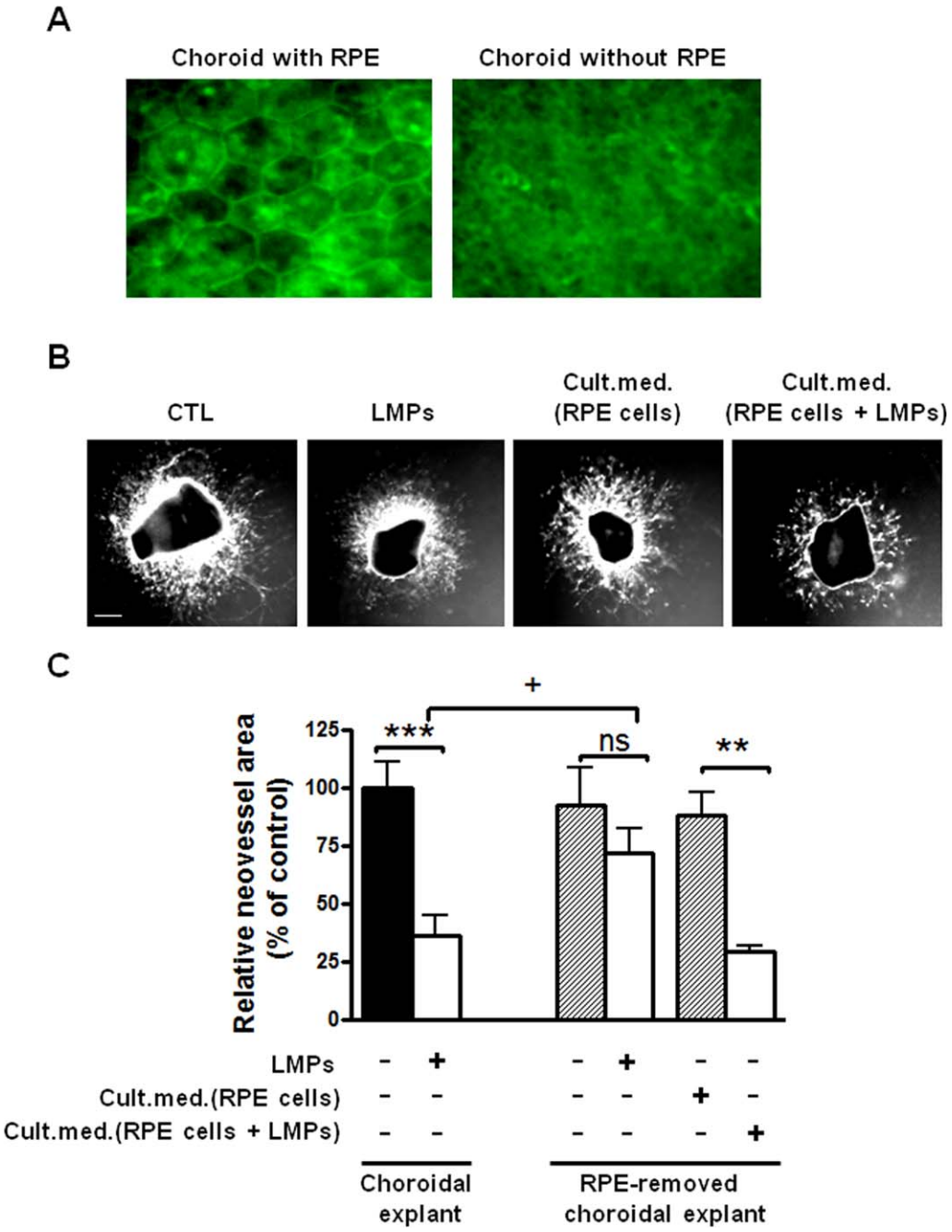

FIGURE 2. (**A**) Representative images of fluorescent phalloidin-stained choroidal explants with and without the RPE. (**B**) Representative images of neovessels sprouting from RPE-removed choroidal explants cultured in normal medium (*control*), with 50 μg/mL of LMPs, with RPE-conditioned culture medium (*cult.med.*), or RPE-conditioned culture medium plus 30 μg/mL LMPs for 48 hours. *Bar*: 400 μm. (**C**) Histogram representing the relative neovessel area of RPE-removed choroidal explants for each condition versus control choroidal explants without LMPs treatment (neovessel area for control choroidal explants at 48 hours was set at 100%). Values are mean ± SEM of 5 separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus LMPs treatment; $+P < 0.05$, difference in LMPs treatment between RPE-removed choroidal explants and choroidal explants.

assembly and shRNA encoding plasmids into 293FT cells (Invitrogen), as described previously.[35]

Western Blot Analysis

RBMVEC cells were collected after infection with lentiviruses for 24 hours. Extraction of soluble proteins and fractionation by SDS-PAGE were performed as described previously.[15] An anti-p75NTR antibody (1:1000) was used to reveal the protein levels of p75NTR. β-actin was used as a loading control. Proteins were visualized using the ECL Western blotting detection system (PerkinElmer, Inc., Waltham, MA). Densitometry values were measured in terms of pixel intensity by Fluorchem software (Protein Simple, Santa Clara, CA).

Statistical Analysis

All experiments were repeated at least 3 times. Values are presented as means ± SEM. Data were analyzed by 1-way ANOVA

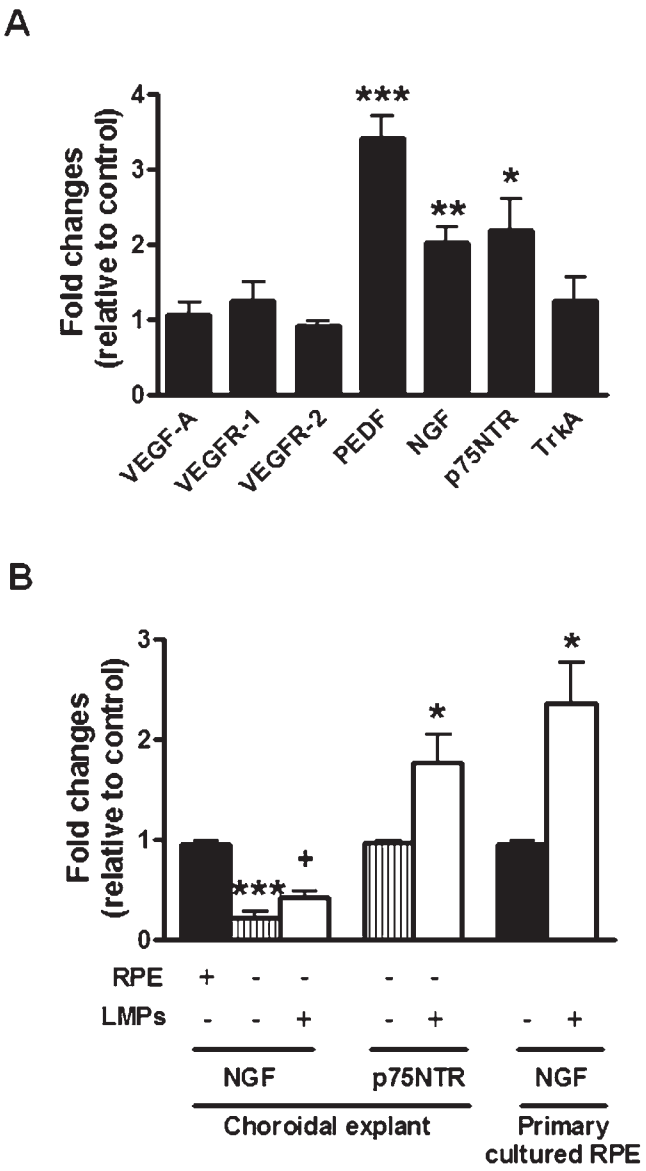

FIGURE 3. (**A**) mRNA levels of VEGF-A, VEGFR-1, VEGFR-2, PEDF, NGF, p75NTR, and TrkA in choroidal explants after 48-hour treatment of LMPs. mRNA expression was assessed using real-time qPCR and presented as fold change in gene expression in the LMPs-treated group compared to the control group (choroidal explants without LMPs treatment), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. (**B**) RPE-removed choroidal explants and primary culture RPE cells were treated with or without LMPs for 48 hours and processed for real-time qPCR. mRNA levels of NGF and p75NTR subsequently were assessed, and presented as fold change over mRNA levels in the control group $^{***}P < 0.001$ versus control choroidal explant without LMPs treatment; $^{+}P < 0.05$ versus RPE-removed choroidal explants without LMPs; $^{*}P < 0.05$ versus control without LMPs treatment.

followed by post hoc Bonferroni tests for comparison among means. Statistical significance was set at $P < 0.05$.

RESULTS

LMPs Suppress Neovessel Sprouting From Cultured Choroidal Explants

We reported previously that LMPs inhibit pathophysiological ocular neovascularization in animal models of inflammatory corneal neovascularization and oxygen-induced retinopathy.[15,17] In our study, we investigated the effects of LMPs on neovessel sprouting from cultured choroidal explants. Our results demonstrated that LMPs treatment (50 μg/mL) significantly reduced neovessel formation by 44% and 64% after 24 and 48 hours of treatment, respectively (Figs. 1A, 1B).

Inhibition of Choroidal Neovessel Formation by LMPs is Dependent Largely on the RPE

The importance of the RPE in the development of the choroidal vasculature has been described[36]; however, its role in mediating the inhibitory effects of LMPs on choroidal angiogenesis has not been investigated to our knowledge. We explored this premise using normal choroidal explants and explants in which the RPE had been removed. Of note, RPE-deficient choroidal explants lacked the characteristic hexagonal shape of RPE cells (Fig. 2A). Compared to control, LMPs did not significantly suppress neovessel sprouting from RPE-deficient explants. Conversely, conditioned medium from LMPs-stimulated RPE primary cells caused a 75% reduction in the neovessel area (Figs. 2B, 2C).

LMPs Increase mRNA Levels of PEDF, NGF, and p75NTR in Choroidal Explants

The RPE cells secrete numerous growth factors that are essential for maintaining the choroidal vasculature. To elucidate the molecular program contributing to the LMPs-induced suppression of choroidal angiogenesis, we assayed choroidal explants for expression of major pro- and antiangiogenic factors by real-time qPCR. Relative to control, LMPs significantly upregulated the mRNA levels of the neurotrophins PEDF (3.4-fold), NGF (2-fold), and p75NTR (2.6-fold). However, we did not detect any significant changes in the mRNA levels of VEGF-A, VEGFR-1, VEGFR-2, and TrkA (Fig. 3A). Removal of the RPE from choroidal explants caused a 70% decrease in NGF expression ($P < 0.001$). Upon LMPs treatment, mRNA levels of NGF and p75NTR were increased markedly in RPE-deficient explants ($P < 0.05$) compared to control. In RPE primary cell cultures, LMPs upregulated the expression of NGF ($P < 0.05$, Fig. 3B); however, expression of p75NTR was undetectable (data not shown).

Blockade of p75NTR and PEDF Promotes Neovessel Formation in Choroidal Explants and Abrogates Effects of LMPs

Based on our data showing increased expression of PEDF and p75NTR in LMPs-treated explants, we questioned the specific involvement of these factors in the antiangiogenic activities of LMPs. Blockade of PEDF and p75NTR signaling with functionally blocking antibodies significantly attenuated the LMPs-mediated inhibition of choroidal angiogenesis (Fig. 4A). Similarly, downregulation of p75NTR with shRNA targeting p75NTR—as indicated by a 66% reduction of mRNA level in RPE-removed choroidal explant (Fig. 4B) and 49% decrease of protein expression in endothelial cells (Fig. 4C)—significantly prevented neovessel formation in LMPs-treated choroidal explants with RPE compared to shRNA-scrambled control. Again, LMPs did not significantly suppress neovessel sprouting from RPE-deficient explants with or without shRNA-p75NTR (Fig. 4D).

p75NTR Mediates LMP-Induced Apoptosis of Choroidal Microvascular Cells

Because p75NTR is known to induce apoptosis in many cell systems, we evaluated whether it could induce apoptosis of

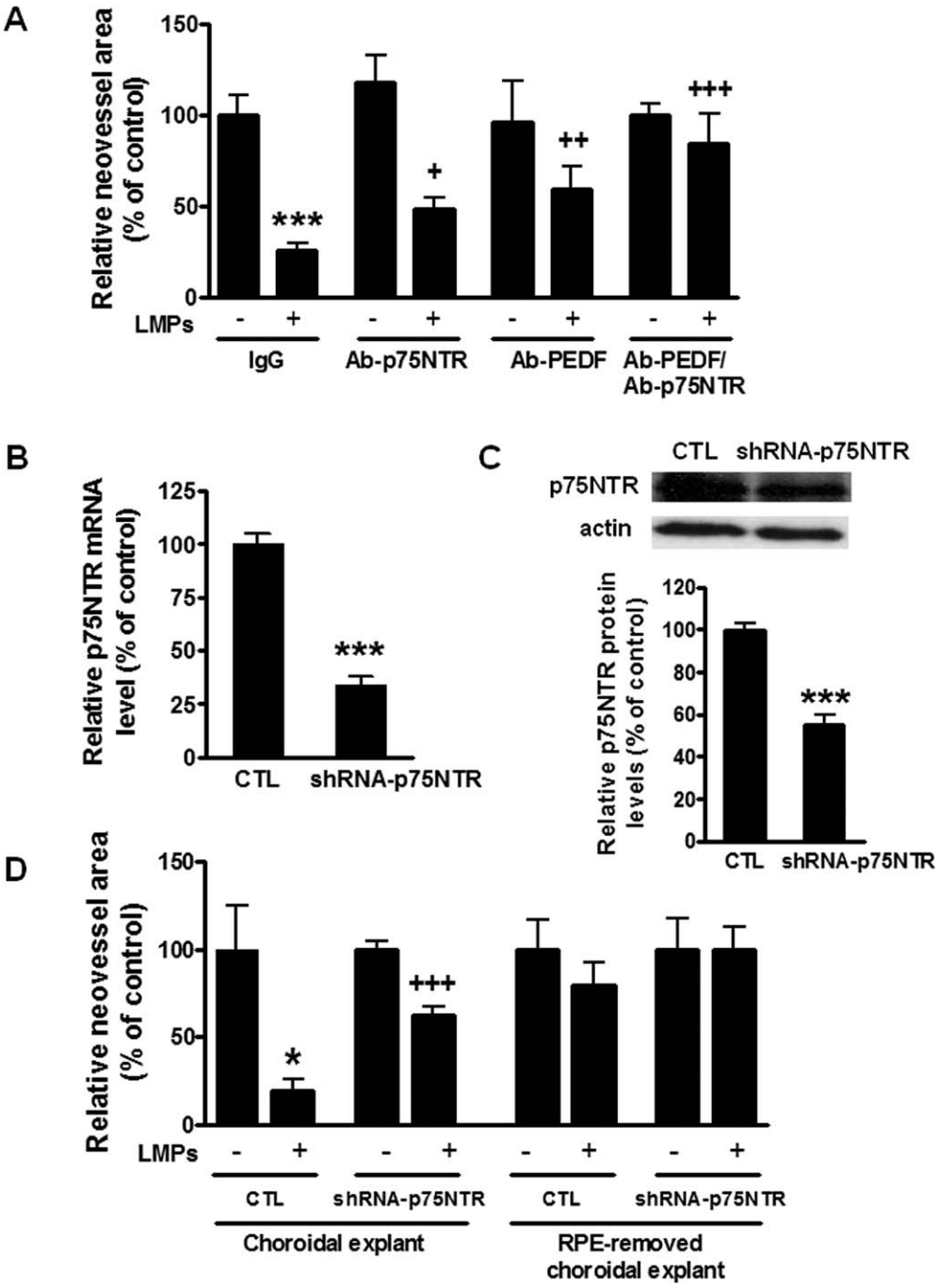

FIGURE 4. (**A**) Choroidal explants were pretreated with antibodies against p75NTR or PEDF followed by incubation with or without LMPs (50 μg/mL) for 48 hours. The choroidal neovessel area was quantified and normalized to control. (**B**) The RPE-removed choroidal explants were infected with lentiviral-scrambled shRNA (control) or lentiviral-shRNA-p75NTR for 24 hours. The p75NTR mRNA was quantified by real-time PCR and presented as percentage of that in control group. (**C**) Western blot and accompanying histogram showing knockdown p75NTR expression by lentiviral-shRNA–p75NTR in brain microvascular endothelial cells (RBMVEC). (**D**) Choroidal explants with or without RPE were infected with lentiviral-shRNA–p75NTR followed by LMPs treatment for 48 hours, and the neovascular area was quantified and normalized to control. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control; $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$ versus LMPs group.

choroidal microvascular cells. Apoptosis was assessed after 48 hours of LMPs treatment via immunoreactivity to activated caspase-3 and annexin V binding. Compared to IgG-pretreated explants, LMPs treatment caused a 5.5-fold increase in apoptosis, an effect that was abrogated significantly in Ab-p75NTR–pretreated explants (Figs. 5A, 5B).

DISCUSSION

The pathogenic mechanisms underlying CNV are complex and largely unknown. Accumulating evidence indicates that inflammatory and neovascular events contribute significantly to the development of this pathology. We demonstrated previously that LMPs can serve as tools for suitable

therapeutic intervention in animal models relevant to human disease, including inflammatory and ischemia-induced ocular neovascularization.[15] We report herein for the first time to our knowledge that LMPs significantly and time-dependently suppress choroidal angiogenesis. Our results showed that the antiangiogenic actions of LMPs are dependent on the integrity of the RPE and are mediated by the apoptotic activities of the neurotrophin receptor p75NTR. (Fig. 6).

The integrity of RPE cells is critical for proper vision and for maintenance of the choroidal vasculature.[36,37] In our studies, removal of the RPE abolished the antiangiogenic actions of LMPs, suggesting that factors secreted by the RPE are involved in this mechanism (Fig. 2). Of note, RPE cells secrete numerous growth factors, including neurotrophins.[38] which are necessary for RPE cell survival.[25] Neurotrophins act via 2 different transmembrane receptors: the Trk and p75NTR receptors.[39–41] It is well established that the overall levels of neurotrophins determine the balance between cell survival and apoptosis during development.[42] Alterations in the levels of neurotrophins and their receptors also has profound effects on the function of NGF-sensitive cells (e.g., retinal cells and Müller cells),[41] on the visual system,[43] and on neuron development and survival.[44] Herein, we showed that LMPs significantly increase choroidal expression of PEDF, NGF, and p75NTR. Moreover, the induction of NGF by LMPs was blunted in RPE-depleted choroids, indicating that the integrity of the RPE is important in this process (Fig. 3).

The PEDF is a potent endogenous inhibitor of angiogenesis.[45] Secreted by RPE cells, PEDF can inhibit the growth of blood vessels in the eye induced in a variety of ways.[46] Previous reports have demonstrated that activation of p75NTR inhibits neovascularization.[25] Using functionally blocking antibodies against PEDF and p75NTR, or using shRNA-mediated knockdown of p75NTR, LMPs treatment resulted in a partial, albeit significant reduction in choroidal angiogenesis. This observation was confirmed using combination of anti-PEDF and anti-NGF antibodies resulted in a more significant suppression of the antiangiogenic effect of LMPs on CNV (Fig. 4). Therefore, our results suggested that inhibition of choroidal angiogenesis by LMPs is mediated significantly by PEDF and p75NTR. Regulation of apoptosis is multifaceted and comprises numerous mediators. It is well established that endothelial cell apoptosis has a major role during vascular development, remodeling, and regression,[47,48] and that the balance between pro- and anti-apoptotic factors is critical for regulating ocular neovascularization.[3,49] We provided evidence that LMPs act via p75NTR to induce microvascular cell apoptosis and inhibit choroidal angiogenesis (Fig. 5). In endothelial cells, p75NTR is known to induce apoptosis via ligand-dependent and independent mechanisms, as well as suppress the VEGF-A/Akt/eNOS/NO pathway.[25] It also has been reported that PEDF can stimulate apoptosis in retinal endothelial cells.[50] Although many findings suggest a receptoric nature of PEDF action, the receptor responsible for its antiangiogenic effect still remains to be identified.[51] In addition, several antiangiogenic factors, including thrombospondin-1 and endostatin, exert their inhibitory effects by triggering endothelial cell apoptosis via downregulation of bcl-2 expression.[52,53] Whether these downstream signaling mediators are implicated in the apoptotic effects of LMPs remains to be determined.

The LMPs are membrane-derived vesicles released from cells during activation or cell death. The composition of LMPs has not yet elucidated completely, although evidence shows that they can serve as mediators of intercellular cross-talk[26,54] and deliver mRNA or proteins to take part in vascular function.[29,55] We observed recently that heat-denatured LMPs still have a significant inhibitory effect on endothelial cell growth (Yang C, Tahiri H, Qiu Q, Hardy P, unpublished data, 2012), suggesting that in addition to the proteins carried by LMPs, lipids or other heat-stable components might contribute to the effects of LMPs as well. Of note, very little is known about the role of LMPs during apoptosis; however, macrophages are said to undergo apoptosis after phagocytosing micro-

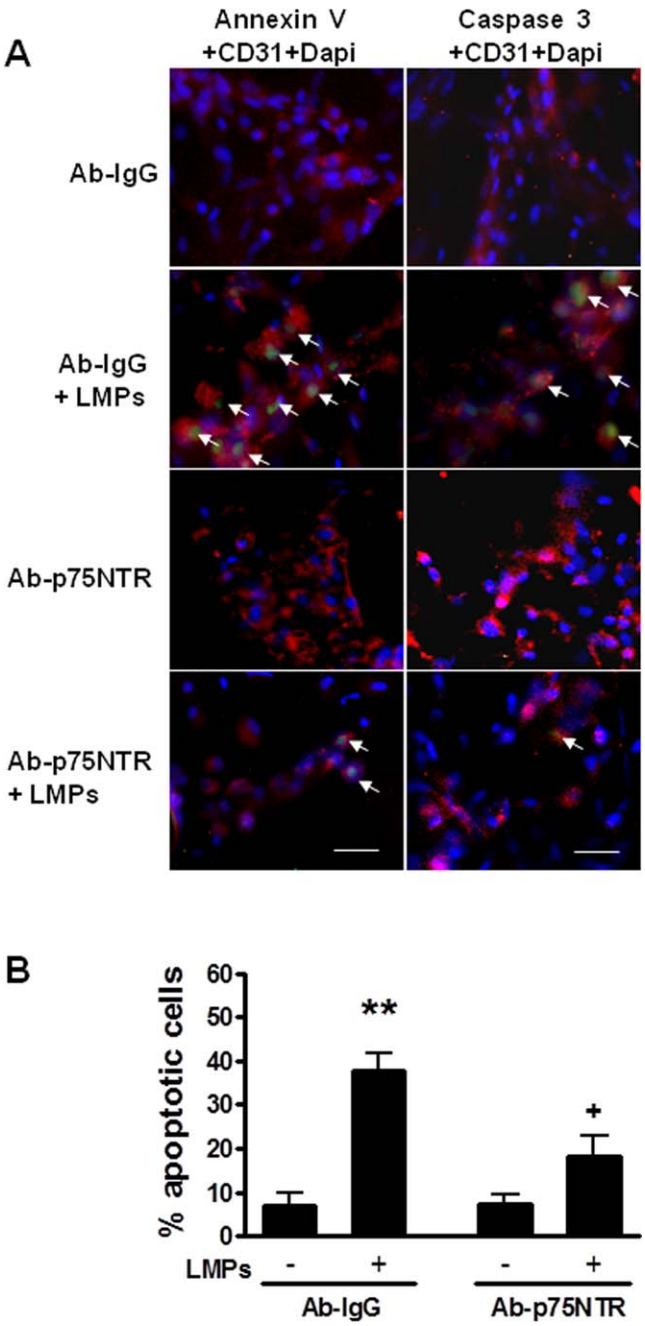

Figure 5. (**A**) High magnification micrographs of choroidal endothelial cells stained with CD31 antibody (*red*) and apoptotic cells using either annexin V binding (*green*), or immunohistochemical staining of activated caspase-3 (*green*) and DAPI (*blue*) in choroidal explants with or without 50 μg/mL of LMPs plus an IgG isotype control or antibody against p75NTR (Ab-p75NTR). *Magnification*: ×200. *Bar*: 30 μm. *Arrows* point to either the annexin V- or activated caspase 3-positive cells (*green*). (**B**) Quantitative assessment of apoptotic cells in choroidal explant sections. The average percentage of annexin V- and caspase-3–positive cells was presented as mean ± SEM from 6 individual choroidal explants per condition. $^{**}P < 0.01$ versus control Ab-IgG, $+P < 0.05$ versus Ab-IgG + LMPs.

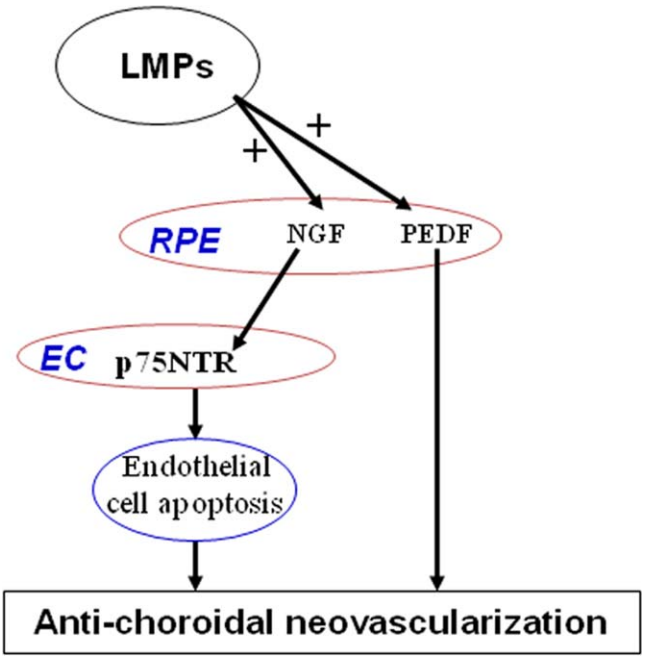

FIGURE 6. Summary of the signaling pathways altered by LMPs during the choroidal neovascularization. LMPs, via the upregulation of PEDF and NGF in RPE, activate p75NTR to induce choroidal endothelial cells apoptosis and suppress choroidal neovascularization. EC, endothelial cell.

particles.[20] Our results showed that LMPs induce apoptosis via mechanisms requiring activation of p75NTR and caspase-3 (Figs. 5A, 5B). This is in line with observations by others demonstrating that p75NTR promotes endothelial cell apoptosis via caspase-3 activation.[25] Whether LMPs transfer caspase-3 to other cells, thus contributing to the induction of endothelial cell dysfunction, remains to be elucidated.

In summary, we demonstrated that LMPs are important candidates for antiangiogenic therapy. We identified p75NTR and PEDF as crucial mediators of the inhibitory actions of LMPs on choroidal angiogenesis. Examination of the role of the RPE and the downstream signaling mechanisms triggered by activation of p75NTR by LMPs may unveil key pathways for pharmacologic intervention of CNV. Therapeutic strategies involving apoptotic clearance of excessive inflammatory or endothelial cells also represents a novel clinical approach. Findings also suggested that effective treatments to counter CNV should consider preservation of RPE integrity.

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