(33)

The effect of intravitreal ranibizumab on apoptosis induction in rat retinal tissue

Wpływ doszklistkowego podania ranibizumabu na indukcję apoptozy w komórkach siatkówki oka dorosłych szczurów

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Abstract:

Purpose: Despite the rapidly accumulating knowledge on pharmacokinetic properties and dosage of ranibizumab, the influence of this vascular endothelial growth factor inhibitor on retinal cell survival/apoptosis homeostasis remains unclear. The aim of this study was to investigate the biological effects of a single intravitreal injection of ranibizumab on retinal tissue with a focus on apoptosis-related signaling pathways in the rat retina.

Material and methods: Male Wistar rats were treated with an intravitreal injection of ranibizumab or anti-rat vascular endothelial growth factor antibody in the right eye. The left eyes were injected with the same volume of physiological saline. On the 3rd and 7th day post-injection, the eyes were enucleated, and the retinas were isolated for further molecular analysis of the expression of selected apoptosis-related molecules at mRNA (*BAX, BCL-2*) and protein (caspase-3) levels using quantitative RT-PCR and western blot techniques, respectively.

Results: Following a 3-day-exposure to ranibizumab at the established concentration, the *BAX/BCL-2* mRNA expression ratio was significantly increased compared to the saline-treated controls and the healthy control eyes. Furthermore, on day 3. post ranibizumab injection, caspase-3 cleavage, detected qualitatively using western blotting, confirmed potential activation of the irreversible phase of apoptosis. In contrast, on day 7. post-injection, there were no significant differences in the *BAX/BCL-2* mRNA expression ratios or caspase-3 cleavage between different groups.

Conclusions: Intravitreal administration of ranibizumab leads to a transient induction of apoptosis in retinal cells, with an onset directly after the vascular endothelial growth factor inhibitor administration and apparent down-regulation shortly afterwards. These results must be considered when intravitreal injections of ranibizumab are administered to treat retinal diseases.

Key words: apoptosis, ranibizumab, retinal degeneration, vascular endothelial growth factor.

Abstrakt:

Cel pracy: pomimo coraz większej wiedzy na temat farmakokinetyki oraz dawkowania ranibizumabu wciąż niezbadany pozostaje jego wpływ na indukcję procesu apoptozy w komórach siatkówki oka. Celem niniejszego badania była ocena wpływu przeciwciała na aktywację sygnałowych szlaków związanych z procesem apoptozy, wywoływanych przez pojedyncze doszklistkowe podanie ranibizumabu, w modelu szczurzym.

Materiał i metody: szczurom rasy Wistar do oka prawego podano doszklistkowo ranibizumab lub przeciwciało skierowane przeciwko szczurzej formie czynnika wzrostu śródbłonka naczyniowego. Do oka lewego podano taką samą objętość roztworu soli fizjologicznej. W trzecim oraz siódmym dniu od podania pobrano siatkówki i oceniono ekspresję wybranych czynników związanych z procesem apoptozy.

Wyniki: współczynnik ekspresji BAX/BCL-2 oceniany na poziomie mRNA w komórkach siatkówki w 3. dobie od podania ranibizumabu był istotnie podwyższony w porównaniu z tym samym współczynnikiem w komórkach siatkówki pozyskanych z oka, do którego podano roztwór soli fizjologicznej, oraz w komórkach siatkówki pozyskanych z oczu szczurów z grupy kontrolnej, do których nie podawano iniekcji. Ponadto w 3. dobie od iniekcji ranibizumabu w analizie na poziomie białka potwierdzono obecność aktywnej formy kaspazy-3, to może odpowiadać indukcji potencjalnie nieodwracalnej fazy wykonawczej apoptozy. W 7. dniu od iniekcji nie stwierdzono różnic w wartościach współczynnika ekspresji BAX/BCL-2 ani w poziomie aktywacji kaspazy-3 u badanych szczurów z poszczególnych grup.

Wnioski: doszklistkowe podanie ranibizumabu prowadzi do istotnej krótkotrwałej indukcji odwracalnej fazy inicjacji apoptozy w komórkach siatkówki oka oraz może aktywować kaskadę kaspaz związaną z indukcją nieodwracalnej fazy wykonawczej apoptozy. Wyniki naszego badania przedstawione w niniejszej pracy powinny być brane pod uwagę podczas planowania leczenia ranibizumabem u pacjentów z chorobami siatkówki oka.

Słowa kluczowe: apoptoza, ranibizumab, degeneracja siatkówki, czynnik wzrostu śródbłonka naczyniowego.

Introduction

Ranibizumab is a recombinant, humanized antigen-binding fragment (Fab) of the monoclonal IgG1 antibody for ophthalmic use; it is specifically designed to bind and inhibit all active forms of human vascular endothelial growth factor (VEGF). Ranibizumab binds with high affinity to the VEGF-receptor binding

site on multiple isoforms of VEGF, such as $VEGF_{110}$, $VEGF_{121}$, and $VEGF_{165}$, thereby preventing the binding of VEGF to its receptors VEGFR-1 and VEGFR-2. All three VEGF isoforms have been demonstrated to stimulate human endothelial cell proliferation, whereas ranibizumab neutralized this process in a dose-dependent manner (1). VEGF inhibitors, including ranibizumab, currently constitute the first-line treatment of the neovascular form of age-related macular degeneration (AMD), diabetic macular edema and retinal vein occlusion.

Vascular endothelial growth factor promotes proliferation, migration and survival of vascular endothelial cells, increases vascular permeability, dilates blood vessels and attracts endothelial cell precursors and monocytes (2). Despite the initial identification of VEGF as an endothelial cell mitogen, it has recently been demonstrated to have a direct biological effect on nonvascular cells. Several reports have documented that VEGF is expressed in a variety of adult retinal cells, including retinal pigment epithelium (RPE), as well as the Muller and neuronal cells that comprise photoreceptors, amacrine cells and ganglion cells (3–6). Similarly, a number of non-vascular retinal cells express VEGFR-2, the primary VEGF signaling receptor, and VEGF has been demonstrated to act on these cells to mediate their proliferation, differentiation and or survival (7, 8). A growing number of reports have supported a major neuroprotective role of VEGF for neuronal growth, differentiation, and survival. In a rat model of ischemia-reperfusion retinal injury, it was demonstrated that VEGF exposure resulted in a dose-dependent reduction in retinal neuron apoptosis (9). VEGF has also been reported to have neurotrophic effects on the developing retina (10). Moreover, it has been demonstrated that mice, which lack the soluble isoforms of VEGF, developed age-dependent, degenerative changes in the RPE-choroid complex, which mimicked the classic features of dry AMD (11).

Thus, some concerns have arisen that pharmacological strategies targeting VEGF reduction in the retina may represent a double-edged sword: the inhibition of VEGF reduces the edema, inflammation, and neovascularization associated with retinal vascular diseases, such as diabetic retinopathy or age-related macular degeneration at a given site; however, depressed VEGF expression levels may also reduce its innate neuroprotective capabilities, which directly impact neural and pigment epithelium cell survival.

Therefore, we aimed to analyze the influence of the VEGF inhibitor ranibizumab on the induction of the apoptotic process in retinal cells. Hence, we conducted a molecular analysis of the expression of selected apoptosis-related signal transduction pathway components at the mRNA and protein levels to depict the effects of ranibizumab administered via an intravitreal injection at a therapeutic dose in the rat retina through a 1-week observation period.

Methods

Animals and tissue collection

Pathogen-free 10–12-week-old mature male Wistar rats (Polish Academy of Sciences, Białystok, Poland) weighing 200–300 g were used in the experiment. The rats were housed in a standard laboratory environment with a 12-hr/ 12-hr light--dark cycle at 21°C. All animal procedures were performed according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local ethics committee.

For intravitreal injection, the animals were anesthetized with an intraperitoneal injection of ketamine (40.0 mg/kg) and xylazine (4.0 mg/kg) (all from Biowet, Puławy, Poland). The rats were randomly assigned to two groups (n=20/group): group 1 received an intravitreal injection of ranibizumab, and group 2 received an anti-rat VEGF antibody. Furthermore, to exclude the possibility of the iatrogenic side effects associated with an intravitreal injection, a group of animals (n=10), which did not receive an injection, was also included. The experimental eye of each rat in group 1 was injected with ranibizumab solution (Lucentis, Novartis, Horsham, UK) in a concentration of 10.0 mg/mL (original vial concentration). Similarly, the experimental eye of each rat in group 2 was injected with polyclonal goat anti-rat VEGF antibody (R & D Systems®, Wiesbaden, Germany) in the concentration of 0.4 μL/mL because the neutralization dose (ND_{50}) of this antibody is 0.2–0.6 μL/mL. For each rat, ranibizumab or anti-rat VEGF antibody was injected into the right (experimental) eye, and a similar volume of physiologic saline was injected into the left eye. The volume of solution injected in each eye was 5 μL. Intravitreal injections were performed via the insertion of a 31-gauge needle, which was attached to a 10-μl Hamilton syringe (HAMILTON® 701N, Nevada, USA). On days 3. and 7. following the intravitreal injection, the eyes were enucleated and dissected, and the anterior segment along with the lens were removed. The retinas were subsequently placed into 2 mL plastic tubes, disrupted in Cell Disruption Buffer (PARIS Kit, Life technologies Paisley, UK) using an Omni Tissue Homogenizer (TH) (Omni International, Kennesaw, USA) and assigned for molecular analysis.

Real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

To analyze the mRNA levels for *BAX* and *BCL2*, total RNA was isolated from retinas at 3, and 7th day post anty-VEGF/ PBS treatment (10 mice per time point) using the PARIS Kit (Life Technologies, Paisley, UK). The RNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, formerly Fermentas International Inc., Waltham, MA, USA). The quantitative assessment of mRNA levels was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A 15-μl reaction mixture containing 7.5 μl iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA), 10 ng of complementary (c)DNA template, and 0.9 μM of the following primers: BAX (f): 5′-AAACTGGTGCTCAAGGCCCT-3′, reverse (r): 5′-AGCAGCCGCTCACGGAG-3′, BCL2 (f): 5′-CCGGGAGAACAGGGTATGATAA-3′, reverse (r): 5′-CCCACTCGTAGCCCCTCTG-3′, and GAPDH (f): 5′-ATGACTCTACCCACGGCAAG-3′, reverse (r): 5′-GGAAGATGGTGATGGGTTTC-3′. The cDNA was amplified under the following conditions: 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 61°C for 30 and 60°C for 60 s. All gene expression analyses were done in duplicate. Relative target gene mRNA expression was quantified using the comparative Ct method.

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The relative quantity of a target, normalized to the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle, ΔCt = (Ct of target genes) – (Ct of endogenous control gene, GAPDH), and $\Delta\Delta Ct$ = (ΔCt of samples for target gene) – (ΔCt of calibrator for the target gene). The calculations were performed using the software, Bio-Rad CFX Manager Gene Study (Bio-Rad, Hercules, CA, USA).

Western blot analysis

For western blot analysis, proteins were extracted from retinas on days 3. and 7. following anti-VEGF/PBS treatment (5 mice per time point) using the PARIS kit (Life Technologies, Paisley, UK), following the manufacturer's instructions with some modifications. Briefly, retinas were homogenized in the Cell Disruption Buffer containing protease and phosphatase inhibitors (10.0 μg/ml leupeptin, 10.0 μg/ml aprotinin, 1.0 μg/ml pepstatin A, 1.0 mM sodium fluoride, and 2 mM Na_3VO_4) (all from Sigma Aldrich, St. Louis, MO, USA). The mixture was centrifuged, supernatants were collected, and protein concentrations were determined using the Bradford protein assay (Sigma Aldrich, St. Louis, MO, USA). Equal amounts of proteins (20.0 μg/well) were loaded and separated by 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, mini-PROTEAN II electrophoresis system, Bio-Rad, Hercules, USA) and then transferred to a 0.2-μm polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with a primary IgG antibody – rabbit anti-CASP3 polyclonal antibody (at 1: 600 dillution, LifeSpan Biosciences, Seattle, WA, USA) which detects activated caspase-3, resulting from cleavage adjacent to Asp1745. Immunoreactive bands were detected using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Chemiluminescence detection was performed using the ECL Select Detection Kit (GE Healthcare, formerly Amersham Life Sciences, Little Chalfont, UK), and bands were subsequently visualized using a UVP camera (Gel DOC-It Imaging system; Bio-Rad, Hercules, CA, USA). Equal loading in the lanes was evaluated by stripping the blots for 2 h at 37°C and then overnight at room temperature (IgG Elution Buffer; Thermo Fisher Scientific, Waltham, MA, USA). Re-probing was then performed in an identical manner with a murine anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1: 1000 dilution, followed by an HRP-conjugated secondary antibody as described above.

Statistical methods

The significance of differences between experimental groups was assessed with the Kruskal-Wallis test followed by the Mann-Whitney test. $P<0.05$ was considered statistically significant.

Results

Intravitreal anti-VEGF therapy induces apoptosis-related gene expression changes

To study the transcriptional response of retinal cells to anti-VEGF therapy, the rats were intravitreally injected with ranibizumab or physiological saline. Because ranibizumab is a humanized monoclonal IgG1 antibody, we simultaneously used a positive control, i.e., the injection of antibody against rat VEGF to corroborate ranibizumab/rat VEGF receptor affinity. Additionally, to exclude the possibility of the iatrogenic side effects associated with an intravitreal injection, non-injected rats were included as a complementary negative control.

To determine whether anti-VEGF treatment is associated with increased apoptosis in retinal cells, the collected eyes were enucleated on the days 3. and 7. post-injection, and the whole retinas were isolated. Apoptosis is tightly controlled by the balance between pro- and anti-apoptotic members of the Bcl-2 protein family. Changes in the relative expression levels of such molecules ultimately decide cell fate (12). Therefore, we determined the relative mRNA expression ratio for the pro-apoptotic gene *BAX* and the anti-apoptotic gene *BCL-2* using a quantitative RT-PCR (qRT-pPCR) technique. Based on the qRT-PCR analysis of the retinal tissues collected on the 3rd day of the experiment, we observed a significant increase in the *BAX/BCL-2* mRNA expression ratio in the group of rats injected with ranibizumab compared with the rats injected with physiological saline (Fig. 1A). This result may indicate an induction of the initial, reversible phase of the intrinsic pathway of apoptosis at the transcriptional level in retinal cells exposed to ranibizumab. Importantly, a similar increase in the *BAX/BCL-2* mRNA expression ratio was observed in the anti-rat VEGF antibody group, with no significant differences in *BAX/BCL-2* mRNA expression between the ranibizumab and anti-VEGF injected animals; these findings suggest that human ranibizumab antibody has an affinity for relevant VEGF receptors in the rat eye (Fig. 1A). Interestingly, we also identified a significant increase in the *BAX/BCL-2* mRNA expression ratio in the retinas collected from rats exposed to physiological saline as compared to rats without any invasive manipulation of the eye (negative control rats) (Fig. 1A). This result indicates that invasive procedures on the vitreous might potentially activate (to some extent) the initial, reversible phase of apoptosis in retinal cells of a rat.

In a similar comparative approach, we also quantified the mRNA expression of *BAX* and *BCL-2* genes in the retinas obtained on day 7. after the injection, finding no significant differences in the *BAX/BCL-2* mRNA expression ratio between the analyzed groups at this time point, which were all similar to the negative control group (Fig. 1B). This finding indicates that initially observed proapoptotic signals may be down-regulated along with a gradual elimination of the active biological compound from the vitreous. Furthermore, to investigate the time-dependent apoptotic effects of different VEGF antagonists used in the experiment, we quantitatively compared the mRNA expression ratios for the *BAX* and *BCL-2* genes in the retinas collected on days 3 and 7 post-injection. As shown in Fig. 1C, a significant difference in the *BAX/BCL-2* mRNA expression ratio was observed between days 3. and 7. in the group of animals treated with ranibizumab. Hence, we assumed that a single injection of VEGF inhibitor resulted in a transient, yet significant, induction of the initial, reversible phase of the intrinsic apoptosis pathway, shortly after the ranibizumab administration, and which was terminated at day 7 post-injection. The tenden-

cy of the activation of reversible phase of apoptosis to decrease with time was also observed in the other groups of rats treated with rat anti-VEGF antibody or physiological saline.

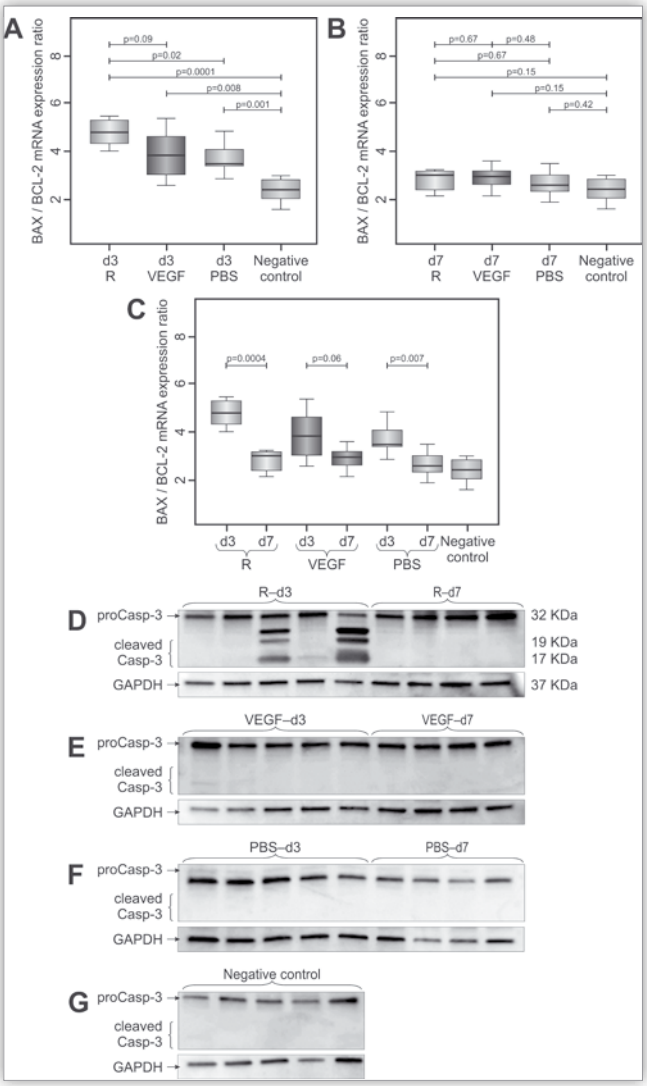

Fig. 1. Quantitative PCR analysis for relative quantification of *BAX/BCL-2* mRNA expression ratio on day 3. (A), day 7. (B) and on days 3. and 7. post-injection (C). The western blot analysis of the active caspase-3 protein expression at selected time points in retinas exposed to ranibizumab (D), anti-rat VEGF antibody (E), physiological saline (F), as well as in retinas of healthy, non-injected rat eyes (G). The 37 kDa band is GAPDH, which was used as an internal control. R – rats exposed to ranibizumab, VEGF – rats exposed to anti-rat VEGF antibody, PBS – rats exposed to physiological saline, Negative control – non-injected rat eyes.

Ryc. 1. Analiza ilościowa qRT-PCR współczynnika *BAX/BCL-2* na poziomie mRNA, 3. dnia (A), 7. dnia (B) oraz 3. i 7. dnia od podania (C). Analiza Western Blot poziomu aktywnej formy kaspazy-3 w badanych odstępach czasowych w siatkówkach oka poddanych działaniu ranibizumabu (D), antyszczurzemu przeciwciału skierowanemu przeciwko czynnikowi VEGF (E), soli fizjologicznej (F), jak również w zdrowej tkance pozyskanej od osobników kontrolnych (G). Kontrolę wewnętrzną stanowiło GAPDH, którego masa wynosi 37 kDa. R – szczury poddane działaniu ranibizumabu, VEGF – szczury poddane działaniu szczurzego przeciwciała anty-VEGF, PBS – szczury poddane działaniu soli fizjologicznej, Negative control – zdrowe osobniki kontrolne nie poddane iniekcjom.

Intravitreal anti-VEGF therapy may transiently activate executive caspase-3

To gain insight into the activation of the irreversible executive phase of apoptosis in response to ranibizumab treatment, we analyzed the expression of active form of caspase-3 protein in collected retinas. The detection of active caspase-3 may potentially indicate the initiation of irreversible phase of apoptosis once the cell has been committed to die via pro-apoptotic factors. The activation of caspase-3 requires the cleavage of its 32-kDa precursor, pro-caspase, into 19-kDa and 17 kDa subunits to exert executive protease activity (13). Accordingly, we used western blot analysis of the retinal lysates harvested on days 3. and 7. post-injection to determine whether the caspase-3 cleavage occurred in the eyes exposed to ranibizumab treatment. As shown in Figure 1d, the analyzed tissues showed a transient expression of the cleaved products of caspase-3 on day 3. post-injection of ranibizumab, which discontinued on day 7. of the experiment. Notably, the cleaved fragments of caspase-3 were detected in only 40% of the animals treated with ranibizumab. In addition, in Fig. 1E and Fig. 1F, we demonstrated that no products of the caspase-3 cleavage were detectable in the retinas exposed to anti-rat VEGF antibody or physiological saline. Similarly, the retinas from the non-injected rat eyes were negative for activation of caspase-3, as shown in Fig. 1G. The results show that the previously observed pro-apoptotic signaling induced by ranibizumab also included the transient activation of caspase-3 at an early time point post-injection, which can indicate the activation of irreversible, executive phase of apoptosis in retinas treated with ranibizumab. However, the biological effect of such treatment was not long-lasting and declined till day 7 post-injection.

Discussion

Vascular endothelial growth factor is a central mediator of angiogenesis and appears to represent a major stimulus for the development and growth of choroidal neovascularization in AMD. The good efficacy, relative safety and reasonable costs of intravitreal administration of ranibizumab have ultimately led to its widespread use worldwide for treatment of wet AMD and macular edema; however, questions have been raised concerning the cellular mechanisms that underlie this effective inhibition of ocular angiogenesis. Numerous studies investigated the potential cytotoxic effects of different clinically approved VEGF inhibitors on retinal cells. Several lines of evidence indicated that an anti-VEGF agent such as bevacizumab, which is a full-length monoclonal antibody against all VEGF isoforms, did not cause functional or morphologic retinal toxicity when administered intravitreally (14). Experimental investigation performed in rabbit eyes demonstrated that a repeated dose of an intravitreal bevacizumab injection did not cause apoptosis, oxidative stress activation, or lipid peroxidation in retinal cells (15). Similarly, bevacizumab applied to animal eyes did not induce functional changes (16), and bevacizumab or ranibizumab did not change the functional physiological parameters in isolated vertebrate retina (17, 18). *In vitro* studies performed in retinal neuron cell lines and retinal pigment epithelium cells demonstrated that the VEGF inhibitor aflibercept, which is a novel type of soluble decoy VEGF receptor, did not induce changes

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in cell morphology, apoptosis, or a permanent decrease in cell viability, proliferation or density (19). Similarly, ranibizumab did not alter ARPE19 cell proliferation in vitro (20).

In contrast, several reports documented clear detrimental effects of anti-VEGF agents on retinal homeostasis. Although the in vivo investigation demonstrated normal retinal function and structure following the injection of intravitreal bevacizumab in rabbits, the detailed histological analysis revealed mitochondrial disruption in the inner segments of the photoreceptors and pro-apoptotic molecules expression (21). In other studies investigating the retinal toxicity of VEGF inhibitors in rabbits, the 5.0 mg dose of bevacizumab was found to induce transient inflammation, ultrastructural abnormalities, and apoptosis of photoreceptors (22, 23). It should be noted that a dose-dependent increase in apoptosis was identified in photoreceptors and other cells following bevacizumab treatment (23). Jee et al. documented that the process of apoptosis in retinal cells following an intravitreal bevacizumab injection is mediated by the down-regulated NGF, which suggests the existence of a paracrine loop between neurotrophic factors and VEGF in the retina (24). Furthermore, serious consideration must be given to the potential negative effects of VEGF neutralization on choroidal circulation. VEGF-A inhibition results in the reduction of choriocapillary fenestration, which may impede nutrient supply in the retina (25). The results of animal studies revealed that fenestrated vascular beds are uniquely vulnerable to VEGF blockage and exhibit clear vessel regression (26, 27). Indeed, a dramatic harmful effect on photoreceptors was described in the absence of VEGF-A (28). Thus, the long-term therapeutic neutralization of VEGF-A may lead to the unexpected and unintentional degeneration of choroidal circulation.

These experimental observations have raised a number of important safety questions regarding the clinical use of anti-VEGF therapy in AMD patients. Retinal pigment epithelial atrophy is frequently observed at presentation in patients with AMD and choroidal neovascularization, and it has commonly been detected following treatment with anti-VEGF. Approximately one fifth of patients enrolled in the *Comparison of Age-related Macular Degeneration Treatments Trial* (CATT) developed geographic atrophy (GA) within 2 years of treatment (29). Seven years after the initiation of intensive ranibizumab therapy in AMD patients, macular atrophy was detected in 98% of eyes, and the area of atrophy was significantly correlated with poor visual outcome (30). However, there are no long-term follow-up studies of these atrophic lesions, and it is not known whether their morphology, local growth patterns, or functional effects are similar compared with *de novo* GA lesions that develop in areas where no neovascularization was previously present. Although the pathogenesis of AMD has not been fully elucidated, ischemia is often postulated as a major pathogenic factor, which raises concerns whether anti-VEGF treatment may exacerbate ischemia-induced retinal damage. The 2-year-data analysis from the inhibition of VEGF in age-related choroidal neovascularization (IVAN) trial demonstrated that the new GA changes were detected significantly more often during follow-up in the participants on monthly compared with discontinuous (PRN) anti-VEGF regimens, which suggests that greater exposure to anti-VEGF medications may have a role in the development of this pathologic condition (31). Similarly, the 2-year-period of anti-VEGF treatment in the CATT trial based on monthly injections, regardless of the type of anti-VEGF agent, was associated with a 59% increase in the risk of GA development compared with PRN treatment (29). Moreover, several recent reports have confirmed the observations obtained from large prospective, randomized trials. In a treat-and-extend protocol, significant associations between the progression of both RPE and choroidal atrophy and the number of anti-VEGF treatments were observed (32). A retrospective study by Lois et al also identified that the development of RPE atrophy, as measured using fundus autofluorescence imaging, was significantly associated with the number of intravitreal ranibizumab injections (33). This finding may indicate that more frequent injections of anti-VEGF agents may be associated with increased incidence and extent of GA, which supports the notion of a potential direct effect of the anti-VEGF therapy on the progression of retinal atrophy.

In our study, we used a single injection of ranibizumab to assess whether it may induce apoptosis in retinal tissue. Following the intravitreal injection of ranibizumab in rabbit eyes, the drug had a half-life of 2.6–2.88 days in the vitreous cavity (34, 35). Similarly, in monkeys, the half-lives of ranibizumab and a related Fab were 3 and 3.2 days, respectively (36). Therefore, to investigate the time-dependent changes in the apoptotic activity of retinal cells following ranibizumab injection, we selected days 3. and 7. post-injection for ocular tissue collection from the experimental animals. As a result, we detected a transient induction of both the initial and executive phase of apoptosis in the retinal tissue on day 3. following ranibizumab injection, which might be a potential biological effect of VEGF neutralization. However, both the initiation and the executive phases of apoptosis were temporary active and terminated prior to day 7. following ranibizumab injection. Importantly, although the induction of apoptosis at the transcriptional level was predominately observed on day 3. post injection, the irreversible, executive phase of programmed cell death was only detected in 40% of rats exposed to ranibizumab at this time point. Therefore, we suggest that the extent of ranibizumab-triggered cell death of retinal cells may well depend on other accompanying factors, such as environmental factors, immune status, and metabolic causes. Indeed, it has been strongly documented that the fast progression of GA in AMD patients is more frequent among current smokers at baseline, subjects with the specific *CFH* or *ARMS2* risk genotypes, and in pseudophakic eyes (37).

In conclusion, despite the intravitreal use of VEGF inhibitors may result in the transient activation of programmed cell death in retinal cells, it still remains the most effective and unique therapeutic option available for patients with an exudative form of AMD. Notably, this is a preliminary, pilot study in a small animal sample, which has substantial limitations. Therefore, no significant conclusions about side effects of ranibizumab could be drawn directly from this work, especially due to the fact, that performed analysis of apoptosis was based mainly on the detection of changes in the expression of the components related to the intrinsic apoptotic pathway. Moreover, methodology of this in vivo experiment would not permit to assess the final fate of the retinal cells affected by the all procedures performed in the rat eyes during the experimental period.

Consequently, additional studies of other cellular mechanisms, including the death receptor-mediated and caspase-3 independent apoptotic pathways, as well as the innovative intravitreal *in vivo* microscopy for studying the cell fate, are imperative to unequivocally demonstrate the safety of ranibizumab-based treatment. Thus, further studies in preclinical and clinical settings are required to examine the potential consequences of prolonged exposure to VEGF inhibitors; the real balance of risks and benefits should be subsequently established in each patient individually. In addition, our current results using a single intravitreal injection of ranibizumab require further investigation to test the biological effects after repeated multiple intravitreal injections of the drug, as it gives the possibility to more accurate imitate the clinical practice. Consequently, our results should be cautiously extrapolated to clinical practice. The establishment of safe doses and regimens of administration of anti-VEGF therapy are still under investigation. Large, randomized, controlled clinical trials are essential to provide rationale of different treatment protocols to be used in different stages of retinal disease.

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