Graefes Arch Clin Exp Ophthalmol (2009) 247:1487–1492
DOI 10.1007/s00417-009-1139-x

BASIC SCIENCE

Constitutive and oxidative-stress-induced expression of VEGF in the RPE are differently regulated by different Mitogen-activated protein kinases

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Received: 9 December 2008 / Accepted: 24 June 2009 / Published online: 15 July 2009

Abstract
Purpose Vascular endothelial growth factor (VEGF) is a fundamental factor for angiogenesis. It plays important roles in pathological conditions (e.g. the development of wet AMD), but also in the healthy organism) e.g. in maintaining the vasculature and supporting the retina). Recent therapies to treat the wet AMD focus on neutralizing VEGF indiscriminately. VEGF is constitutively expressed in the retina, but its expression is upregulated by various (noxious) stimuli, e.g. oxidative stress or hypoxia. Discrimination between constitutive expression of VEGF and its pathological upregulation might provide the possibility of focusing on inhibiting the pathological expression only. Here, we focused on the influence of different mitogen-activated protein kinase (MAPK) (p38, Erk, JNK) on the secretion and expression of VEGF, with or without being challenged by oxidative stress.
Methods VEGF secretion was measured using a perfusion organ culture model; expression was examined in primary RPE culture and Western blotting.
Results Constitutive VEGF expression and secretion can be diminished by inhibiting p38, while inhibiting Erk or JNK does not show a significant effect. When challenged with oxidative stress (250 μM t-butylhydroperoxide), VEGF expression and secretion increases and the influence of the MAPK changes: While p38 still accounts for about 30% of the secretion, Erk shows a similar influence. Inhibiting JNK presents conflicting results. In organ culture, inhibiting JNK significantly increases VEGF secretion after stimulation with 250 μM tBH, while with regard to VEGF expression in RPE cell culture, this effect could not be seen.
Conclusion Constitutive and oxidative stress induced VEGF secretion, and expression is differently regulated, which might offer an opportunity to selectively inhibit pathological VEGF expression only.

Keywords VEGF · Oxidative stress · MAPK · JNK · Erk · p38

Introduction

Age-related macula degeneration is one of the major challenges for the aging population, and the main reason for legal blindness in the developed countries. While the therapy of this disease has made enormous progress in the recent past, there is still no cure. VEGF antagonists can halt progression of AMD in a high percentage of patients, reversing vision loss in some cases, but the treatment has to be applied regularly, which is a strong financial burden to the healthcare system, and can not cure the condition [1, 2].

VEGF is the major physiological growth factor in angiogenesis in the developing organism. In the adult, it is involved in the female cycle and in wound healing. Additionally, it maintains the existing vasculature by inhibiting apoptosis of the endothelial cells [3], and has a neuroprotective function in the retina [4]. Inhibiting these physiological functions in the eye over a prolonged period of time might have yet undiscovered side-effects. While most papers see no toxic effects of either Avastin or

No financial relationships. This work was financially supported by the CAU Medical Faculty Intramural Research Grant.

The authors have full control of all primary data, and agree to allow Graefe's Archive for Clinical and Experimental Ophthalmology to review their data upon request.

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Lucentis [5, 6], recent publications see morphological changes after treatment with VEGF antagonists [7]. In order to find a treatment for AMD, a means of distinguishing between physiological and pathological VEGF expression would be desirable. The regulation of VEGF expression is complex. It depends on the stimulus as well as on the cell type, and a variety of signalling molecules are involved [8]. A family of important signal transducers involved in the regulation of VEGF expression are the mitogen-activated protein kinases (MAPK): c-Jun-activated kinase (JNK), p38 and Erk. In RPE cells, JNK and p38 have an important role in stabilizing VEGF mRNA [9]; and the insulin-like-growth factor (IGF-1)-induced VEGF expression is dependent on JNK [10]. The secretion of VEGF by insulin and TGF-ß is mediated by p38 [11, 12], while Erk is involved in the upregulation of VEGF-induced by advanced glycation endproducts and, together with p38, by TGF-ß [12, 13]. However, the involvement of the MAPK on the constitutive VEGF expression in RPE cells is not very well understood.

With the perfusion organ culture, we have a system which produces considerate amounts of VEGF without stimulation, and which is easily accessible for manipulation [14]. Here, we compare the influence of p38, Erk and JNK on the expression of VEGF with or without oxidative stress, in order to distinguish between physiological and pathological regulation of VEGF expression.

Methods

Organ culture

Organ culture was prepared as described previously [14]. In brief, for the preparation of retina–RPE–choroid sheets, freshly slaughtered pig eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, retina–RPE–choroid sheets were separated from sclera, and prepared tissue was fixed between the lower and upper part of a fixation ring. Organ sheets were cultivated in a perfusion chamber (Minucells & Minutissue, Bad Abbach, Germany). The chamber was placed on a heating plate and perfused with medium.

Treatment of the organ culture

On the second day of cultivation, unchallenged or oxidative stressed (250 µM t-butylhydroperoxide (tBH)) tissue sheets were exposed to 10 µM U0126 (inhibitor of Erk), 10 µM SB203580 (inhibitor of p38) or 20 µM SP600125 (Inhibitor of JNK) respectively. The perfusion of the tissue was interrupted, the medium was removed from the chamber with a syringe and transferred to a falcon tube, where the respective inhibitor was added to the medium. The medium was transferred back into the chamber and incubated for 20 min. The perfusion was restarted, and the supernatant was collected. Tubes collecting the medium were changed every hour.

Evaluation of VEGF content

The VEGF content was measured by a VEGF-Elisa (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The Elisa detects all isoforms of VEGF-A.

RPE isolation and cell culture

RPE cells were isolated as previously described [14]. In brief, freshly slaughtered pig eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, as well as lens, vitreous and retina. In each eye cup, trypsin was added, and incubated for 5 min at 37°. Trypsin solution was removed and substituted with trypsin-EDTA for 45 min at 37°. RPE cells were gently pipetted of the choroid, collected in media and washed. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) and Ham F12 medium (1:1) supplemented with penicillin/streptomycin (1%), L-glutamine, amphotericine B (0,5 µg/ml), HEPES (25 mM), sodium-pyruvat (110 mg/ml) and 10% porcine serum.

Treatment of cells

For all experiments, confluent RPE cells of passage one or two were used. Cells were treated with 10 µM U0126 (inhibitor of Erk), 10 µM SB203580 (inhibitor of p38) or 20 µM SP600125 (Inhibitor of JNK) respectively, 30 min prior to being challenged with 250 µM tBH.

Whole cell lysate

Whole cell lysate of RPE was generated as previously described [15]. In brief, confluent RPE cells of first or second passage after treatment were scraped off in PBS, centrifuged and the pellet resuspended in DLB buffer (Tris pH 7,4 10 mM, 1% SDS, protease inhibitor, phosphatase inhibitor). Samples were heated at 95° for 5 min, sonicated by ultrasound and centrifuged. The protein concentration of the supernatant was determined by the BioRad protein assay with BSA as standard.

Western blotting

Western blotting was performed as described elsewhere [14]. In brief, cell lysate was separated under reducing

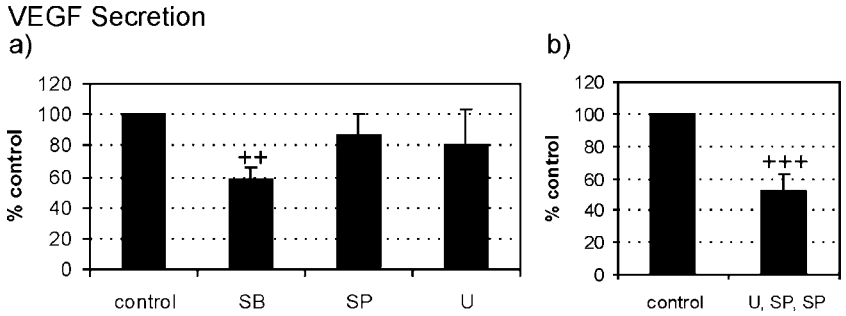

Fig. 1 Influence of different MAPK on VEGF secretion in organ culture. Of the three inhibitors applied (p38 inhibitor SB2035980 "SB", JNK inhibitor SP600125 "SP", Erk inhibitor U0126 "U"), only the inhibition of p38 displays a significant decrease in VEGF secretion (**a**). All three inhibitors together display a similar result (**b**). Data measured in Elisa, displayed as % of control. Significances were evaluated with *t*-test, ++ $p<0.01$; +++ $p<0.001$

conditions on 15% SDS polyacrylamid gel, and transferred onto PVDF membranes. The blot was blocked by 4% skim milk in Tris buffered saline with 0.1% Tween for 1 h at room temperature, and incubated overnight at 4° with a primary antibody against VEGF (R&D Systems). After washing with TTBS, blots were incubated with peroxidase-conjugated donkey-anti-goat secondary antibody for 30 min at RT. Following the final wash, the blot was incubated with Immobilon chemiluminscence reagent (Millipore) and the signal was detected with Amersham Hyperfilm. To evaluate Western blots, GeneTool software was used. Each band's density was measured and related to a non-specific protein control band which is detected by the secondary antibody alone, and whose intensity correlates only to the protein content visualized by Ponceau staining. Each band is displayed as a percentage of untreated control.

Statistics

Each experiment was independently repeated at least three times. Significances were calculated with *t*-test, calculated with Sigma Plot software. A p value less than 0.05 was considered significant.

Results

Constitutive expression and secretion of VEGF is dependent on p38, but not JNK or Erk

In perfusion organ culture, adding of SB203580 resulted in a significant decrease to 58 % of control in VEGF secretion after 6 h of incubation ($p<0.01$, SA: 8,5) (Fig. 1a)). In Western blot, using primary RPE cells, the results concerning VEGF expression displayed a reduction to 68 % of control ($p<0.01$, SA: 24.2) (Fig. 2). When inhibiting Erk with U0126 or JNK with SP600125 without prior oxidative challenge, no significant changes in VEGF secretion (organ culture, Fig. 1a)) or expression (cell culture, Fig. 2) could be obtained. Additionally, when applying all three inhibitors together, the result on VEGF secretion resembles that of SB203580 only, with a reduction to 51.8 % of control (SA: 10.5) (organ culture, Fig. 1b)).

250 µM tBH increase VEGF expression and secretion

When challenged with 250 µM tBH, a moderate but highly significant increase in VEGF secretion could be found in the perfusion organ culture after 4 h (117% of control; $p<0.001$, SA:12.5) and 6 h (121% of control; $p<0.001$, SA:12.7). Lesser concentration (125 µM) showed no significant effect on VEGF secretion (data not shown). When VEGF expression was assessed in RPE cells in Western Blot 6 h after a challenge with 250 µM tBH, an overall increase in VEGF expression can be seen, (132.4% of control, $p<0.01$, SA:32.9) (Fig. 3).

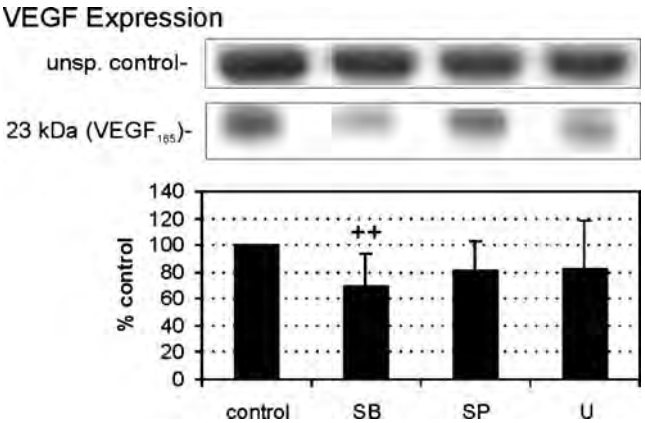

Fig. 2 Influence of different MAPK on VEGF expression in RPE cell culture. Of the three inhibitors applied (p38 inhibitor SB2035980 "SB", JNK inhibitor SP600125 "SP", Erk inhibitor U0126 "U"), only the inhibition of p38 displays a significant decrease in VEGF expression. Data displayed as % of control, densitometrically evaluated from different blots. Additionally, a representative blot is shown. Significances were evaluated with *t*-test, ++ $p<0.01$

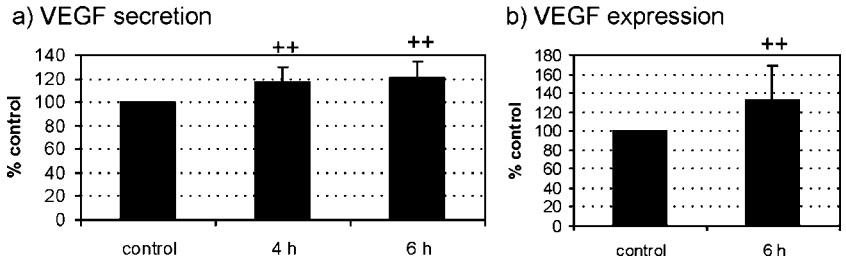

Fig. 3 Increase of VEGF secretion (organ culture, **a**) and expression (cell culture, **b**) after challenge with t-butylhydroperoxide. **a** Data assessed in Elisa, displayed as % of control. **b** Data displayed as % of control, densitometrically evaluated from different blots. Significances were evaluated with *t*-test, ++ $p<0.01$

Oxidative stress induced VEGF is dependent of p38 and Erk

Under oxidative stress, p38 still influences the VEGF secretion in organ culture. When inhibiting p38 with SB203580, the increase seen under tBH is completely diminished (to 97.7% of control, $p<0.01$, SA:17.9). The increase of VEGF expression in primary cell culture is also significantly diminished by SB203580, to 76.9% of control ($p<0.05$, SA:27.3). Contrary to the results obtained in the constitutive culture, Erk significantly influences VEGF secretion. When oxidative stress is induced, inhibition of Erk diminishes VEGF secretion under tBH (82 % of control, $p<0.05$, SA:12.4) (Fig. 4). In primary cell culture, Erk strongly diminishes the VEGF expression after oxidative challenge (57.8 % of control, $p<0.01$, SA:3.8)

Under oxidative stress, inhibition of JNK significantly elevates VEGF secretion

Interestingly, when JNK is inhibited after a challenge with oxidative stress, VEGF levels in organ culture significantly increase to 148% of control ($p>0.001$, SA:8.4), suggesting a role of JNK as a repressor of pathological VEGF expression (Fig. 4). Considering expression, the upregulation of VEGF expression in cell culture under JNK inhibition is similar to the results obtained in Elisa (143.4 % of control, SA: 16.4), yet the upregulation of VEGF expression under oxidative stress is only slightly stronger then the increase of the secretion (132.4% of control, SA:27.3), so no statistical differences between could be found.

Discussion

VEGF is induced by a variety of stimuli, most importantly hypoxia, inflammatory cytokines or oxidative stress [16]. Oxidative stress is a common denominator for various retinopathic disorders, e.g. age-related macular degeneration or proliferative diabetic retinopathy [17, 18]. In our model, choroid, RPE and retina are cultured and secrete considerate amounts of VEGF [14]. By challenging the cells with 250 μM tBH, a significant increase in VEGF expression and secretion can be detected. There is no significant difference between the increase in expression and secretion of VEGF.

It is worthy of note that the regulation of the VEGF expression and secretion differs between cells challenged with oxidative stress and unchallenged cells. In both

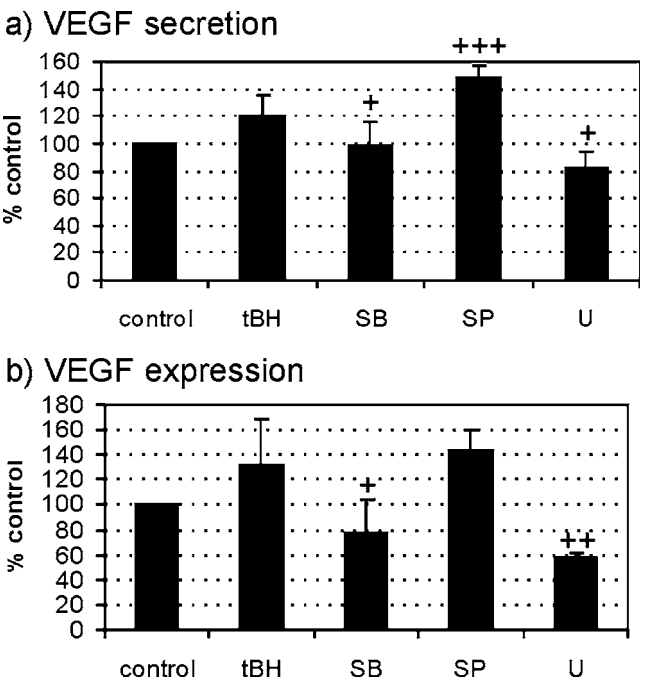

Fig. 4 Influence of different MAPK on VEGF secretion (organ culture), **a**) and expression (cell culture), **b**) after challenge with 250 μM tBH. Of the three inhibitors applied (p38 inhibitor SB2035980 "SB", JNK inhibitor SP600125 "SP", Erk inhibitor U0126 "U"), the inhibition of p38 and of Erk displays a significant effect on VEGF secretion (**a**) and expression (**b**). Inhibition of JNK significantly increases VEGF secretion (**a**) but not expression (**b**). **a** Data assessed in Elisa, displayed as % of control. **b** Data displayed as % of control, densitometrically evaluated from different blots. Significances were evaluated with *t*-test; + $p<0.05$; ++ $p<0.01$; +++ $p<0.001$

challenged and unchallenged cells, p38 plays an important role in VEGF expression. The importance of p38 in VEGF expression in RPE cells has been shown for thrombin and TGF-ß induced VEGF expression [19, 20], but not for regulation of VEGF in unchallenged or oxidatively stressed cells. These data indicate that p38 is an important factor both for the physiological VEGF expression in RPE cells and for the (pathologically) induced. Other cell types (endothelial cells, chondrocytes) also exhibit a p38-dependent VEGF induction after hypoxia or IL-1 challenge [21, 22], so this might be a universal feature of p38.

The inhibition of JNK induces an increased secretion of VEGF after oxidative stress, but not in unchallenged cells. This is an interesting finding, as JNK is supposed to have a stabilizing effect on VEGF mRNA, thus prolonging its survival time in the cell [9]. On the other hand, a transient activation of JNK has been shown to be important for JunD expression [23], the lack of JunD induces an increased secretion of VEGF in mice [24] and the overexpression of JunD downregulates VEGF [25]. It is plausible that JNK, which is activated by oxidative stress, induces the expression of JunD, which in turn suppresses VEGF secretion, especially as JunD is part of the antioxidant defense system [25]. The effect gets lost when VEGF expression in RPE cells is assessed. While the increase of VEGF expression under combined JNK inhibition and oxidative stress is identical to what is seen in secretion, the overall increase of VEGF in RPE cells is slightly stronger and exhibits a higher standard deviation, so the significance of this effect is lost. The influence of JNK on VEGF expression is cell type and stimulus dependent, as e.g. in chondrocytes; JNK-inhibition has an inhibitory effect after IL-1 induced, but not hypoxia-induced VEGF elevation [21].

The MAPK Erk, on the other hand, is only involved in the oxidative stress-induced VEGF secretion, and this effect is also seen in expression. Erk has been shown to be an important factor in VEGF expression in fibroblasts [26], especially under hypoxia [27]. In RPE cells, TGF-ß and thrombin induce VEGF via Erk activation [12, 13]. The underlying pathways of the Erk mediated VEGF elevation are not known to date. An interesting candidate might be hypoxia-inducible factor 1, as Erk has been shown to be involved in HIF-1 upregulation [28, 29]. This transcription factor is not only induced by hypoxia [30], but also by oxidative stress [31], TGF-ß [32] and thrombin [33].

As Erk seems to be only involved in induced, but not in physiological VEGF expression, this pathway might be of some interest for pharmacological intervention. In fact, the Ras/Raf/MEK/Erk pathway also has a profound role in cancer biology, and small molecule inhibitors of Erk have been developed and are assessed in clinical trials [34]; additionally, the use of cancer agents in fighting age-related macular degeneration is precedented [35]. It has to be considered, however, that Erk is an ubiquitose kinase that has many functions [36], so that an inhibition of Erk creates a high risk of unwanted side-effects. The development of the Erk inhibitor compound P0325901, for example, has been stopped due to toxicity [34]. Still, the inhibition of pathological pathways provides a great chance for developing new agents against AMD, which might be applied through a less invasive method than repeated intravitreal injections. Also, the eye is a defined area, and localized application might strongly reduce side-effects seen in systemically used agents.

In conclusion, to discriminate between physiological and pathological VEGF secretion might help to find a cure to VEGF-induced diseases. For the first time, we were able to show differences in MAPK signalling in physiological and pathological VEGF secretion.

Acknowledgements We would like to thank K. Lange for her excellent technical assistance. This work was financially supported by the CAU Medical Faculty Intramural Research Grant.

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