Propranolol Attenuates Proangiogenic Activity of Mononuclear Phagocytes: Implication in Choroidal Neovascularization

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PURPOSE. Targeting β-adrenergic receptor signaling with propranolol has emerged as a potential candidate to counteract choroidal neovascularization (CNV). Little is known of its effect on macrophages, which play a critical role in CNV. We investigated the effect of propranolol on angiogenic response of mononuclear phagocytes (MPs).

METHODS. The angiogenic effect of propranolol was evaluated in laser-induced CNV model. Mice received intraperitoneal injections of propranolol (6 mg/kg/d) or vehicle. CNV area and inflammatory cells were determined respectively by using lectin staining and an anti–IBA-1 antibody on RPE/choroid flat mounts. Inflammatory gene expression was evaluated by quantitative (q) PCR analysis. Mechanisms of propranolol was studied in MP cell lines J774 and RAW264.7 and in primary peritoneal macrophages. Expression of pro- and antiangiogenic mediators was studied. In addition, effects of propranolol treatment of MPs was assessed on choroidal explant.

RESULTS. CNV was attenuated by propranolol and concomitantly associated with decreased inflammatory mediators IL-6 and TNFα, albeit with accumulation of (β-adrenoceptor harboring) MPs in the CNV area. Conditioned media from MPs preincubated with propranolol exerted antiangiogenic effects. Treatment of J774 confirmed the attenuation of inflammatory response to propranolol and increased cleaved caspase-3 on choroidal explant. We found that propranolol increased pigment epithelium-derived factor (PEDF) expression in MPs. Trapping of PEDF with an antibody abrogated antiangiogenic effects of propranolol. PEDF was also detected in CNV-associated MPs.

CONCLUSIONS. We hereby show that propranolol confers on MPs antiangiogenic properties by increasing PEDF expression, which complements its effects on vascular tissue resulting in inhibition of choroidal vasoproliferation in inflammatory conditions. The study supports possible use of propranolol as a therapeutic modality for CNV.

Keywords: mononuclear phagocytes, CNV, propranolol, PEDF

choroidal neovascularization (CNV) is a serious complication of various eye diseases, including wet AMD, Stargardt’s disease, ocular histioplasmosis, degenerative myopia, and choroidal hemangioma. Pathologic neovascularization has been described to result from an imbalance in the expression of multiple proangiogenic factors, such as VEGF, FGF, platelet-derived growth factor (PDGF), and antiangiogenic factors, such as pigment epithelium-derived factor (PEDF) and thrombospondin 1 (TSP-1).1–3 The loss of retinal vascular homeostasis in favor of a proangiogenic microenvironment promotes proliferation and leakage of new vessels leading ultimately to blindness. Anti-VEGF antibodies (e.g., bevacizumab, ranizumab) are effective in interfering with angiogenesis. However, refractori-
ness and variable response to such treatment endorses alternative strategies.

Over the last decade, antiangiogenic properties of propranolol, a nonselective β-adrenoceptor blocker, have been demonstrated in capillary hemangioma. Following evidence for expression of β-adrenergic receptors in the choroid, Lashbrook et al. has uncovered the involvement of sympathetic innervation in potential choroidal remodeling; interestingly, this effect was exerted via PEDF in RPE. More recently, the antiangiogenic properties of propranolol have been demonstrated in a model of CNV. Although these studies pointed to an effect of propranolol in reducing VEGF, others have questioned this mechanism to fully explain the effects of propranolol, while proposing concomitant respective activation of prodeath and suppression of prosurvival factors in β-adrenoceptor-bearing vascular cells, suggesting that antiangiogenic mechanisms for propranolol may differ depending on the type of environment and tissue/cell incurring vasoproliferation. In this context, CNV as observed in various clinical conditions as well as in the laser-induced photocoagulation model, is associated with an inflammatory component. Of relevance, mononuclear phagocytes (MPs) attracted to pathologic sites in β-adrenergic receptors, has yet to be explored; moreover, the mechanism by which β-adrenoceptor inhibition in MPs could convey antiangiogenic properties to the choroid is also not known. We herein show, for the first time, that propranolol can modulate the angiogenic properties of MPs favoring the release of the major proapoptotic/antineovascular agent PEDF, which as a consequence decreases choroidal endothelial cells sprouting.

**Materials and Methods**

**Animals**

Adult male 6-week-old C57BL/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed and maintained at local animal facilities under a 12:12-hour light/dark cycle. All procedures in this study were approved by the Maisonneuve-Rosemont Hospital Animal Welfare Committee and are in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research published by the US National Institutes of Health.

**Mouse Laser-Induced CNV**

Mice were anesthetized using intraperitoneal administration of ketamine hydrochloride (10%)/xylazine (1%). Their pupils were dilated with tropicamide (1%). A coverslip was placed over the cornea, and Argon laser photoocoagulation (50 μm, 400 mW, 0.05 seconds) was performed to rupture Bruch’s membrane at four locations in each eye. Mice were randomly grouped to receive intraperitoneal propranolol (6 mg/kg/d) or vehicle (saline solution) daily for 10 days, starting on day 4 after laser burn and until death (day 14). The time corresponds to that of immune cell recruitment as reported and relevant herein. Mice were killed 14 days after the laser photoocoagulation, and the eyes were rapidly enucleated. The choroids were prepared for immunofluorescence, RT-PCR, and Western blot analysis.

**Cell Culture**

**Cell Lines.** The murine macrophage cell line J774 and RAW264.7, purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM;11995-065; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; 085-150; Wisent Bioproducts, Saint-Jean-Baptiste, QC, Canada), and 1% penicillin/streptomycin (450-201-EL; Wisent Bioproducts). SIM-A9 (microglia) cell line purchased from ATCC, was cultured in DMEM F12 (11330-032; Gibco, Thermo Fisher Scientific) supplemented with 5% horse serum (HS, 26050-070; Gibco, Thermo Fisher Scientific), 10% FBS, and 1% penicillin/ streptomycin.

**Primary Cells.** Primary peritoneal macrophages were isolated from 10-week-old mice. We injected 5 mL of cold PBS (with 3% FBS) into the peritoneal cavity using a 27-G needle. After 2 minutes of gently massage of the peritoneum, we inserted a 25-G needle into the peritoneal cavity to collect the fluid avoiding contamination with blood. Cells were isolated by centrifugation (475g, 8 minutes) and resuspended in DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin.

**Quantitative RT-PCR**

Choroidal tissues and J774 macrophages were collected to extract the total RNA using an RNA extraction kit (74104; Qiagen, Toronto, ON, Canada). DNase-treated RNA was then converted into complementary (c) DNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (28025021; Thermo Fisher Scientific). Quantitative analysis of gene expression was performed on an ABI Prism 7500 sequence detection system with SYBR Green Master Mix Kit (172-5124; Thermo Fisher Scientific). Gene expression levels were normalized to 18S universal primer (AM1718; Thermo Fisher Scientific), and the percentage of change was calculated according to a previously described formula. PCR primers were synthesized by Alpha DNA based on the sequences presented in the Table.

**Preparation of Conditioned Media**

J774, RAW264.7, SIM-A9 cells, and peritoneal macrophages (PM) were cultured in 24-well plates containing DMEM or DMEM F12 supplemented with 10% FBS or combination of 10% FBS, 5% HS according to the cell type and 1% penicillin/ streptomycin at 37°C in 5% CO2. The cells were incubated with propranolol (10 μM; P0884; Sigma-Aldrich, Oakville, ON, Canada) or a combination of different beta-adrenoceptor (AR)-selective antagonists (1 μM [effective dose]): Betaxolol, ICI 118.551 and L748.337, respectively, β1-, 2-, and 3-AR antagonist (0906, 0821, and 2760, respectively; Tocris Bioscience, Oakville, ON, Canada), after 24 hours of incubation the media was discarded and cells were washed twice with PBS to ensure that propranolol or β-AR-selective antagonists were completely removed. The cells were incubated with DMEM or DMEM F12 supplemented with 1% FBS. After 24 hours, the media were collected, centrifuged at 5000g for 10 minutes and filtered through 0.22-μm pore filters (85.1826.001; Sarstedt, Montreal, QC, Canada). Conditioned media (CM) from macrophages were termed macrophage CM when incubated with PBS or macrophage X CM with X identified as the compound incubated with macrophage cells (i.e., for J774 incubated with propranolol, J774 Pro CM).
Complex sclera/choroid was used to generate choroidal explants containing 10% FCS and 1% penicillin/streptomycin. The rate of one RPE cell from one eye per well in DMEM-F12 and seeded on fibronectin-coated well (83.3920; Sarstedt) at a density of 10^5 cells/ml. Pigmented RPE cells were collected by pipetting up and down. Pigmented RPE cells were collected and seeded on fibronectin-coated well (85.3920; Sarstedt) at a density of 10^5 cells/ml. Complex sclera/choroid/RPE was cultured at 37°C Ci n5 %C O 2 for a gentle digestion. Enzymatic digestion is inhibited by adding DMEM-F12 containing 10% fetal calf serum (FCS). The eye was opened under the ora serrata to remove the anterior part (lens and cornea) and the neuroretina carefully removed. The complex sclera/choroid/RPE was cultured at 37°C Ci n5 %C O 2 for a gentle digestion. Enzymatic digestion is inhibited by adding DMEM-F12 containing 10% fetal calf serum (FCS). The eye was opened under the ora serrata to remove the anterior part (lens and cornea) and the neuroretina carefully removed. RPE cells were gently detached by pipetting up and down. Pigmented RPE cells were collected and seeded on fibronectin-coated well (85.3920; Sarstedt) at a density of one RPE cells from one eye per well in DMEM-F12 containing 10% FCS and 1% penicillin/streptomycin. The complex sclera/choroid was used to generate choroidal explant devoid of RPE cells.

**Primary RPE Culture**

Primary RPE cells were isolated from an 11-day-old mouse obtained as described previously.25,26 Briefly, after dissection the eyes are maintained overnight at room temperature in DMEM-F12, and then incubated for 45 minutes with 2 mg/ml trypsin/collagenase I at 37°C for a gentle digestion. Enzymatic digestion is inhibited by adding DMEM-F12 containing 10% fetal calf serum (FCS). The eye was opened under the ora serrata to remove the anterior part (lens and cornea) and the neuroretina carefully removed. RPE cells were gently detached by pipetting up and down. Pigmented RPE cells were collected and seeded on fibronectin-coated well (85.3920; Sarstedt) at a density of one RPE cells from one eye per well in DMEM-F12 containing 10% FCS and 1% penicillin/streptomycin. The complex sclera/choroid was used to generate choroidal explant devoid of RPE cells.

**Treatment of Choroidal Explants and Measurement of Neovascularization**

Choroidal explants were obtained from 6-week-old mice. The choroidal explants were prepared according to a previously described procedure.25,26 Briefly, mice eyes were dissected in a Petri dish containing 1× Hank’s balanced salt solution (HBSS; 02-0121-0500; VWR, Mont-Royal, QC, Canada). The eye was opened under the ora serrata to remove the anterior part (lens and cornea) and the neuroretina carefully removed. The complex sclera/choroid/RPE was cultured at 37°C Ci n5 %C O 2 for 4 days in endothelial cell growth basal medium (EBM-2) supplemented with Microvascular Endothelial SingleQuots kit (EGM-2MV; respectively, CC-3156 and CC-4147; Lonza Biosciences, Basel, Switzerland). The culture medium was changed on day 5 with DMEM or DMEM F12 and explants were incubated with PBS, 10 μM propranolol, CM from J774, RAW264.7, SIM-A9, or PM. For some experiments, anti-PEDF antibody or recombinant PEDF were added to the CM. Photographs of individual explants were taken before (T0) and 24 hours after the treatment using an Axiosview 200 M inverted microscope (Zeiss, Oberkochen, Germany). The neovessel areas were determined using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry Staining**

Cryosection, choroidal flat-mounts, and choroidal explant were prepared as described previously.27 In brief, the eyes of mice were enucleated and fixed in 4% PFA. After the cornea, lens and retina were removed, the choroid-sclera complex was permeabilized in 1.0% Triton X-100 and blocked in 10% normal goat serum. Primary antibodies used were Rabbit IgG, goat and rabbit antibody against PEDF (1:300; sc-25994; Santa Cruz Biotechnology, Dallas, TX, USA), sheep PEDF receptor antibody (1:100; AF5365; R&D Systems, Minneapolis, MN, USA), rabbit β1-AR, β2-AR, and β3-AR antibodies (1:200; sc-568, sc-9042, and sc-50436, respectively; Santa Cruz Biotechnology), rabbit PEDF receptor antibody (1:100; AF5365; R&D Systems, Minneapolis, MN, USA), rabbit β1-AR, β2-AR, and β3-AR antibodies (1:200; sc-568, sc-9042, and sc-50436, respectively; Santa Cruz Biotechnology), and rabbit activated caspase-3 antibody (1:300; Apo175; New England Biolabs, Whitby, ON, Canada). The following corresponding Alexa secondary antibodies were used: Alexa-488-conjugated goat anti-mouse (1:500; A11070; Invitrogen, Thermo Fisher Scientific), Alexa-594-conjugated donkey anti-mouse (1:500; A21203; Invitrogen, Thermo Fisher Scientific), and Alexa 647-conjugated goat anti-rabbit (1:500; 41888; New England Biolabs) to reveal the primary antibodies. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:5000; D9542; Sigma-Aldrich). Labeled flat-mounts were examined with a laser scanning confocal microscope (Zeiss LSM 510).

**Western Blot**

Cells were collected and lysed with lysis buffer. Protein samples were extracted as described previously.27 Briefly the samples were centrifuged, and 50 μg of the pellet was loaded on an SDS-PAGE gel and subsequently electroblotted onto nitrocellulose membrane (162-0115; BioRad). After blocking, the membranes were probed with specific primary antibodies against PEDF (1:300; sc-25994; Santa Cruz Biotechnology), β1-AR, β2-AR, β3-AR antibodies (1:200; A11070; Invitrogen, Thermo Fisher Scientific), Alexa-594-conjugated donkey anti-mouse (1:500; A21203; Invitrogen, Thermo Fisher Scientific), and Alexa 647-conjugated goat anti-rabbit (1:500; 41888; New England Biolabs) to reveal the primary antibodies. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAP) (1:5000; D9542; Sigma-Aldrich). Labeled flat-mounts were examined with a laser scanning confocal microscope (Zeiss LSM 510).

**Antibody Array**

Mouse angiogenesis array kit (AAM-ANG-1-2; RayBiotech, Peachtree Corners, GA, USA) was used to detect proteins expression in conditioned media of J774 incubated with or without propranolol. The procedure was followed as described by the manufacturer. The intensity of the protein signal was compared with the relative positive signals using the ImageJ software.

**Dot Blot Analysis**

The procedure was followed as described by the manufacturer. Briefly, 100 μl of CM from J774, RAW264.7, or PM incubated with or without propranolol were spotted on a nitrocellulose membrane and subsequently blocked for 30 minutes with 5% BSA and incubated with anti-PEDF (1:300; sc-25994; Santa Cruz Biotechnology, Dallas, TX, USA). The procedure was followed as described by the manufacturer. The intensity of the protein signal was compared with the relative positive signals using the ImageJ software.
Propranolol Alters Macrophage Angiogenic Response

Enzyme-Linked Immunosorbent Assay
Conditioned media from J774 incubated with or without propranolol was collected and centrifuged (2588g for 10 minutes at 4°C). The levels of PEDF and TSP-1 were monitored in CM (3 times) by using commercial ELISA (MBS265201 and MBS264008; Mybiosource, San Diego, CA, USA) according to manufacturer instructions. The medium calibration curves were prepared using purified standards for assessed protein.

Quantification of Cleaved Caspase-3 on Choroidal Explant
Choroidal explant stimulated with CM from J774 incubated with or without propranolol was fixed after 24 hours of stimulation and prepared for immunostaining with anti-cleaved caspase-3 antibody. Vessels were visualized with TRITC-lectin and nuclei were counterstained with DAPI. Endothelial cells positive to cleaved caspase-3 staining were counted on the whole explant. Results were expressed as the mean of cleaved caspase-3–positive cells per explant.

Quantification of Mononuclear Phagocytes in CNV
MPs associated to CNV were evidenced by immunofluorescence with anti–Iba-1 antibody on RPE/choroid flat mounts stained with FITC-lectin to visualize the CNV. RPE were counterstained with rhodamine-phalloidin. IBA-1–positive cells were counted on flat mounts from mice receiving laser burn treatment with or without propranolol. Cell numbers were expressed as the mean number of IBA-1–positive cells per CNV spot.

Statistical Analysis
All experiments were performed in triplicate and repeated independently at least three times. Values are presented as means ± SEM. Statistical analysis was performed using non-parametric Mann-Whitney U test to compare two conditions. Data with three or more conditions were analyzed using Kruskal-Wallis test. Statistical significance was set on the basis of P value (*P < 0.05, **P < 0.01, ***P < 0.001).

Results
Propranolol Limits CNV While Increasing Infiltration of Inflammatory Cells
In a model of inflammation-associated photocoagulation-induced CNV, daily administration of propranolol decreased the extent of CNV detected 14 days after the photocoagulation (Figs. 1A–C), consistent with previous reports. The effect of propranolol was associated with reduced IL-6 and TNF expression (Fig. 1E), both of which promote cell proliferation, respectively, through the MAPK cascade and through augmentation of VEGF transcription. Interestingly, these effects of propranolol were accompanied by accumulation of β-adrenergocort–expressing Iba-1–positive cells (MP) (Fig. 1D) likely attracted to the injured choroid by increased MCP-1 (CCL2) (Fig. 1E). All three types of β-adrenergocort co-localized on MPs in CNV (Fig. 1F), while no clear colocalization of the β-AR was found in resident microglia in physiologic condition (Supplementary Fig. S1A). On the other hand, the three types of β-adrenergocort were detected in vivo in RPE/choroid complex by Western blot and immunofluorescence and ex vivo in intact murine choroid endothelium (Supplementary Figs. S1B–D), as previously documented. However, while β1- and β2-AR were confirmed by immunofluorescence analysis in RPE primary cells, β3-AR was faintly detected (Supplementary Fig. S1E). These data suggest that β3-AR expression evidenced by Western blot mainly arise from choroid.

Propranolol Restricts Inflammation and Angiogenesis Through Actions on Mononuclear Phagocytes
Given that β-adrenocortones have been localized on macrophages, we investigated the role of the β-adrenocort blocker propranolol on inflammatory cells associated to CNV; these mechanistic studies were performed on murine J774 and RAW264.7 macrophage cell line and on primary peritoneal macrophages. As seen in vivo, MPs expressed the three different isotypes of β1-, β2-, and β3-AR, as attested on immunocytochemistry and immunoblot (Figs. 2A–C). We preincubated the different MPs with or without propranolol, and collected the conditioned media to study their effects on choroidal explant vascular sprouting (Figs. 2D–F). Treatment of cell lines or primary MPs with propranolol markedly interfered with neovascularization of choroid explants. The antiangiogenic effect of CM from all three MPs tested (J774, RAW264.7, and peritoneal macrophages) after propranolol treatment demonstrated effective reduction in choroidal explant neovascularization. In view of determining if resident microglia are affected by propranolol, choroids were treated with conditioned media of microglia cell line SIM-A9; the latter hardly affected choroidal explant neovascularization, which was unaltered by propranolol (Supplementary Fig. S2A).

In contrast to effects of propranolol (which antagonizes all three β-adrenocort subtypes), antagonism of single or dual β-adrenergocort on macrophages did not affect choroidal neovascularization (Supplementary Figs. S2B–C); antagonism of all three β-adrenergocort was required to prevent choroidal neovascularization (Supplementary Fig. S2C). These findings indicate that actions of propranolol on MPs depend on antagonism of more than one or two β-adrenergocort, in contrast to other cell types.

To elucidate the antiangiogenic properties of propranolol via MPs, we measured inflammatory factors from incubated macrophages J774 cells, which were found to exert the most robust CNV reduction after 24 hours treatment with propranolol (10 µM; compared with other macrophages). Because the effects of propranolol were associated with caspase 3 activation (cleaved caspase 3) on endothelial cells (Fig. 3A), we attempted to identify potential mediators. Propranolol treatment reduced the expression of proinflammatory inducible nitric oxide synthase (iNOS) and IL-6, and marginally that of TNFα whereas it increased the expression of the anti-inflammatory IL-10 (Fig. 3B). Together in vivo (Fig. 1) and in vitro (Fig. 3B) data reveal that propranolol attenuates the inflammatory response.

Propranolol Induces Expression of the Antiangiogenic Factor PEDF in Mononuclear Phagocytes
Antiangiogenic properties of propranolol also infer possible inhibition in the release of proangiogenic factors and/or release...
of proapoptotic/antiangiogenic factor(s) from the MPs. A proangiogenic protein array failed to identify prominent propranolol-affected factors (Fig. 3C). We thus justifiably focused on expression of major antiangiogenic TSP-1 and especially PEDF as both have been found to be upregulated by propranolol. TSP-1 was negligibly expressed in J774 CM and did not change in response to propranolol treatment; whereas PEDF protein expression was found to be markedly increased in propranolol-incubated J774 macrophages on dot-blot, Western blot, ELISA assay, and immunohistochemistry (Figs. 4A–D); these changes in MPs were independent of VEGF, which was hardly affected by propranolol (Fig. 3C). Noteworthy, the modulation of PEDF expression following propranolol treatment was also observed in RAW264.7 cells and primary peritoneal macrophages (Supplementary Figs. S2D–E). As anticipated, PEDF-receptor was detected on choroidal vascular sprouting (Fig. 4E). To ascertain that PEDF is a key regulator of the antiangiogenic effect driven by propranolol-incubated J774 macrophages, we added recombinant PEDF to the J774 CM at the level measured (1–1.5 ng/mL) in conditioned media of vehicle-exposed J774 cells, and then stimulated choroidal explants. PEDF inhibited in a dose dependent–manner the endothelial cell sprouting of choroidal explants induced by J774 CM, mimicking the effect of propranolol exposure to J774 cells (referred to as J774 Pro CM) (Fig. 4F). Moreover, anti-PEDF antibody added to conditioned media of propranolol-incubated macrophages interfered fully with the antiangiogenic effects of propranolol (Fig. 4G). Hence, propranolol confers an antiangiogenic property on macrophages by inducing release of PEDF. Interestingly, stimulation of primary RPE with J774 Pro CM also induced PEDF expression but suppressed that of VEGF (Supplementary Fig. S2F), thus complementing the direct effects of propranolol on macrophages. It should however be pointed out that propranolol did not change either PEDF or VEGF expression in primary RPE (Supplementary Fig. S2F); correspondingly, choroid devoid of RPE consistently responded to conditioned media of J774 cells exposed to propranolol by antiangiogenesis (Supplementary Fig. S2G).

Propranolol Induces PEDF and PEDF-R Expression in CNV Induced by Laser

Ex vivo induction of PEDF and PEDF-R by propranolol was corroborated in vivo in CNV elicited by laser photocoagulation. PEDF-R was most expressed in choroidal endothelial cells (Fig. 5A); PEDF-R and PEDF mRNA expression were increased by propranolol (Fig. 5B) and the ratio of PEDF-R/PEDF mRNA expression (Fig. 5C) reveals that propranolol treatment increases choroidal sensitivity to PEDF. CNV was associated with apparent augmented PEDF expression co-localized with MPs (CD11b+ cells) in propranolol-exposed animals (Fig. 5D) that exhibit less CNV (Figs. 1A, 1C); coincidentally anticipated cleaved caspase-3 immunodetection was found in CNV areas (Fig. 5E), along with upregulation in caspase 3 mRNA expression (Fig. 5F) in propranolol-exposed tissues.
FIGURE 2. Propranolol modulates MPs angiogenic function. (A–C) Representative images of J774 (A), RAW264.7 (B), and mouse primary peritoneal (C) macrophages immunostained with anti-beta-adrenergic receptor (β1, 2, and 3-AR) antibodies (green), rhodamine phalloidin (red) or CD11-b (white) and DAPI (blue). Scale bar = 20 μm. (D–F) Representative images of endothelial cells sprouting from choroidal explant incubated for 24 hours with CM from J774 (D), RAW (E), or PM (F) preincubated with PBS or propranolol (10 μM), basal DMEM was used for CTL media. Quantification of vascular area was performed with ImageJ software and are presented in histogram (N = 4–6, *P < 0.05, **P < 0.01 versus CTL media).
DISCUSSION

A contribution of β-adrenoceptors in cancer progression is well-documented. More recently, antiangiogenic properties of propranolol have surged as β-AR antagonists have been established as therapy for hemangiomas. In this context, propranolol was later found to suppress CNV in murine laser-induced photocoagulation, by reducing VEGF generation. But CNV is often associated with inflammation, as is the case for photocoagulation-induced CNV. The present work demonstrates, for the first time, the modulatory effect of propranolol on the angiogenic activity of MPs in a choroidal explant model; suppression of PEDF action was sufficient to abrogate antineovascular effects of propranolol in choroidal explant. These findings uncover an additional element of complementarity related to actions of propranolol in CNV by acting not only on RPE, choroidal pericytes, and endothelium, but also on invading MPs.

In order to assess the in vitro effect of propranolol on the angiogenic property of MP, we required to establish conditions that reproduce MP-induced neovascularization. Stimulation of choroidal explants with conditioned media from different MPs revealed robust vascular sprouting from J774 CM compared with CM from RAW264.7 and primary MP—ideal to conduct pharmacologic studies. The use of the macrophage cell line J774 has been supported in the literature by studies investigating the role of MPs in mouse models of CNV. Shaw et al. showed in a mouse model that systemic depletion of MPs using clodronate blocks choroidal neovascularization. Conversely, others have demonstrated antiangiogenic effects of macrophages, as these cause regression of an abnormal vasculature of the eye. Macrophages are not all alike and subtypes have been proposed based on their inflammatory and vasoproliferative profile; β-adrenergic signaling has been shown to promote accumulation of classically and alternatively activated macrophages in a murine model of breast cancer; but the ensuing in vivo role of these myeloid cells on CNV has not yet been investigated. Data in the present study favor an intermediate effect of propranolol on CNV through suppression of (some) inflammatory mediators and induction of antivasoproliferative agent, notably PEDF.

Macrophages can produce diverse angiogenic factors, such as VEGF, PDGF, TGFβ, FGF, and PEDF. Collectively these results led us to use J774 cells as a reliable model to explore the mechanisms of propranolol-induced choroidal antiangiogenesis. Concordantly, efficacy of propranolol in reducing choroidal vascular sprouting and in inducing PEDF in J774 cells was reproduced in RAW264.7 and primary MPs.

MPs may exert opposing effects on local tissue. Sakurai et al. showed in a mouse model that systemic depletion of MPs using clodronate blocks choroidal neovascularization. Conversely, others have demonstrated antiangiogenic effects of macrophages, as these cause regression of an abnormal vasculature of the eye. Macrophages are not all alike and subtypes have been proposed based on their inflammatory and vasoproliferative profile. β-adrenergic signaling has been shown to promote accumulation of classically and alternatively activated macrophages in a murine model of breast cancer; but the ensuing in vivo role of these myeloid cells on CNV has not yet been investigated. Data in the present study favor an intermediate effect of propranolol on CNV through suppression of (some) inflammatory mediators and induction of antivasoproliferative agent, notably PEDF.
Different combinations of β-AR antagonist failed to mimic the antiangiogenic effect on choroidal explant induced by propranolol. However, the combination of the three β-AR antagonist all together induced similar effect than propranolol suggesting that all of them were involved in the antiangiogenic effect. Thus, the question about a potential synergy between β-AR isoforms deserved to be raised, especially the hetero-oligomerization of β-AR 1/β-AR 2 and β-AR 2/β-AR 355,56 could be investigated to explain the regulation of PEDF induced by propranolol in MPs.

Although an inverse relation and important decreased ratio of PEDF/VEGF has been reported to explain retinal neovascularization,13 our findings point to an effect of propranolol on PEDF/VEGF has been reported to explain retinal neovascularization,13 our findings point to an effect of propranolol on MP–RPE interaction regarding PEDF (and VEGF) release, as alluded to by others.61 Accordingly given the complexity of interactions, we cannot exclude the contribution of other factors produced by RPE and endothelial cells in response to propranolol-incubated MPs associated with CNV.

β1, β2, and β3 adrenergic receptors have been localized in the choroidal microvasculature and Müller cells59 or lipopolysaccharide for the cortex or microglia, have been documented to modulate β-AR expression.60 These results suggest that the activation state of microglia should have an important role to modulate their β-AR expression.

We substantiated the role of PEDF by trapping it in conditioned media of macrophages incubated with propranolol, resulting in complete interference with its choroidal antiangiogenic properties. Our observations additionally support a MP–RPE interaction regarding PEDF (and VEGF) release, as alluded to by others.61 Accordingly given the complexity of interactions, we cannot exclude the contribution of other factors produced by RPE and endothelial cells in response to propranolol-incubated MPs associated with CNV.

**Figure 4.** Propranolol (Pro) increases PEDF expression in J774 macrophages leading to antiangiogenic effect on choroidal explant. PEDF and TSP-1 present in J774 CM following 24-hours stimulation with propranolol was assessed using dot blot (A) and ELISA-based assay (B) (N = 3, *P < 0.05 versus CTL). (C) Representative images of J774 cells incubated with or without propranolol (10 μM) immunostained with anti-PEDF antibody (green) and DAPI (blue). Scale bar: 40 μm. (D) Western blot analysis for PEDF and beta-actin expression in J774 cells incubated with or without propranolol (10 μM). (E) Endothelial cells sprouting from choroidal explant immunostained with anti-PEDF receptor antibodies (green), lectin (red), and DAPI (blue), scale bar: 30 μm. (F) Representative images of endothelial cells sprouting from choroidal explant incubated for 24 hours with recombinant PEDF (1, 1.5, and 2 ng/mL) added to CM from J774; or incubated for 24 hours with CM from J774. Basal DMEM was used for CTL media. Quantification of vascular area was performed with ImageJ software and are presented in histogram (N = 4–5, **P < 0.01 versus J774 CM). (G) Representative images of endothelial cells sprouting from choroidal explant incubated for 24 hours with basal DMEM (CTL media), CM from J774 incubated with or without propranolol (10 μM), and with or without anti-PEDF antibody. Quantification of vascular area was performed with ImageJ software and are presented in histogram (N = 3–5, *P < 0.05, **P < 0.01 versus CTL media).
inhibition of certain factors (cytokines) and activation of others (notably PEDF) and that these were not generated upon concomitant exogenous stimulation of β-ARs, inverse agonist properties of propranolol67 along with positive allosteric modulatory properties68 appear to be supported.

To date effects of propranolol have been attributed to antagonism of β-adrenoceptors in endothelium, pericytes, and RPE. The present study highlights for the first time the contribution of MP on antiangiogenic effects of the non-selective β-AR antagonist propranolol in choroidal explant. Our in vivo observations underline the interest to study as a future perspective the action of propranolol on CNV-associated MPs. We surmise that propranolol is a potential candidate for choroidal vasoproliferation. We propose the anti-VEGF therapy.

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References


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