Role of receptor-mediated endocytosis in the antiangiogenic effects of human T lymphoblastic cell-derived microparticles

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Submitted 19 September 2011; accepted in final form 8 February 2012

Yang C, Xiong W, Qiu Q, Shao Z, Hamel D, Tahiri H, Leclair G, Lachapelle P, Chemtob S, Hardy P. Role of receptor-mediated endocytosis in the antiangiogenic effects of human T lymphoblastic cell-derived microparticles. Am J Physiol Regul Integr Comp Physiol 302: R941–R949, 2012. First published February 15, 2012; doi:10.1152/ajpregu.00527.2011.— Microparticles possess therapeutic potential regarding angiogenesis. We have demonstrated the contribution of apoptotic human CEM T lymphocyte-derived microparticles (LMPs) as inhibitors of angiogenic responses in animal models of inflammation and tumor growth. In the present study, we characterized the antivasculature endothelial growth factor (VEGF) effects of LMPs on pathological angiogenesis in an animal model of oxygen-induced retinopathy and explored the role of receptor-mediated endocytosis in the effects of LMPs on human retinal endothelial cells (HRECs). LMPs dramatically inhibited cell growth of HRECs, suppressed VEGF-induced cell migration in vitro experiments, and attenuated VEGF-induced retinal vascular leakage in vivo. Intravitreal injections of fluorescently labeled LMPs revealed accumulation of LMPs in retinal tissue, with more than 60% reductions of the vascular density in retinas of rats with oxygen-induced neovascularization. LMP uptake experiments demonstrated that the interaction between LMPs and HRECs is dependent on temperature. In addition, endocytosis is partially dependent on extracellular calcium. RNAi-mediated knockdown of low-density lipoprotein receptor (LDLR) reduced the uptake of LMPs and attenuated the inhibitory effects of LMPs on VEGF-A protein expression and HRECs cell growth. Intravitreal injection of lentivirus-mediated RNA interference reduced LDLR protein expression in retina by 53% and significantly blocked the antiangiogenic effects of LMPs on pathological vascularization. In summary, the potent antiangiogenic LMPs lead to a significant reduction of pathological retinal angiogenesis through modulation of VEGF signaling, whereas LDLR-mediated endocytosis plays a partial, but pivotal, role in the uptake of LMPs in HRECs.

VEGF; angiogenesis; retinopathy; endocytosis

RETNOPATHY OF PREMATUREITY (ROP) is the major ocular disorder of the neonate, and the dominant cause of severe visual impairment in childhood in North America and Europe (13). The oxygen-induced retinopathy (OIR) model of ischemic retinopathy, which reproduces the human vasoobliterative and neovascularization phases of ROP and accurately assesses treatment outcome, has provided substantial insight into the pathogenesis of this condition (32). Present therapeutic modalities to limit the adverse consequences of aberrant vascularization are invasive and/or tissue-destructive. While antivasculature endothelial growth factor (VEGF) agents hold promise as a strategy to counter ROP (30), they have not been completely successful in treating this pathology. Consequently, a variety of emerging treatments aimed at supplementing or competing with anti–VEGF therapies are currently under development (9, 16, 38).

Lymphocytes-derived microparticles (LMPs) are naturally occurring membrane-coated vesicles released from lymphocytes during activation or apoptosis (7, 18, 25, 27, 29, 37). Our previous studies have demonstrated that LMPs generated from apoptotic human CEM T lymphoblast cells possess strong antiangiogenic effect, which was evidenced by suppressing aortic ring microvessel sprouting and in vivo corneal neovascularization (42). On endothelial cells, this effect was linked to downregulation of VEGF receptor type 2 (VEGFR-2) and phosphorylated ERK1/2 expression, increased production of reactive oxygen species, and upregulation of CD36 protein levels, a known negative regulator of VEGF/VEGFR2 signaling pathway (31, 35, 42).

Like other microparticles, LMPs can stimulate targeting cells directly or transfer important biological signals (4, 7, 10, 24, 26, 28, 36, 37). Consistent with these properties, we have demonstrated that the low-density lipoprotein receptor (LDLR) mediates the anti-VEGF effects of LMPs by translocating LMPs into lung carcinoma cells (41). The mechanism(s) by which LMPs alter retinal angiogenesis and evidence for their role in cell-to-cell communication in retinal endothelial cells are unknown. Here, we demonstrate that the inhibition of pathological retinal vascularization by LMPs is mediated, in part, via LDLR-mediated endocytosis, such that silencing LDLR significantly attenuates the antiangiogenic effects of LMPs during OIR. Through their potent antiangiogenic effects, LMPs may potentially serve as valuable tools for therapeutic interventions aimed at regulating ocular pathological angiogenesis (41, 42).

MATERIALS AND METHODS

LMP production. Human T-cell lymphoblast-like cell line (CEM) T cells were purchased from American Type Culture Collection (Manassas, VA) and cultured with X-VIVO medium (Cambrex, Walkersville, MD). LMPs were generated and characterized as previously...
described (42). Briefly, CEM T cells were treated with 0.5 μg/ml actinomycin D (Sigma-Aldrich, St. Louis, MO) for 24 h. A supernatant was obtained by centrifugation at 750 g for 15 min, then 1,500 g for 5 min to remove cells and large debris. LMPs from the supernatant were washed after three centrifugation steps (50 min at 12,000 g) and recovered in saline. Washing medium from the last supernatant was used as a control for all in vitro experiments. To generate Dil-labeled LMPs (DiI-LMPs), fluorescent lipophilic membrane dye dialkylcarbocyanine (DiI) (Molecular Probes, Eugene, OR) was added to CEM T cells 24 h before actinomycin D treatment (21, 41). LMPs concentrations were determined using the Bio-Rad (Hercules, CA) protein assay.

**Cell culture and cell growth assays.** Human retinal endothelial cells (HRECs) were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and cultured as recommended. HRECs were seeded into 24-well plates, and different concentrations of LMPs were added when cells reached 60% confluence. After 24 h of treatment, cell growth was assessed by measuring the activity of enzymes that reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan dye; cell proliferation was evaluated by [3H]thymidine incorporation assay, as described previously (41, 42).

**Cell migration assay.** Cell migration was determined using a coverslip border migration assay (42). Briefly, 0.5 × 10⁶ HRECs were seeded onto 12-mm coverslips in a 24-well plate. Cells were serum starved for 4 h, and proliferation was inhibited by adding 10 μg/ml mitomycin C (Fluka Biochemika, Buchs, Switzerland) for 30 min. Next, coverslips were carefully removed, washed with fresh media, and transferred into a 12-well plate containing 10 ng/ml of human recombinant VEGF (hrVEGF; Calbiochem, La Jolla, CA) in the presence or absence of 10 μg/ml LMPs. After 72 h, coverslips were removed, and the number of cells that migrated off the coverslip was quantified by MTT assay. Images were captured after 72 h using an Axiovert 200 M inverted microscope (Zeiss). Results were given as the means ± SE of 3 separate experiments.

**Western blot analysis.** Cells were plated at a density of 1 × 10⁶ cells per 100-mm dish and cultured in the medium containing 10 ng/ml human recombinant VEGF. Cells were collected after they were incubated with LMPs at different time points (0, 10, 20, and 60 min). Extraction of soluble proteins and fractionation by SDS-PAGE were performed as described previously (42). The following antibodies were used to investigate the effects of LMPs on phospho-ERK and phospho-Akt expression in HRECs: anti–phospho–ERK1/2, anti–phospho-Akt, and anti-Akt (all from Cell Signaling, Danvers, MA); for phospho-Akt expression in HRECs: anti–phospho–ERK1/2, anti–phospho-Akt, and anti-Akt (all from Cell Signaling, Danvers, MA); and anti-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA). For LDLR knockdown studies, silencing RNA (siRNA; Santa Cruz Biotechnology) or scramble siRNA was transfected into HRECs using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) 24 h before LMPs treatment. To assess the effectiveness of LV-shRNA LDLR in vivo, lentivirus and control lentivirus were intravitreally injected into the vitreous using a 27-gauge Hamilton syringe (Hamilton, Reno, NV) once every 2 days for 3 total injections. On day 6, retinas were collected and subjected to radial cyrosectioning or retinal whole mounts, followed by staining of microvessels with FITC-conjugated lectin (Sigma-Aldrich).

**Preparation of short hairpin RNAs (shRNAs) and lentivirus production.** Both lentiviral pLKO.1 vector expressing short hairpin RNA (shRNA) against rat LDLR and control pLKO.1 vector containing scramble shRNA were obtained from OpenBiosystems (Huntsville, AL). Infectious lentiviruses (LV-shRNA-LDLR) were generated by transiently cotransfecting plasmids encoding proteins essential for viral assembly and shRNA encoding plasmids into 293FT cells (Invitrogen), as previously described (11). The following shRNA constructs were used for determining the wet weight of the retinas. Evans blue dye was extracted by incubating each retina in 120 μl of formamide (Sigma-Aldrich) for 18 h at 70°C. To precipitate proteins, the extract was ultracentrifuged at 70,000 rpm for 45 min at 4°C. The absorbance of the supernatant (60 μl) at 620 nm was then determined using a spectrophotometer (UV-1600 PC; Shimadzu, Kyoto, Japan). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide.

**Model of OIR.** One-day-old Sprague-Dawley litters with their nursing mothers and 6- to 8-wk adult Sprague-Dawley rats were purchased from Charles River (St-Constant, Quebec, Canada) and used according to a protocol approved by the Sainte Justine Research Center Animal Care Committee. The well-established animal model of OIR was reproduced as described previously (6, 32). Briefly, central retinal capillary loss was induced by exposing newborn rats and their mothers to 24-h alternating cycles of hyperoxia (50% oxygen) In the endocytosis inhibition experiment, HRECs were seeded onto 12-well plates with coverslips. The following day, 20 μL vehicle-containing (Fluka) cells were added 2 h before incubation with DiI-LMPs (10 μg/ml). After incubation at 37°C for 4 h, HRECs were fixed, and nuclei were stained with DAPI. Fluorescence microscopy was performed on a Zeiss LSM510 laser scanning confocal microscope attached to a Zeiss Axiovert 200 microscope using a Zeiss Plan-Apo 63×1.40 NA oil immersion lens.

To determine the knockdown efficacy of siRNA in HRECs, cells at ~50% confluence were transfected using Lipofectamine 2000 with scrambled siRNA (control) or sequence-specific siRNA targeting LDLR (siRNA-LDLR; Santa Cruz Biotechnology). Western blot analysis revealed that a 40-nM concentration of siRNA-LDLR produced >50% knockdown efficiency; therefore, this concentration was used in subsequent Dil-LMPs uptake experiments. To investigate whether LDLR mediated the uptake of LMPs, 10 μg/ml Dil–LMPs were added to siRNA-LDLR-transfected HRECs, followed by a 24-h incubation and then measurement of Dil fluorescence intensity in intact cells, membrane, and cytosolic fractions.

**Localization of LMPs in retinal tissue and assessment of retinal vasoproliferative ability.** To localize Dil-LMPs within retinal tissue, adult Sprague-Dawley rats were subjected to general anesthesia and 2.8 μL DiI-LMPs were injected into the vitreous using a 27-gauge Hamilton syringe (Hamilton, Reno, NV) once every 2 days for 3 total injections. On day 6, retinas were collected and subjected to radial cyrosectioning or retinal whole mounts, followed by staining of microvessels with FITC-conjugated lectin (Sigma-Aldrich).

**Preparation of VEGF-induced blood-retinal barrier breakdown was performed as described by Xu et al. (40). Briefly, after adult rats were placed under general anesthesia, one eye was intravitreally injected with 50 ng of recombinant murine VEGF165 (R&D Systems, Minneapolis, MN) in PBS, while the contralateral eye received either the same quantity of PBS or VEGF plus 2.8 μg LMPs. 2.8 μg LMPs makes a final intraocular concentration of LMPs as 50 μg/ml based on estimated eye volume, which is the optimal effective dosage of LMPs being tested. After 24 h, Evans blue was injected at a concentration of 45 mg/kg and allowed to distribute systemically for 90 min. Animals were subsequently perfused with saline via the left ventricle, and retinas were immediately and carefully dissected away under an operating microscope. Aliquots of the retinas were added to 500-fold, and an ELISA kit (Invitrogen) was used for determining the wet weight of the retinas in vivo. The following shRNA sequences were used for determining the wet weight of the retinas. Evans blue dye was extracted by incubating each retina in 120 μl of formamide (Sigma-Aldrich) for 18 h at 70°C. To precipitate proteins, the extract was ultracentrifuged at 70,000 rpm for 45 min at 4°C. The absorbance of the supernatant (60 μl) at 620 nm was then determined using a spectrophotometer (UV-1600 PC; Shimadzu, Kyoto, Japan). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide.
and hypoxia (10% oxygen) from postnatal day 1 (P1) to P14 followed by return to room air (21% oxygen for 4 days). To achieve the above conditions, a bioactives gas controller (Oxycycler, model A820CV; BioSpherix, Redfield, NY) was used. Litter numbers were between 12 and 15 pups for each experiment to ensure consistency in outcomes.

Assessment of developmental and OIR-induced retinal vascularization. To investigate developmental retinal vascularization, rat pups were anesthetized with isoflurane and injected intravitreally using glass capillaries (60 gauge) at P2 and P4 with 5 μl saline or 50 μg/ml LMPs (final intraocular concentration based on estimated eye volume) in the right or left eyes, respectively. Pups were killed at P6, and retinal flatmounts were prepared.

In the OIR model, newborn rat pups were anesthetized with isoflurane and intravitreally injected at P12, P14, and P16 with 5 μl saline in the right eye and 50 μg/ml LMPs in the left eye. Pups were killed at P18, and retinal flatmounts were prepared. For lentiviral knockdown studies, animals were injected with LV.shRNA-LDLR at P10 to allow sufficient time for adequate expression of the shRNA, followed by intravitreal injections of saline or LMPs, as described above. The LV.shRNA-LDLR group was compared with control LV.shScrambled.

Retinal whole mounts. Retinal whole mounts were prepared as described previously (8). Briefly, eyes were enucleated and fixed in 4% formaldehyde. Retinas were dissected free, subjected to 100% methanol (−20°C) for 10 min, and incubated overnight (4°C) in 1% Triton X-100/PBS with TRITC-conjugated lectin (Sigma-Aldrich). Retinas were washed in PBS and mounted. The retinal surface covered by vessels (vascularized area) was measured and quantified as a percentage of the entire retinal area. Vessel density was determined using Image-Pro Plus software (version 4.1; Media Cybernetics, Silver Spring, MD).

Statistical analysis. All experiments were repeated at least 3 times. Values are presented as means ± SE. Data were analyzed by one-way ANOVA followed by post hoc Bonferroni tests for comparison among means. Statistical significance was set at P < 0.05.

RESULTS

LMPs inhibit HRECs proliferation and suppress VEGF-induced cell migration. To confirm that LMPs exert same effects on HRECs as they exhibit on other human endothelial cells (42), we repeated the cell growth assay, cell proliferation assay, and cell migration assay on HRECs. Incubation of HRECs with LMPs resulted in a dose-dependent decrease in cell growth (Fig. 1A). LMPs also strongly inhibited HRECs proliferation as detected by [3H]thymidine DNA incorporation assay (Fig. 1B). In addition, VEGF-induced cell migration was
reduced by 55% in HRECs treated with 10 μg/ml LMPs (Fig. 1, C and D).

To ascertain whether the inhibitory effects of LMPs were specific to LMPs released by apoptotic stimuli, we generated hyperoxia-induced LMPs (Hi-LMPs) by exposing human CEM T lymphocytes to 36 h of hyperoxia (95% oxygen). Effects of Hi-LMPs on endothelial cell growth were comparable to those observed using apoptotic-induced LMPs (Fig. 1A).

LMPs downregulate expression of phospho-ERK and phospho-Akt in HRECs. Our previous studies revealed that LMPs antagonize VEGF/VEGFR-2 signaling events by downregulating VEGFR-2 expression in endothelial cells (42) and VEGF-A expression in lung carcinoma cells (41). We investigated whether LMPs exert similar inhibitory effects on VEGF downstream intracellular signaling in HRECs. Results shown that VEGF-A-activated ERK1/2 phosphorylation was decreased by 33% and 64% after 10- and 60-min incubation with 10 μg/ml LMPs, respectively (Fig. 1, E and F). Phosphorylation of Akt, another downstream molecule of VEGF signaling, was also suppressed by 43%, 48%, and 51% at 10, 20, and 60 min, respectively, after LMP treatment (Fig. 1, G and H).

Intravitreally injected LMPs localize in the retina and suppress VEGF-induced retinal permeability. To visualize the distribution of LMPs in retinas, we produced the fluorescent Dil-labeled LMPs in vitro. Following intravitreal injection of Dil-LMPs in adult rats, Dil staining (red fluorescence) was evident throughout the inner retinal layers and colocalized with lectin-FITC-positive vascular cells (green fluorescence) (Fig. 2A). Dil staining was evident throughout the retina in the retinal whole mount. However, Dil-LMPs seemed to preferentially accumulate in vascular cells, with microvessels demonstrating higher fluorescence intensity compared with the surrounding areas (Fig. 2B).

Because retinal VEGF levels are known to be significantly and positively correlated with retinal vascular leakage (23), we investigated the impact of LMPs on VEGF-induced retinal vascular leakage in vivo. Intraocular injection of VEGF (50 ng/eye) increased retinal plasma leakage in rat eyes by 2.9-fold (P < 0.001; n = 7) relative to an equivalent injection of saline. Co-administration of LMPs (2.8 μg/eye) plus VEGF (50 ng/eye) resulted in a 50% decrease in plasma leakage (P < 0.05, n = 9) compared with VEGF injection alone (Fig. 2C).

LMPs inhibit retinal vascularization in vivo. VEGF-A and its receptors play pivotal roles in retinal development during perinatal vascularization (12). We assessed the effect of LMPs on developmental retinal angiogenesis in newborn rats. Compared with vehicle-treated retinas, intravitreal injections of LMPs at P2 and P4 resulted in a 12% decrease in vascularized retinal area, as determined at P6 (Fig. 3, A and B).

Rodent models of OIR provide important insights into the pathogenesis of ROP. To induce OIR, neonatal rats were exposed to alternating cycles of hyperoxia and hypoxia (15, 33), with a resultant 51.6% induction in retinal vascular density. Compared with vehicle-treated retinas at P18, intravitreal injections of LMPs at P12, P14, and P16 significantly reduced the oxygen-induced retinal vascular density by ~63% (Fig. 3, C and D).

Knockdown of LDLR expression decreases LMPs uptake into HRECs. Our previous studies revealed that LDLR—a key receptor involved in endocytosis—mediates the uptake of LMPs into Lewis lung carcinoma cells (14, 41). Therefore, we used Dil-labeled LMPs to explore more in detail the endocytic internalization of LMPs in HRECs. Quantification of LMPs binding using spectrophotometry revealed that the interaction between HRECs and LMPs is an active process dependent on temperature (Fig. 4A). In addition, LMPs uptake was partially, but significantly, inhibited by extracellular Ca2+ chelation by EDTA (Fig. 4A). The endocytosis process involved in LMPs internalization was further reinforced by the observation that the internalization of LMPs was dramatically decreased by the treatment of cytochalasin D, an inhibitor of microfilament formation required for endocytic processes (Fig. 4B).

Insights into the mechanisms of LMP uptake into HRECs were further explored via LDLR knockdown experiments. LDLR protein expression was decreased by 60% in HRECs transfected with siRNA–LDLR (40 nM), as determined by Western blot analysis (Fig. 4, C and D). Moreover, 24-h incubation of siRNA-LDLR-transfected HRECs with Dil–LMPs significantly reduced Dil–LMPs uptake and distribution in cytosolic compartments (Fig. 4, E and F).

LDLR mediates the anti-VEGF effects of LMPs in HRECs. To test whether LDLR also mediated the anti-VEGF effects of LMPs, VEGF-A protein expression was analyzed by Western
In siRNA–LDLR-transfected HRECs. LMPs suppressed VEGF-A protein expression, an effect that was significantly attenuated in siRNA–LDLR-transfected HRECs (Fig. 5, A and B). In addition, downregulation of LDLR expression abrogated the inhibitory effects of LMPs on cell growth (Fig. 5C). These results were subsequently confirmed following blockade of LDLR using an anti-LDLR antibody; preincubation of HRECs with 15 μg/ml anti-LDLR antibody significantly prevented the cell growth–inhibitory effects of LMPs compared with the isotype control group (Fig. 5D).

Knockdown of LDLR abrogates the antiangiogenic effects of LMPs in OIR. Generation of a lentivirus containing an shRNA targeting LDLR (LV.shRNA-LDLR) enabled effective knockdown of LDLR expression in vivo. In HRECs, this viral particle potently suppressed LDLR protein expression by 53% (Fig. 6, A and B). We then assessed the role of LDLR in mediating the effects of LMPs during OIR. LV.shRNA-LDLR was given intravitreally to rat pups at P10 and retinal tissue was collected at P18. LV.shRNA-LDLR partially, yet significantly, blocked the inhibitory effects of LMPs on retinal vascularization in the OIR model. The control lentivirus containing scramble shRNA was ineffective (Fig. 6, C and D).

**DISCUSSION**

The present study demonstrated the contribution of LMPs in the impediment of pathological retinal angiogenesis during OIR. The antiangiogenic effects of LMPs on retinal vascularization are, at least in part, dependent on the inhibition of anti-VEGF effects. Because VEGF is considered a principal mediator of developmental and pathological retinal angiogenesis, it was important to explore the impact of LMPs on these processes. In vitro, LMPs in pharmaceutical dose suppressed human retinal endothelial cell proliferation and VEGF-induced cell migration, the two pivotal processes involved in retinal angiogenesis. Moreover, the downstream events of VEGF-A signaling pathway were also downregulated by LMPs in HRECs, which indicated that LMPs antagonized the VEGF/VEGFR-2 signaling pathway, and this result is consistent with our previous work using other types of endothelial cells (42).

The antiangiogenic effect of LMPs was further elucidated in the OIR animal model, which mimics contemporary severe ROP in North American patients (15). LMPs substantially diminished oxygen-induced retinal vascularization, whereas they decreased developmental retinal vascularization modestly. This finding may be deemed advantageous because LMPs could potentially inhibit pathological retinal vascularization without extensively interfering with physiological development of the retinal vasculature. Overall, the conspicuous antagonizing effects of LMPs in the OIR model may be attributed to inhibition of VEGF bioactivity pathways, observations that have been supported by previous studies (41, 42).

The importance of VEGF as a therapeutic target derives from its role in basic processes within a typical ROP lesion, including its involvement in vascular permeability (2, 12, 23). The profound vascular permeability induced by VEGF is important in the treatment of established neovascular retinal lesions, in which leakage of fluid from new vessels causes visual loss through retinal edema, exudation, and hemorrhage (43). LMPs ability to interfere with VEGF signaling pathway was further evidenced by abrogation of VEGF-induced retinal

**Fig. 3.** LMPs inhibit developmental retinal angiogenesis and vascularization in oxygen-induced retinopathy (OIR). A: retinal flatmounts taken at postnatal day 6 (P6) after intravitreal injections (at P2 and P4) with saline (CTL) or 50 μg/ml LMPs. B: retinal surface area covered by vessels (vascularized area) was measured and quantified as a percentage of the entire retinal area, and these values were presented as relative to saline-treated group, which was set as 100%. C: images of retinal flatmounts from OIR–exposed rats at P18 following three total intravitreal injections (at P12, P14, and P16) with saline (CTL) or LMPs (50 μg/ml final vitreous concentration). D: microvascular density presented as a percentage of the vessel density from P18 normoxia-exposed retinas (WT). **P < 0.01 vs. control. ***P < 0.001 vs. WT (normoxia). #P < 0.05 vs. control in OIR.
vascular permeability (Fig. 2C). However, the mechanisms underlying LMPs-mediated barrier protection require further investigation. Several mediators capable of decreasing endothelial permeability have been identified, raising the possibility that alterations in endothelial barrier function are determined by the presence of permeability–increasing mediators (e.g., VEGF, thrombin) and barrier-stabilizing factors (e.g., angio-poetin-1, sphingosine-1-phosphate) (19). In this context, LMPs could provide novel therapeutic strategies for inflammatory and angiogenic diseases. By inhibiting permeability–increasing mediators, LMPs could reverse the defects in vessel wall permeability associated with these neovascular diseases (34). To achieve the effect on vascularization, LMPs were demonstrated to be able to penetrate and spread within the retina following intravitreal injections. LMPs distributed preferentially in the outer retinal layers and colocalized with microvascular cells. However, our in vivo data did not indicate that LMPs solely interact with capillary endothelial cells; it should be taken into consideration the possibility that LMPs indirectly affect angiogenesis in this model by interacting with nonendothelial cells. This differential distribution of LMPs in the retinal cell population and the ensuing effects on cell function are currently being investigated in our laboratory. Of vital importance, the cell viability of staurosporine-induced terminal differentiated retinal ganglion cells (RGC-5) and that of rat cortical neuronal cells in primary cultures was not affected by LMPs (unpublished data).

The mechanisms by which LMPs interact with target cells were investigated in current studies. It has been suggested that LMPs released from Jurkat T cells are transferred to macrophages by endocytosis (17). In our study, LMPs were successfully carried into retinal tissue and endothelial cells. Uptake experiments conducted with DiI demonstrated that interaction between LMPs and endothelial cells is an active process dependent on temperature and on an intact actin cytoskeleton (Fig. 4, A and B). LMPs transfer to endothelial cells was significantly dependent on calcium (Fig. 4A), suggesting a role for receptor-mediated endocytosis. Members of the LDLR

Fig. 4. LDLR partially mediates the uptake of LMPs by HRECs. A: presence of Dil-LMPs in HRECs was determined by spectrofluorometry after cells were incubated at different conditions: 37°C, 35°C, and 22°C temperatures, with additional 0.5 mM CaCl2 or 2.5 mM EDTA for 4 h, and presented as relative Dil-LMP uptake compared with control (regular culture medium at 37°C, set as 100%). **P < 0.01, ***P < 0.001 vs. control. B: representative fluorescence microscopy showing uptake of LMPs by HRECs. Photos were taken 4 h after HRECs were incubated with 10 µg/ml Dil-LMPs with and without 20 µM cytochalasin D pretreatment. ×40 magnification; scale bar: 30 µm. LDLR protein levels in HRECs transfected with scrambled siRNA (control) or siRNA-LDLR were detected by Western blot (C) and presented as a percentage of control (D). After a 24-h incubation of Dil-LMPs with siRNA-LDLR-transfected cells, the presence of Dil-LMPs in intact cells (E) and in membrane or cytosolic fractions (F) was measured and expressed as a percentage of control. *P < 0.05, **P < 0.01 vs. control.
family consist of endocytic receptors that mediate the uptake of biologically active molecules into the cell (22) and participate directly in signal transduction, including a suggested link between LDLR and VEGFR (39). Bearing in mind the plasma membrane components of LMPs and the physiological function of LDLR, the critical role of LDLR mediating the anti-proliferative and antiangiogenic effects of LMPs was suggested in in vitro and in vivo observations:

1) downregulation of LDLR expression in HRECs significantly dampened the transfer of LMPs to both membrane and cytoplasm components; 2) modulation of LDLR levels in HRECs altered the effect of LMPs on VEGF-A expression; and 3) downregulation

Fig. 5. LDLR mediates the effects of LMPs. A: representative Western blot shown the VEGF-A expression in siRNA-transfected HRECs with LMPs treatment. B: VEGF-A protein levels were determined and normalized to β-actin. Data are presented as a percentage of control (scramble siRNA alone). **P < 0.01 vs. control. #P < 0.05 vs. scramble siRNA plus LMPs. C: cell growth was assessed in HRECs transfected with siRNA-LDLR and treated with 10 μg/ml LMPs for 24 h. Data are presented as a percentage of control (scramble siRNA). *P < 0.05 vs. CTL; #P < 0.05 vs. scramble siRNA plus LMPs. D: HRECs were preincubated with 15 μg/ml of LDLR antibody or rat IgG (isotype control), cell growth was determined after 24-h incubation with 10 μg/ml LMPs. Data are presented as a percentage of control; **P < 0.01 vs. CTL; #P < 0.05 vs. LMPs.

Fig. 6. Knockdown of LDLR reduces retinal vascularization during OIR. A: Western blots from two animals, #1 and #2, of LDLR protein levels in retinas infected with LV.scramble shRNA (control) or LV.shRNA-LDLR (shRNA-LDLR). B: LDLR protein levels are presented as a percentage of control. **P < 0.01 vs. control. C: representative images of retinal flatmounts in OIR-exposed rats at P18 after three intravitreal injections (at P12, P14, and P16) with scramble shRNA plus saline (control), scramble shRNA plus LMPs, shRNA-LDLR plus saline, or shRNA-LDLR plus LMPs. D: microvascular density was measured on the retinal flatmounts and the relative retinal vessel density presented as a percentage of control. ***P < 0.001 vs. control, #P < 0.05 vs. scramble shRNA + LMPs.
of LDLR expression or blockade of LDLR activity attenuated the inhibitory effect of LMPs on cell growth and in vivo retinal pathological angiogenesis. Nevertheless, other receptors or factors in HRECs may also contribute to the uptake of LMPs, since partial inhibition of LMPs internalization resulted in a limited reverse of their effects observed when LDLR expression or its activity was down-regulated. Multiple receptors may also be involved in microparticles binding to target cells. This was suggested from a study of microparticles generated by stimulated T cells, because only part of these microparticles binding to monocytes was inhibited by high-density lipoproteins (7).

With regard to the effects of LMPs on cell growth, the postulate indicating that different effects evoked by LMPs are from different active stimulations (1, 3, 5, 20, 25) is not supported by this result. Hence, LMPs generated from hyperoxia-mediated oxidative stress, exhibited comparable anti-cell growth effect as that of LMPs from apoptotic human T-lymphocytes following actinomycin D stimulation. In addition, from our current and previous studies, we observed that LMPs are able to suppress angiogenesis in different tissues, including aorta, cornea, retina, and tumor. Taken together, all these findings strongly indicated that the antiangiogenic effect of LMPs is not tissue specific.

Perspectives and Significance

Our present observations suggest that LMPs transfer into retinal tissue may suppress proangiogenic responses during pathophysiological conditions. In addition, the antiproliferative and antiangiogenic effects of LMPs on HRECs and retinal vasculature are, at least in part, dependent on LDLR-mediated endocytosis. Additional studies are necessary to delineate the components of LMPs involved in triggering this process, as well as the specific receptors and ensuing events mediating the effects of LMPs. These findings may have broader implications for pathological angiogenesis-related eye disorders besides ROP, such as corneal neovascularization and the subretinal neovascularization that occurs in the wet form of macular degeneration.

ACKNOWLEDGMENTS

The authors wish to thank Carmen Gagnon and Bupe R. Mwaikambo for invaluable technical and editing assistance, respectively. Sylvain Chemtob holds a Canada Research Chair (Vision Science) and the Leopoldine Wolfe Chair in translational research in age-related macular degeneration.

GRANTS

Chun Yang is a recipient of a Fellowship from the Eye Network Fonds de la recherche en santé du Québec. Houda Tahiri is a recipient of a Fellowship from the Eye Network Fonds de la recherche en santé du Québec and a Canada Graduate Scholarsfip Master’s Award from the Canadian Institutes of Health Research. This work is supported by a grant from the Canadian Institutes of Health Research (MOP-86631).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Chun Yang, Wei Xiong conceived and designed the research, acquired, analyzed and interpreted the data, performed statistical analysis, and drafted the manuscript; Qian Jiu, Zhiu Shao, David Hamel, Houda Tahiri acquired the data; Grégoire Leclaire, Pierre Lachapelle and Sylvain Chemtob made critical revision of the manuscript for important intellectual content; Pierre Hardy conceived and designed the research, handled funding and supervision and made critical revision of the manuscript.

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