



The Effect of Intravitreal Bevacizumab on Apoptosis of Rat Retina Cells

Intravitreal Bevacizumabın Sıçan Retina Hücrelerinde Apoptozis Üzerine Etkileri

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Summary

Purpose: To investigate the apoptotic effects of intravitreal bevacizumab on rat retinal cells.

Material and Method: Thirty-six male adult Swiss albino rats were randomly divided into two groups. The first group was applied 0.25 mg bevacizumab in 0.01 ml saline, and the second group received the same amount of saline intravitreally. Each group was divided into 3 subgroups. The animals were sacrificed and their globes were enucleated at the 3rd, 24th, and 72nd hours of the experiment. Enucleated eyes were preserved for histological analysis, immunohistochemical analysis of caspase-3, caspase-8, caspase-9, Fas/Fas L, VEGF and VEGF receptors (Flt-1, Flk-1), and TUNEL staining.

Results: Histological evaluation showed no sign of retinal toxicity in both groups. In TUNEL staining, TUNEL(+) cells were detected in all subgroups, but the number of positive cells was relatively lower in bevacizumab treatment group that reached statistically significant level at 24 and 72 hours of treatment ($p < 0.001$). Immunohistochemical analysis revealed that in saline-treated subgroups, immunoreactivity was more pronounced for all apoptosis-inducing proteins compared to bevacizumab-treated group. Also immunoreactivity of VEGF was prominent in saline treated group. For VEGF receptors, staining was only positive for Flt-1 at the 3rd hour in the control group.

Discussion: Bevacizumab did not have apoptosis-inducing potential compared to saline solution in short term, which was documented with TUNEL and immunohistochemical staining. (*Turk J Ophthalmol* 2013; 43: 39-44)

Key Words: Apoptosis, bevacizumab, caspase, VEGF

Özet

Amaç: Sıçan retina hücrelerinde intravitreal bevacizumabın apoptotik etkilerinin incelenmesi.

Gereç ve Yöntem: Otuz altı erişkin erkek İsviçre-Albino sıçan randomize olarak iki gruba bölündü. İlk gruba 0,01ml salin içinde 0,25mg bevacizumab, ikinci gruba aynı miktar salin intravitreal olarak uygulandı. Her grup üç alt gruba ayrıldı. Hayvanlar deneyin 3, 24 ve 72inci saatlerinde feda edildi ve göz küreleri enükle edildi. Enükle gözler histolojik analiz, kaspaz-3, kaspaz-8, kaspaz-9, Fas/Fas L, VEGF ve VEGF reseptörleri (Flt-1, Flk-1) ve TUNEL boyama için hazırlandı.

Sonuçlar: Histolojik değerlendirme her iki grupta da retinal toksisite işaretleri göstermedi. TUNEL boyamada, TUNEL (+) hücreler tüm alt gruplarda izlendi, ancak pozitif hücre sayısı bevacizumab tedavi grubunda tedavinin 24 ve 72 inci saatlerinde istatistiksel anlamlı düzeye erişecek derecede göreceli olarak daha düşüktü ($p < 0,001$). İmmünohistokimyasal analizler sonucunda salin ile tedavi gören alt gruplarda apoptoz indükleyen proteinlerin immünreaktivitesinin bevacizumab uygulanan gruba göre daha belirgin olduğu ortaya çıktı. VEGF immünreaktivitesi de salin uygulanan grupta belirlendi. VEGF reseptörleri için boyanma sadece Flt-1 için kontrol grubunda üçüncü saatte pozitif.

Tartışma: Bevacizumab kontrol grubu ile karşılaştırıldığında TUNEL ve immünohistokimyasal boyamalarda kısa dönemde apoptotik indüksiyon potansiyeline sahip değildir. (*Turk J Ophthalmol* 2013; 43: 39-44)

Anahtar Kelimeler: Apoptozis, bevacizumab, kaspaz, VEGF

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Introduction

Angiogenesis is the formation of new blood vessels associated with sprouting or splitting from existing vessels.¹ This abnormal vascular proliferation is the major pathologic process in numerous ophthalmologic disorders including age-related macular degeneration (ARMD) and diabetic retinopathy. Vascular endothelial growth factor (VEGF) has been implicated as the key angiogenic stimulus responsible for the pathologic neovascularization in these disorders.^{1,2}

Bevacizumab, an antibody that binds human VEGF with high affinity, is approved for treating colorectal cancer patients. It is a humanized monoclonal antibody that binds all isoforms of VEGF and interferes with its binding to receptors, thus inhibiting its signal. VEGF binds two highly related receptor tyrosine kinases, Flt-1 and Flk-1. Flt-1 expression is up-regulated by hypoxia. Flk-1 binds VEGF, albeit with lower affinity relative to Flt-1. The key role of this receptor in developmental angiogenesis and hematopoiesis is evidenced by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice, resulting in death in utero. There is a general consensus that Flk-1 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.³ Previously several studies have reported beneficial therapeutic effects of intravitreal injection of bevacizumab at various clinical conditions.⁴⁻¹⁰

In laboratory studies, human cell line and animal cell line studies showed very high tolerability of retina for bevacizumab even at very high concentrations.¹¹ Similarly, despite its off-label clinical use, to date, no retinal toxicity has been reported after intravitreal injection of bevacizumab, but limited safety data are available.⁴⁻¹⁰

In current concepts, neural apoptosis is generally believed to be mediated by two distinct pathways: caspase-dependent and caspase-independent pathways.¹² Caspases are cysteine proteases that mediate apoptotic cell death, including neurons. Caspases are activated through extrinsic or intrinsic pathways.¹³

This study is planned to investigate the apoptosis-inducing potential and retinal toxicity of bevacizumab through the apoptotic cell counts with TUNEL staining and immunohistochemical analysis of caspase-3, -8, -9, Fas/FasL and VEGF receptors (Flt-1 and Flk-1) after intravitreal application of bevacizumab in an experimental study model.

Material and Methods

Full-length humanized VEGF antibody which is commercially available (Bevacizumab, Avastin; Genentech Inc., San Francisco, CA) was used in the study. All experiments were conducted in accordance with the guidelines set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Thirty-six male adult Swiss albino rats weighting 150-200 g were used in the study. The study group

was randomly divided into two groups of eighteen rats. In Group I, 0.25 mg bevacizumab diluted in 0.01 ml saline was injected intravitreally with a 30-gauge needle which was placed 1 mm posterior to superotemporal limbus. In Group 2, similar amount of saline was injected. Each group was randomly divided into 3 subgroups of 6 rats in each. The animals of each subgroup were sacrificed and their globes were enucleated at the 3rd, 24th, and 72nd hours of the experiment.

Histological evaluation

The enucleated eyes were fixed in 10% formalin solution for 24-48h and the samples were then put in 100% ethanol for 20 hours. They were incubated 5 hours in chloroform and then 5 hours in paraffin. After processing, tissue specimens were embedded in paraffin wax and then cut into 5µm-thick sections. The 5 µ sections from paraffin blocks were stained with hematoxylin and eosin (H&E) for general histological and morphologic evaluation. Immunohistochemical labelling of VEGF, Flt-1, Flk-1, caspase-3, caspase-9, caspase-8 and Fas/FasL and TUNEL staining for detection of apoptotic cells were performed.

Immunohistochemistry

Sections were incubated at 60°C overnight and then dewaxed in xylene for 30 min. After soaking in a decreasing series of ethanol (100%, 95%, 80%, 70%, and 60%) for 2 min for each concentration, followed by a wash in tap water for 5 min, sections were washed with distilled water. They were then treated with 2% trypsin (ab970, Apcam, Cambridge, UK) in 50mM Tris buffer (pH 7.5) at 37°C for 15 min, and washed with phosphate-buffered saline (PBS). Sections were delineated with an Elite Pap pen (DBS, Pleasanton, CA, USA) and incubated in 3% H₂O₂ solution for 15 min to inhibit endogenous peroxidase activity. They were washed 3 times for 5 min each with PBS and incubated with primary antibodies, anti-VEGF (SC-7269, Santa Cruz, CA) anti-Flt1 (RB-1527, Neomarkers, Fremont, CA) and anti-Flk1 (RB-1526, Neomarkers, Fremont, CA), anti-caspase-3 (600-1235, Novus Biologicals, Littleton, CO, USA), anti-caspase 8 (RB-1200, Neomarkers, Fremont, CA), anti-caspase 9 (RB-1205, Neomarkers, Fremont, CA), and anti-Fas/FasL (600-1236, Novus Biologicals Littleton, CO, USA) all for 18h. Next, the sections were incubated with biotinylated IgG (both anti-mouse and anti-rabbit supplied ready to use by Zymed, San Francisco, CA, USA) for 30 min, followed by three washes in PBS and then with streptavidin-peroxidase conjugate (supplied ready to use by Zymed) for 30 min (Histostain-Plus Bulk Kits; Zymed) and washed with PBS three times. They were then incubated in a solution containing 1:9 concentration of diaminobenzidine (DAB, Zymed) with hydrogen peroxide (Histostain-Plus Bulk Kits; Zymed, San Francisco, CA) for 5 min to visualize immunolabeling, and after rinsing with distilled water counterstained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). The sections were dehydrated with 80% and 95% alcohol and immersed in xylene and covered with mounting media (01730 Surgipath,

Bretton, Peterborough, Cambs., UK). The negative controls received the same treatment as described above, but were incubated with rabbit or mouse IgG instead of the primary antibodies. The negative controls were performed for each of the antibodies and the staining in all cases was negative.

TUNEL Staining

Deparaffinised and rehydrated sections, prepared as described above, were stained using a commercial kit (Cat.No: 1 684 817, Roche, Mannheim, Germany) according to the manufacturer's instructions. They were incubated 30 min with 20 µg/mL proteinase K and rinsed again 3 times for 5 min with PBS. They were then incubated in TdT-enzyme solution at 37°C for 1h. Subsequently, the sections were rinsed three times with PBS at room temperature for 15 min each. The sections were then incubated converter-POD solution for 30 min. They were washed with PBS and dehydrated with graded ethanol and were covered with mounting medium (Scytec Laboratories, Logan, UT, USA). As a negative staining control, TdT was omitted during the tailing reactions.

Evaluation of Sections

The slides were evaluated using a light microscope (Olympus BX40, Tokyo, Japan). The average number of apoptotic cells was determined by counting TUNEL positive cells in five neighboring medium-power fields where the maximum number of stained cells were observed and dividing the total by five. Cells in areas with necrosis, poor morphology or in the margins of sections were not included. Immunolabeling intensity was graded independently by two observers blinded to the experimental conditions with the following scale: mild (+), moderate (++) and strong (+++). The software SPSS 10.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical evaluation. All values in the text are expressed as mean±SEM. Differences among groups were statistically analyzed with non-parametric Kruskal-Wallis test. The results were analyzed by one-way analysis of variance. A p-value of <0.05 was considered as significant.

Results

In histological sections of eyes which received bevacizumab or saline, no sign of retinal toxicity was seen with H&E staining. Both in bevacizumab-treated retinas and in controls, TUNEL positive cells were detected at 3, 24 and 72 hours (Table 1). However, the number of TUNEL positive cells was relatively lower in bevacizumab-treatment group particularly at 24 and 72 hours of treatment ($p<0.001$). The positive cells were mainly observed at ganglion cell layer and inner nuclear layer of retina in both groups (Figure 1).

At the 3rd hour of treatment, mild immunoreactivity of caspase-3, caspase-9 and moderate immunoreactivity of caspase-8 were detected, meanwhile the immunoreactivity of Fas/Fas L was negative (Table 1). In the control group, positive immunoreactivity was detected for all four proteins, but immunoreactivities of caspase-8 and caspase-9 were relatively higher compared to bevacizumab-treatment group.

As shown in Table 1 at the 24th hour in treatment and control groups, the positive immunoreactivity of all four antibodies was detected. In addition, the intensity of caspase-3 immunoreactivity was more in the treated group when compared with the control group. Caspase-9 and Fas/FasL immunoreactivities were weak or negative, while the immunoreactivities of caspase-8 and caspase-3 were positive (Figure 2), at the 72nd hour in the control group, (Table 1). In the treatment group, the intensity of caspase-9 was more pronounced when compared with the other immunoreactivities and control group (Table 1).

Immunohistochemical analysis showed that VEGF staining was positive in both groups, at the 3rd hour of treatment (Table 1). In the control group, it was particularly positive at endothelial cells of newly formed vessels. Staining was only positive for Flt-1 in the control group for VEGF receptors (Table 1). Cytoplasmic VEGF staining was positive in both groups but more pronounced in the control group at

Table 1. Comparison of TUNEL and immunohistochemical results of groups

	Group I (Becavizumab)			Group II (Control)		
	3 hour	24 hour	72 hour	3 hour	24 hour	72 hour
TUNEL +	15.2±0.97	36.2±2.98*¥	29.2±1.02*¥	22.2±1.50	51±2.00	60.2±1.16
Caspase-3	±	++	+	+	++	++
Caspase-8	++	++	+	+++	++	++
Caspase-9	+	+	++	++	++	±
Fas/FasL	-	+	±	+	+	±
VEGF	±	+	+	+	++	+++
Flt-1	-	-	-	+	-	-
Flk-1	-	-	-	-	-	-

* Within bevacizumab treatment group, the number of TUNEL positive cells were statistically significantly higher at 24 and 72 hours compared to 3rd hour ($p<0.05$)

¥ At 24 and 72 hours, bevacizumab treated groups revealed statistically significantly lower TUNEL positive cells compared to controls ($p<0.05$)

24 and 72 hours of treatment (Figure 2). VEGF receptor staining was negative at 24 and 72 hour of treatment in both groups (Table 1).

Discussion

ARMD is one of the most common causes of visual loss in the Western world.^{14,15} As mentioned previously, VEGF is the primary angiogenic stimulus responsible for pathologic neovascularization in ARMD.^{16,17} Recent reports have demonstrated the efficacy of systemic bevacizumab for neovascularization in ARMD and in pathologic myopia.^{18,19}

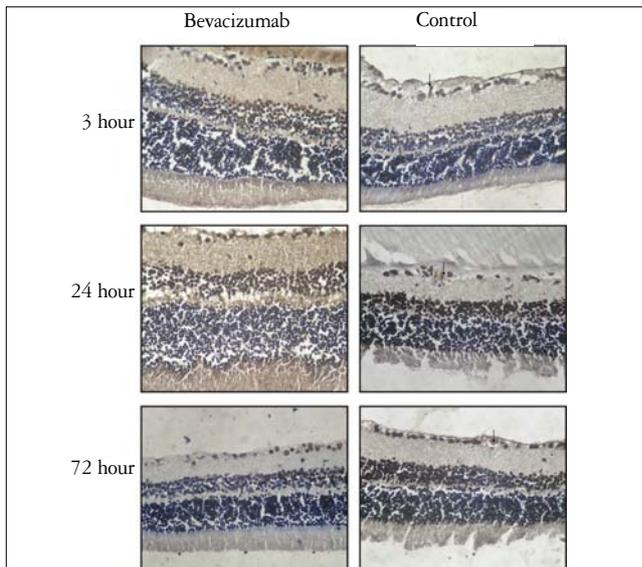


Figure 1. TUNEL staining of retina at 3, 24 and 72h in bevacizumab-treated and control groups

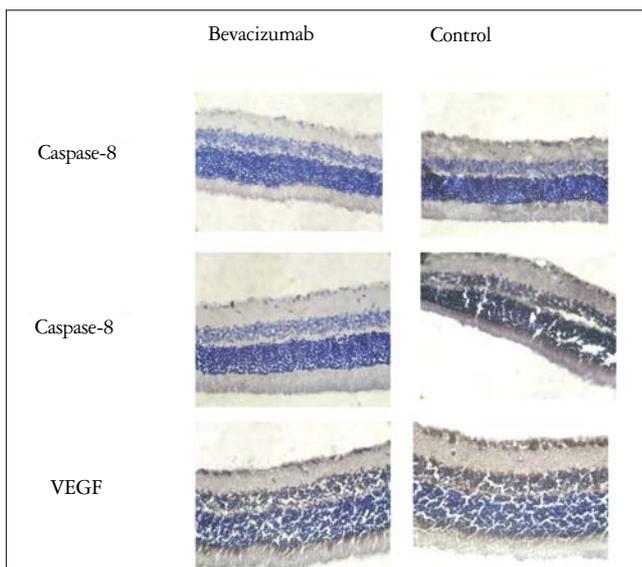


Figure 2. Immunohistochemical staining of caspase-3, caspase-8 and VEGF at 72 hour in bevacizumab-treated and control groups

Spitzer et al.²⁰ compared the efficacy of three commercially available anti-VEGF products on choroidal endothelial cell proliferation, and according to the authors, none of the drugs was superior in respect to endothelial cell growth inhibition. Similarly, a recent study showed that at clinical doses, bevacizumab and ranibizumab are equally potent in neutralizing VEGF.²¹

VEGF is a critical regulator of vasculogenesis and angiogenesis, as well as a potent inducer of vascular permeability.^{22,23} Hence, inhibition of VEGF in the retina may be a double-edged sword: inhibition of VEGF will probably reduce the edema, inflammation, haemorrhage, and neovascularization; however, the same process could also reduce the innate neuroprotective capabilities that directly impact neural cell survival. It has been showed that VEGF has neuroprotective potential against the damage induced by ischemic injury.²⁴

Apoptosis is the programmed cell death with release of certain mitochondrial and cell membrane proteins via extrinsic or intrinsic pathways. Numerous cell damaging agents including cytotoxic drugs, DNA-damaging agents, heat-shock, and hypoxia induce permeabilization of mitochondrial membranes and release of these proteins. The exact mechanism of apoptosis induction is still unknown but would be predicted to involve activation of the mitochondrial pathway and subsequent activation of downstream caspases. Caspase-8 lies at the apex of an apoptotic cascade and initiates mitochondrial activation of downstream caspase family members. Cytochrome-c is the first apoptogenic protein released from mitochondria during apoptosis. Following release from mitochondria, cytochrome-c, together with the apoptosis protease-activator factor 1, dATP and procaspase-9, forms a high molecular-weight caspase-activating complex, termed the "apoptosome". Once assembled, the apoptosome processes and activates procaspase-9 as the initiator caspase, which in turn proteolytically activates the executioner procaspase-3 and procaspase-7. A proteolytic cascade is then initiated in which caspase-3 activates other procaspases, resulting in a feedback amplification of the apoptotic signal. On the other hand, Fas/FasL protein plays a role through extrinsic pathway which activates different signal proteins.²⁵

In addition to angiogenesis, VEGF is also a survival factor for endothelial cells, both in vitro and in vivo.³ Also, it has been shown that VEGF induces expression of the antiapoptotic proteins in endothelial cells.²⁶ In vivo, the prosurvival effects of VEGF are developmentally regulated.³ Gerber et al.²⁷ showed that VEGF inhibition results in extensive apoptotic changes in the vasculature of neonatal, but not adult, mice. Furthermore, a marked VEGF dependence has been demonstrated in endothelial cells of newly formed vessels.²⁸ Safety profile of bevacizumab has been previously documented by different methods including electrophysiologic tests, light microscopy, electron microscopy and immunohisto-

chemical analysis.²⁹⁻³² Shabar et al.³⁰ reported nontoxicity with 2.5 mg intravitreal bevacizumab in both the short and long term. Also, the safety of intravitreal bevacizumab injection has been also shown by an electrophysiological study in humans.³¹ A recent study showed that bevacizumab at concentrations of 0.125 mg/mL, 0.25 mg/mL, 0.50 mg/mL, and 1 mg/mL (at or above the dose normally used in clinical practice) is safe, in short term for human RPE cells, rat neurosensory retinal cells, and human microvascular endothelial cells in vitro.¹⁵ Bevacizumab has half life of 17–21 days in humans³³, and Bakri et al.³⁴ did not detect any histological finding in favor of retinal or optic nerve toxicity or inflammation in any of the rabbit eyes even 4 week after injection.

We observed that apoptotic process was induced in both bevacizumab- and saline-treated groups in our study. However, in the bevacizumab-treated group, induction of apoptosis was slightly delayed compared to the control group which was demonstrated by both TUNEL and immunohistochemical stainings. In the bevacizumab group, less immunoreactivity of caspase-3 and caspase-8 compared to the control group supported that the apoptotic pathway may not be aggravated with treatment. So that, we did not observe any increase in apoptosis in retinal tissues after anti-VEGF treatment compared to control group. As shown in Figure 1, TUNEL positive cell nuclei were stained brown and dispersed over the retina in both groups. In contrast to our findings, Inan et al. detected intensive expression of caspase-3, caspase-9, and bax cytoplasmic protein in the photoreceptor cell layer after bevacizumab injection at both 1.25- and 3.00-mg doses in rabbit eyes in long term.²⁹ This study was performed in rabbit eyes, and long-term effects were evaluated. However, others did not detect any sign of apoptosis in cell cultures of retina-RPE-choroid complex and neural retinas alone even at very high concentrations.³⁵

As shown in Figure 2 and Table 1, treatment with bevacizumab did not suppress VEGF expression totally. VEGF immunoreactivity was positive but relatively weak in the bevacizumab group compared to the control group for all periods. On the other hand, positivity of Flt-1 at the 3rd hour of treatment in the control group, which was not seen in following investigations and in the bevacizumab-treatment group, arose suspicion that bevacizumab treatment could suppress the expression of VEGF receptors at early period.

Bevacizumab has been reported to cause a dose-dependent suppression of DNA synthesis in choroidal endothelial cells as a result of moderate antiproliferative activity.³⁶ It has been suggested that bevacizumab at concentrations higher than 0.8 mg/mL may be harmful to the retinal pigment epithelium and should not be used in concentrations higher than 0.25 to 0.3 mg/mL (equaling a total dose of bevacizumab of 1.25 mg in 4 mL vitreous volume of humans).³⁶ In a dose escalation study by Avci et al.,³⁷ intravitreal injection of bevacizumab and

pegaptanib sodium caused a significant increase in apoptotic activity in rabbit photoreceptor cells, which was evident especially in higher doses of bevacizumab. In our study, only 0.25mg of bevacizumab was used in rat eyes and apoptotic activity was not detected with the described methods. We did not observe any toxicity to RPE at 0.25 mg injection dosage in rat eyes with light microscopic investigation.

In summary, our results showed that intravitreal bevacizumab treatment in rat eyes had a relatively lower apoptosis-inducing potential at 0.25 mg dosage compared to intravitreal saline. Decreased expression of apoptotic proteins and VEGF was detected by immunohistochemical assessment. We believe that further studies will put forward apoptotic potential of different anti-VEGF treatment alternatives in long term on receptor levels and apoptotic proteins and combine this critical knowledge with clinical success in ARMD cases.

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