Crenolanib inhibits retinal fibrosis associated with resistance to anti-VEGF in neovascular age-related macular degeneration in retinal Müller cells

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Research Article

Keywords: Anti-VEGF, crenolanib, fibrosis, Müller cell, PDGF

Posted Date: March 16th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2580274/v1

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Abstract

Background

Anti-vascular endothelial growth factor (VEGF) treatment for neovascular age-related macular degeneration (NVAMD) may cause fibrosis in primary cultured mouse retinal Müller cells (PMCs), resulting in resistance to treatment. This study aimed to confirm the decrease in the expression of fibrosis-related proteins after treatment with platelet-derived growth factor receptor (PDGFR) inhibitor.

Methods

PMCs were treated with anti-VEGF, bevacizumab, and PDGF, for 24 and 72 h, and the expression of fibrosis-related proteins, inflammatory cytokines, and PDGFR was confirmed. After 72 h of co-treatment of PMCs with bevacizumab and crenolanib, a PDGFR inhibitor, fibrosis-related protein expression was confirmed.

Results

When PMCs were treated with 1 mg/mL bevacizumab for 72 h, the expression of VEGF and inflammatory cytokines decreased, and the expression of fibrosis-related proteins and PDGFR-β increased. When PMCs were treated with 100 ng/mL PDGF-B for 72 h, the expression of VEGF and inflammatory cytokines increased, and the expression of fibrosis-related proteins and PDGFR-β decreased. When PMCs were treated with 1 mg/mL bevacizumab and 5 nM crenolanib, the expression of fibrosis-related proteins decreased.

Conclusion

If PMCs are treated with anti-VEGF agents for a long time, fibrosis may occur that may lead to resistance to NVAMD treatment. When PMCs are treated with anti-VEGF and crenolanib, a PDGFR inhibitor, the expression of fibrosis-related proteins is reduced.

Background

Age-related macular degeneration (AMD) is a retinal degenerative disease that causes blindness in elderly patients in developed countries.[1] Vascular endothelial growth factor A (VEGF-A) has been identified as a key regulator of angiogenesis and vascular permeability within the VEGF family, and its role in the pathogenesis of neovascular AMD (NVAMD) is well recognized.[2] At present, the mainstay of therapy is intravitreal injection of anti-VEGF, which is successful in preventing further angiogenesis and reducing exudation.[3] As effective intravitreal anti-VEGF therapy is usually not durable, repeat injections are required.[4] It has been reported that despite anti-VEGF treatment, 25–35% of patients have evidence of
active exudation on either angiography or optical coherence tomography (OCT) after 1 year of therapy.[5, 6] The mechanism of anti-VEGF treatment resistance in NVAMD patients has not been elucidated. Anti-VEGF resistant NVAMD is defined as (A) persistent intraretinal, subretinal, or sub-retinal pigment epithelium (RPE) fluid; (B) persistent or new hemorrhage; and/or (C) progressive lesion fibrosis, assessed after the initial loading dose or after a period of sustained treatment.[7]

Progressive fibrosis also occurs at an unexpected frequency in NVAMD. Fibrotic scars developed in 24.7% of all eyes treated in the CATT study with an increased risk of scarring associated with baseline characteristics of a type 2 macular neovascularization leakage pattern, larger lesion size, increased foveal retinal thickness, subretinal fluid, and presence of subretinal hyperreflective material on OCT.[8] In NVAMD, choroidal neovascularization begins in the choroid, extends to the subretinal space, progresses further, and remodels into various mixed tissues, eventually becoming a fibrotic scar. Photoreceptors and RPE are gradually destroyed, which is accompanied by neovascular remodeling and subretinal fibrosis progression.[9] This permanent damage to the photoreceptor in patients with NVAMD results in irreversible visual impairment.[10] In patients with NVAMD, fibrotic degeneration that occurs despite anti-VEGF therapy remains a challenge for ophthalmologists, and identifying and addressing the cause is important for NVAMD treatment.

Although VEGF is expressed in astrocytes, RPE cells, ganglion cells, astrocytes, and photoreceptor cells, [11–14] but Müller cells are the primary cell type that produce VEGF in the retina.[15] This Müller cell-secreted VEGF is a major contributor to retinal angiogenesis.[16] Müller cells are radial glial cells of the retina, spanning the entire thickness of the retina and interacting with all retinal cell types.[17] Studies have shown that Müller cells are involved in the promotion of retinal angiogenesis.[18] Therefore, targeting Müller cells to inhibit retinal angiogenesis is an important therapeutic strategy.

Although VEGF is a key molecule in NVAMD, other pro-angiogenic factors can also promote angiogenesis. In particular, platelet-derived growth factor (PDGF) is responsible for pericyte recruitment, maturation, and the development of new vessels.[19, 20] Our previous findings showed that PDGF-stimulated Müller cell proliferation occurs via activation of the c-JNK and PI3K/Akt signaling pathways.[21] In addition, it was confirmed that endothelial-mesenchymal transformation was promoted by PDGF in glioblastoma and downregulation of VEGFR-2 expression occurred through the PDGF/NF-κB/Snail pathway, suggesting that PDGF plays an important role in resistance to anti-VEGF treatment.[22]

One study confirmed that crenolanib improved the recovery of liver fibrosis by inhibiting the proliferation of stellate cells.[23] In another study, crenolanib effectively inhibited the proliferation and migration of fibroblasts, thereby attenuating skin and cardiac fibrosis.[24] Because crenolanib predominantly inhibits PDGF receptor-β (PDGFR-β), we attempted to determine whether it could alleviate retinal fibrosis.

Therefore, this study aimed to confirm the reduction of cell fibrosis that induces resistance to anti-VEGF treatment in NVAMD patients when crenolanib, a PDGF receptor inhibitor, is used in the primary culture of retinal Müller cells of mice.
**Methods**

**Primary cultures of mouse Müller cells (PMCs)**

Mouse retinas were isolated from the eyes of 2–4 weeks old mice using disinfected forceps and scissors and incubated in Dulbecco’s modified Eagle medium containing 2 mM glutamine protected from light for 90 min. For the digestion solution, 5 mL Earle’s balanced salt solution (EBSS) was added to papain (Worthington, LK003150) and incubated in 37°C water baths for 10 min to dissolve papain. EBSS (500 μL) was added to a DNase I vial and mixed with 250 μL DNase I and papain solution. A mixture of papain and DNase I was used as a digestion solution. The retina pieces, as small as possible, were immersed in 15-mL tube containing the digestion solution, triturated by gentle pipetting, and incubated in 37 °C water baths for 10–20 min. The digested retina was re-suspended into single cells by gentle pipetting and centrifuged at 600 × g for 5 min. The supernatant was removed, and the cell pellet was washed with a culture medium containing 10% fetal bovine serum and 1× penicillin/streptomycin. Finally, the supernatant was removed after centrifugation, and the cells were resuspended in culture media and cultured on 35-mm culture dishes. After cultivation for 5–7 days, numerous bipolar spindle-shaped cells were observed in the culture dishes, and the medium was replaced every 3 days.

**Anti-vegf**

PMCs were treated with 0.25 and 1 mg/mL of bevacizumab (Avastin®, F. Hoffmann La-Roche AG, Switzerland) as anti-VEGF.

**Immunocytochemistry**

Muller cells were cultured on sterilized glass coverslips in 24-well plates. After treatment with anti-VEGF (Avastin) for 24 or 72 h, cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT). The cells were washed thrice with PBS, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 5% bovine serum albumin for 1 h at RT. Thereafter, the cells were incubated with 1:100 mouse anti-glutamine synthetase (GS) antibody or 1:100 mouse anti-fibronectin overnight at 4 °C. After washing thrice, the cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:500) for 1 h at RT in the dark. Then, after washing three times, the cells were stained with 5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) for 10 min at RT in the dark. The stained cells were viewed under an LSM 700 laser confocal microscope (Carl Zeiss, Göttingen, Germany). The cell images were obtained using identical exposure settings.

**Western Blot Analysis**

The cells were lysed for total protein using RIPA lysis buffer (Thermo Fisher Scientific, #89900), and the protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, #23227).
according to the manufacturer’s protocol. A total of 30 µg of protein from the lysates was separated using SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) for 2–3 h and incubated overnight at 4 °C with various primary antibodies. After primary washing with 1× TBST, the membrane was incubated with appropriate secondary antibodies specific for either rabbit or mouse IgG for 2 h and then washed with 1× TBST. For protein detection, immunoblots were developed using SuperSignal West Pico PLUS ECL reagent (ThermoFisher, #34580). Images were taken using an iBrightTM FL1500 imaging system (ThermoFisher). An antibody-specific band on the image was measured using the ImageJ software and normalized to β-tubulin levels.

Enzyme Linked Immunosorbent Assay (Elisa)

Cell culture media were centrifuged at 1000 × g for 10 min to remove particulates, such as cell debris, and supernatants were collected by Amicon Ultra-15 centrifugal Filter 10 kDa unit (Millipore, UFC9010) for concentration and were immediately measured using mouse VEGF, IL-6, TNF-α, and CCL2 ELISA Kit (Abbkine, KET7016, KET7009, KET7015, and KET7001, respectively) according to manufacturer’s instructions or stored samples at −80 °C. Briefly, the standards and samples were diluted with 1× sample buffer, and 100 µL of the diluted standard or sample was added to each well in duplicate. The plate was covered with a film and incubated for 2 h at RT. The standards and samples were removed by inverting the plate, and each well was washed thrice with 250 µL of 1× wash buffer. One hundred microliters of the diluted detection antibody were added to each well and incubated for 1 h at RT. After repeating the wash step, 100 µL of the working dilution of streptavidin–horseradish peroxidase (HRP) was added to each well and incubated for 30 min at RT in the dark. After repeating the washing process five times, 100 µL of HRP substrate (TMB) was added to each well and incubated for 15 min at RT by protecting the plate from light. After the last incubation, 50 µL of stop solution was added to each well, and the optical density of each well was determined using a microplate reader set to 450 nm.

Statistical Methods

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS) software 25.0 (IBM, Armonk, New York, USA). Comparisons between groups were performed using the Mann–Whitney U test. Statistical significance was defined as p < 0.05. Results in the graphs are expressed as the mean ± standard deviation.

Results

Primary cultures of mouse Müller cells (PMCs)

As mouse Müller cells proliferated, they became increasingly flattened, showed epithelioid morphology, and were passaged to fresh culture dishes. The cells proliferated rapidly, and adherent Müller cells
became relatively pure after 3–4 passages. We identified the cells by morphology and immunostaining with GS and DAPI at 3–4 passage, and nearly all cells were positive for GS. Consequently, we confirmed the successful culture of the mouse Müller cells (Fig. 1).

**Induction Of Fibrosis Of Pmcs After Anti-vegf Treatment**

Immunocytochemical staining was performed for fibronectin, a high-molecular-weight glycoprotein of the extracellular matrix (ECM) that has been associated with fibrosis to study changes in fibronectin after bevacizumab treatment in four passages PMCs (Fig. 2). Fibronectin was predominantly expressed in PMCs treated with 1 mg/mL bevacizumab after 24 and 72 h.

We performed western blotting to study changes in the expression of other fibrosis-related proteins [N-cadherin and α-smooth muscle actin (α-SMA)] when PMCs were treated with anti-VEGF. We confirmed that the expression of N-cadherin and α-SMA increased after 72 h of treatment with 1 mg/mL bevacizumab. In the case of α-SMA, a statistically significant increase was observed (p < 0.05) (Fig. 3).

**Reduced Expression Of Inflammatory Cytokines Of Pmcs After Anti-vegf Treatment**

We performed western blotting and ELISA to determine the changes in the expression levels of inflammatory cytokines in four passages PMCs after anti-VEGF treatment. We analyzed the expression levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphorylated NF-κB (pNF-κB) by western blotting and analyzed the expression levels of VEGF, IL-6, and CCL2 by ELISA. When PMCs were treated with 1 mg/mL bevacizumab, the expression of NF-κB was reduced after 72 h (p < 0.05). The expression of pNF-κB decreased after 72 h (p < 0.05) (Fig. 4A). The concentrations of VEGF, IL-6, and CCL-2 in PMCs were measured by ELISA, and the expression levels decreased at 72 h rather than at 24 h. Treatment with 1 mg/mL bevacizumab led to a significant decrease in the production of VEGF, IL-6, and CCL-2 protein after 24 and 72 h (p < 0.05) (Fig. 4B).

**Increased Expression Of β Subunits Of Pdgfr Of Pmcs After Anti-vegf Treatment**

We treated four passages PMCs with 0.25 and 1 mg/mL of bevacizumab to determine effect of anti-VEGF on PDGFR expression by western blotting. The expression of PDGFR-β was confirmed to be significantly increased (p < 0.05), while PDGFR-α expression was slightly increased (Fig. 5).

**Changes In Pmcs Upon Pdgf Treatment**
We performed ELISA to determine the changes in the expression levels of VEGF, IL-6, and MCP-1 in four passages PMCs after treatment with 100 ng/mL of PDGF-AA, -AB, and -BB. When PDGF-AB and -BB were used to treat PMCs, VEGF, IL-6, and MCP-1 levels were significantly higher than those with PDGF-AA and the control group. In particular, it was confirmed that treatment with PDGF-BB resulted in a greater increase than treatment with PDGF-AB. When the cells were treated with PDGF-AA, no significant difference was observed compared to that with the control group (Fig. 6).

We confirmed the expression levels of PDGFR-α and -β subunits by western blotting, when four passages PMCs were treated with 100 ng/mL PDGF-AA, -AB, and -BB. PDGF-BB decreased the expression levels of PDGFR-α and -β. In particular, PDGFR-β was significantly decreased (p < 0.05). Even when PDGF-AB was used, the expression level was slightly decreased (Fig. 7).

We confirmed the expression levels of fibrosis-related proteins (bronectin, N-cadherin, and α-SMA) using western blotting when four passages PMCs were treated with PDGF-AA, -AB, and -BB. When PDGF-BB was used, the expression levels of bronectin and N-cadherin significantly decreased compared to those in the control group after 72 h (p < 0.05). The expression level of α-SMA was slightly decreased (Fig. 8).

**Protein Signaling By Crenolanib As A Pdgfr Inhibitor In Pmcs**

We performed western blotting to study changes in the signaling pathway of proteins associated with fibrosis (N-cadherin, bronectin, vimentin, and α-SMA) at 72 h after induction with anti-VEGF and PDGFR inhibitor (5 nM of crenolanib) in four passages PMCs. We confirmed that PDGFR-β, bronectin, and vimentin protein expression was increased by bevacizumab (p < 0.05) and that the expression of PDGFR-β and vimentin was significantly decreased when crenolanib was treated with bevacizumab (p < 0.05). Although not statistically significant, we also observed a slight decrease in bronectin and α-SMA levels when bevacizumab and crenolanib were combined (Fig. 9).

**Discussion**

The results of our study can be summarized as follows: (1) when PMCs were treated with 1 mg/mL bevacizumab after 72 h, levels of VEGF and inflammatory cytokines decreased, while fibrosis-related protein expression increased. (2) Conversely, when PMCs were treated with PDGF-AB and -BB, VEGF and inflammatory cytokines were increased, and fibrosis-related protein expression decreased. (3) Finally, when PMCs were treated with bevacizumab and crenolanib, PDGFR inhibitors, the expression of fibrosis-related proteins was reduced.

First, we confirmed that PMCs secrete VEGF, IL-6, and CCL-2, and the levels of these cytokines were higher when cultured for three days than when cultured for one day (Fig. 4B). Previous studies have shown that Müller cell-derived VEGF is an essential factor for retinal inflammation.[26] In this study, we confirmed that VEGF increased with increasing incubation time and that the inflammatory cytokines IL-6 and CCL-2...
increased as VEGF increased. We confirmed that PMCs secrete VEGF independently without other retinal cells.

Second, we confirmed that the concentration of Müller cell-derived VEGF decreased when the PMCs were treated with anti-VEGF. However, the expression of fibrosis-related proteins (fibronectin, N-cadherin, and α-SMA) increased with a decrease in VEGF concentration. This was more significantly increased after 72 h when 1 mg/mL bevacizumab was administered. (Figs. 2 and 3) Retinal fibrosis occurs when the ECM is overexpressed by activated Müller cells, microglia, transformed RPE cells, fibroblast-like cells, vascular endothelial cells, and pericytes.[27, 28] Müller cells are activated in pathologic conditions and contribute to fibrosis by generating ECM, such as fibronectin, connective tissue growth factor, and α-SMA.[29, 30]

We confirmed that when PMCs were treated with anti-VEGF, the concentrations of IL-6, CCL-2, and NF-κB decreased along with a decrease in the concentration of VEGF. (Fig. 4) Anti-VEGF is known to reduce the levels of inflammatory cytokines that occur in the retina.[26, 31] In this study, when the expression concentration of IL-6, CCL-2, and NF-κB were decreased in the treated group compared to that in the control group, the expression of the fibrosis-related proteins was significantly increased. Previous studies have shown that IL-6 plays a key role in protecting neuroretina.[32–34] In addition, another study found that Müller-cell-derived pigment-epithelium-derived factor by NF-κB activation could play an important role in neuroretinal survival.[35] Therefore, 72 h after treatment with 1 mg/mL bevacizumab, VEGF expression was decreased; however due to the significant decrease in IL-6, CCL-2, and NF-κB, the protective function of the retina was reduced, and we suspected that fibrosis may have occurred.

Third, the treatment of PMCs with anti-VEGF significantly increased PDGFR-β levels. There are two types of tyrosine kinase receptors for PDGFs, PDGFR-α and PDGFR-β.[36] The PDGF-B chain can interact with both PDGFR-α and PDGFR-β, whereas the PDGF-A chain can bind only to PDGFR-α.[37] When PDGF-B was used to treat PMCs, the expression of VEGF, IL-6, and MCP-1 was significantly increased, while that of PDGFR-β and fibrosis-related proteins was significantly decreased. The PDGF family comprises dimeric proteins that play important roles in the development of neural, glial, and vascular cells.[38, 39] This growth factor family includes PDGF-A, -B, -C, and -D, which form disulfide-linked homo- or heterodimers. It has been reported that the PDGF is essential for the disease development of proliferative vitreoretinopathy.[40, 41] Generally, the expression of PDGF-BB in normal retinal tissues is maintained in a low level. Whereas, when it comes to the gliosis condition, the pathogenic retinal tissues tend to express and release huge amounts of PDGF-BB, most part of which is derived from the Müller cells.[40] PDGF-BB induces the Müller glia-derived neurogenic cluster, and PDGF signaling is partially required to initiate the Müller glia-derived regenerative response and contributes to retinal regeneration in zebrafish.[42] Therefore, in this study, the expression of PDGFR-β was increased in a situation where fibrosis increased in retinal Müller cells due to the presence of high concentrations of anti-VEGF in PMCs for a long time. This may have occurred because it became a pathogenic condition in the retinal Müller cells. When PDGF-B were treated with PMCs to create a situation in which the concentration of PDGF-B was increased, the concentrations of VEGF and inflammatory cytokines increased. In contrast, the expression
of fibrosis-related proteins was decreased. This suggests that when a pathogenic condition occurs in retinal Müller cells, PDGF-B secreted from Müller cells can reduce fibrosis but increase angiogenesis.

Finally, when crenolanib, which blocks PDGFR, was treated with anti-VEGF, the expression of fibrosis-related proteins in PMCs was reduced compared to treatment with anti-VEGF alone. These results suggest that PDGFR inhibitors reduce the progression of fibrosis in Müller cells. Studies have shown that crenolanib contributes to the recovery of liver fibrosis in hepatocytes [23] and is also effective in skin and heart fibrosis.[24] Crenolanib can inhibit the activation of receptor tyrosine kinases, such as PDGFR-α/β, FMS-related tyrosine kinase 3, and c-KIT, with the highest inhibitory effect on PDGFR-β.[23, 43] One study revealed that inhibition of both VEGF-A and PDGF-B signaling is more effective than blocking VEGF-A alone for ocular neovascularization.[44] In another study, it was confirmed that the combination of anti-VEGF and PDGFR-β inhibitors was effective in treating drug-resistant breast cancer cells. [45] Therefore, in this study, when anti-VEGF was used alone in retinal Müller cells, fibrosis was induced if the concentration and treatment time were increased. At this time, it was suggested that fibrosis could be reduced when anti-VEGF and crenolanib, a PDGFR inhibitor, were used together.

This study has some limitations. Since the study was conducted with only PMCs, confirmation of the interaction between all retinal cells was insufficient. Further studies are needed to confirm this interaction by testing other cells, such as retinal endothelial cells. In addition, it is necessary to elucidate the signaling pathway through additional studies on the mechanism by which PDGF-B reduces fibrosis when PDGFR inhibition is performed. Compared with an in vivo setting, this in vitro study has several limitations.

**Conclusions**

In summary, anti-VEGF therapy is an excellent treatment option for NVAMD. However, there are cases in which the effect is reduced owing to fibrotic changes. Retinal Müller cells occupy a large portion of the retina and are likely to play a major role in NVAMD progression. Fibrosis increased when Müller cells were treated with anti-VEGF, and this fibrotic change is thought to be related to a signaling system related to PDGF. Therefore, it was confirmed that when the PDGFR inhibitor, crenolanib, was combined with anti-VEGF, the expression of fibrosis-related proteins was reduced. Thus, the possibility of a new treatment to control fibrosis in NVAMD is confirmed.

**Abbreviations**

vascular endothelial growth factor (VEGF)

neovascular age-related macular degeneration (NVAMD)

primary cultured mouse retinal Müller cells (PMCs)

platelet-derived growth factor receptor (PDGFR)
age-related macular degeneration (AMD)
optical coherence tomography (OCT)
retinal pigment epithelium (RPE)
platelet-derived growth factor (PDGF)
Earle’s balanced salt solution (EBSS)
room temperature (RT)
glutamine synthetase (GS)
4,6-diamidino-2-phenylindole (DAPI)
Tris-buffered saline Tween-20 (TBST)
Enzyme linked immunosorbent assay (ELISA)
horseradish peroxidase (HRP)
Social Sciences software (SPSS)
extracellular matrix (ECM)
α-smooth muscle actin (α-SMA)
nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

**Declarations**

**Ethics approval and consent to participate**

All experimental procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocols were approved by Medical Science Institute, Kyung Hee University Hospital at Gangdong, Seoul. This study is reported in accordance with ARRIVE guidelines.

**Consent for publication: Not applicable**

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing interests: None**
Funding

This research was supported by the National Research Foundation of Korea (NRF), funded by the Ministry of Science and ICT (2018M3A9E8078812) and Kyung Hee University (KHU-20191225)

Authors' contributions

J.H.J. contributed to the data acquisition, analysis, and interpretation of this work; and participated in drafting and revising the contents of the study. J.C and J.W.S contributed to the data acquisition and analysis. S.W.M contributed to the data analysis, the concept of this work and approved the submission of the final version. All authors have read and approved the manuscript.

Acknowledgements

We would like to thank Editage (www.editage.co.kr) for English language editing.

Authors' information (optional)

References


**Figures**

![Image](image_url)

**Figure 1**

- **P**: passage
- **GS**: Glutamine Synthetase as muller cell marker
- **DAPI**: for nuclear stain
Culture of primary retinal Müller cells (PMCs) in mice. Mouse retinal Müller cells were cultured by four passages. The cultured cells are stained with Müller cell-specific marker GS (glutamine synthetase) and DAPI (4′,6-diamidino-2-phenylindole) for nuclear stain.

Figure 2
The four passages primary cultured Müller cells (PMCs) were stained with fibronectin, which is associated with fibrosis. The PMCs were treated with distilled water (control) or 1 mg/mL of bevacizumab for 24 h (A) and 72 h (B), and the result showed that fibronectin was increased in bevacizumab-treated cells.

Figure 3
Western blot analysis of N-cadherin and α-SMA protein expression in four passages PMCs treated with 0.25 and 1 mg/mL of bevacizumab for 24 and 72 h (n = 5). Treatment with 1 mg/mL of bevacizumab led to increased production of N-cadherin and α-SMA protein. In particular, α-SMA demonstrated a statistically significant increase (p < 0.05). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 1.

![Western blot analysis](image)

**Figure 4**

Changes in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Vascular endothelial growth factor (VEGF), and inflammatory cytokines (IL-6 and CCL2) at 24 and 72 h after treatment with bevacizumab in four passages PMCs. (A) Western blot analysis of NF-κB and phosphorylated NF-κB (pNF-κB) protein expression in PMCs treated with 0.25 and 1 mg/mL of bevacizumab for 24 and 72 h (n = 5). Treatment with 1 mg/mL of bevacizumab led to statistically significant decrease in the production of NF-κB and pNF-κB protein (p < 0.05). (B) ELISA analysis of the amounts of VEGF, IL-6, and CCL2 in PMCs treated with or without 0.25 and 1 mg/mL of bevacizumab for 24 and 72 h (n = 5). VEGF, IL-6 and CCL-2 decreased after 24 and 72 h of treatment with 1 mg/mL of bevacizumab (p < 0.05). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 2.
Western blot analysis of α and β subunits of platelet-derived growth factor receptor (PDGFR) expression in four passages PMCs treated with 0.25 and 1 mg/mL of bevacizumab for 24 and 72 h (n = 5). Treatment with 1 mg/mL of bevacizumab after 24 and 72 h led to a significantly increased production of PDGFR-β (p < 0.05). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 3.

ELISA analysis of the levels of VEGF, IL-6, and MCP-1 in four passages PMCs treated with or without 100 ng/mL of platelet-derived growth factor (PDGF)-AA, -AB, and -BB for 24 and 72 h (n = 5). Treatment with PDGF-AB and -BB after 24 and 72 h led to significantly increased production of VEGF, IL-6, and MCP-1 (p < 0.05).
Figure 7

Western blot analysis of α and β subunits of platelet-derived growth factor receptor (PDGFR) expression in four passages PMCs treated with 100 ng/mL of PDGF-AA, -AB, and -BB for 24, 48, and 120 h (n = 5). Treatment with PDGF-BB after 24, 48, and 120 h led to significantly decreased production of PDGFR-β (p < 0.05), while that with PDGF-AA after 120 h led to significantly increased production of PDGFR-α (p < 0.05). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 4.

Figure 8

Western blot analysis of fibrosis-related proteins (fibronectin, N-cadherin, and α-SMA) in four passages PMCs treated with 100 ng/mL of platelet-derived growth factor (PDGF)-AA, -AB, and -BB for 24 and 72 h (n = 5). Treatment with PDGF-BB after 72 h led to significantly decreased production of fibronectin and N-cadherin (p < 0.05). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 5.
Western blot analysis of platelet-derived growth factor receptor (PDGFR) and fibrosis-related proteins (N-cadherin, fibronectin, vimentin, and α-SMA) in four passages PMCs treated with 1 mg/mL of bevacizumab (Ava) alone and 1 mg/mL of bevacizumab and 5 nM of crenolanib (iP, inhibitor protein) together (Ava + iP) for 72 h (n = 5) (A). Treatment with bevacizumab after 72 h led to significantly increased production of PDGFR-β, fibronectin, and vimentin (p < 0.05). On the contrary, after 72 h, treatment with bevacizumab in combination with crenolanib significantly reduced PDGFR-β and vimentin compared to treatment with bevacizumab alone (p < 0.05) (B). The samples derive from the same experiment and the gels/blots were processed in parallel for improves clarity and conciseness. The original gel is shown in Supplementary Fig. 6.

Supplementary Files

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- SupplementaryFig.docx