Research Article

Bevacizumab does not affect autophagy clearance during proteasomal inhibition in human retinal pigment epithelial cells

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Abstract: The most common reason for blindness in developed countries is age-related macular degeneration (AMD) that can be classified into two main categories: the atrophic, or dry form and the exudative, or wet form. The diagnostic difference between dry and wet AMD is the development of choroidal neovascularization in wet AMD. A master regulator in the development of neovascularization is Vascular Endothelial Growth Factor (VEGF) A, which is therapeutically inhibited to treat wet AMD. Current VEGF-antagonists are the Fab-Fragment ranibizumab (Lucentis), the fusion protein aflibercept (Eylea) and the off-label used antibody bevacizumab (Avastin). Due to its low costs, Avastin is largely used worldwide in clinics. Recent findings reveal that proteasomal and lysosomal clearance systems including autophagy have been disturbed in AMD. Failure of autophagy in aged postmitotic cells, including retinal pigment epithelial (RPE) cells, can result in accumulation of aggregate-prone proteins, cellular degeneration and finally, cell death that secondarily lead to photoreceptor damage and visual loss. In this study, we show that bevacizumab does not affect autophagy clearance in ARPE-19 cell during proteasome inhibition and protein aggregation.

Keywords: autophagy, bevacizumab, degeneration, macula

Introduction

Age-related macular degeneration (AMD) is the most common reason for legal blindness in the developed countries. Phenotypically, AMD can be divided into two main groups: dry (atrophic) and wet (exudative) type and further subdivided into early and late stage disease [1]. The early stage of dry AMD is asymptomatic, although pigment mottling, accumulation of intracellular lysosomal lipofuscin, and extracellular drusen deposits can be detected in ophthalmological examinations. The late stage of dry AMD, also known as geographic atrophy, is characterized by discrete areas of RPE loss and impairment of the overlying retinal photoreceptor cells and severe visual loss [2, 3]. Today there is no effective treatment for dry AMD, although omega-fatty acid and antioxidants are usually recommended for high risk AMD patients [4, 5]. In wet AMD, aberrant blood vessels sprout from the choroidal capillaries and penetrate through the Bruch’s membrane leading to subretinal membranes, hemorrhage, retinal edema and damage to retinal cells. This proliferative process is called choroidal neovascularisation (CNV). Wet AMD results in a rapid loss of vision if not treated. A major factor in the development of neovascularization is Vascular Endothelial Growth Factor (VEGF) A, which is therapeutically inhibited to treat wet AMD [6]. In addition to its pathological effects,
VEGF-A has important physiological functions both systemically and in the retina. Current VEGF-antagonists are the Fab-Fragment ranibizumab (Lucentis), the fusion protein aflibercept (Eylea) and the off-label used antibody bevacizumab (Avastin). In addition, the aptamer pegaptanib (Macugen) is approved for the treatment of AMD, but rarely used. These molecules differ in molecular size and structure which impacts their pharmacological behavior such as their effects on retinal cells, ocular and systemic clearance, or systemic VEGF-A inhibition. Although all these drugs have been shown to be equally effective in the treatment of wet AMD, the respective therapeutic molecule may have different systemic and/or retinal effects especially after prolonged usage [7, 8, 9].

Fig. 1. Western blotting from the ARPE-19 cells exposed solely to 0.25 mg/ml bevacizumab or 1 µM MG-132 proteasome inhibitor or combination of bevacizumab and MG-132 for 0.5 hours, 3 hours, 12 hours and 24 hours. α-tubulin was used as a loading control.
Bevacizumab is a recombinant full-length humanized antibody with a molecular weight of 149-kDa, binding to all VEGF-A isoforms and reducing angiogenesis and vascular permeability. It was originally developed to target pathologic angiogenesis in malignant tumors and approved by the authorities for the treatment of metastatic colorectal cancer [6]. However, mostly because of economic reasons, bevacizumab is widely used intravitreally as an off-label treatment for proliferative eye diseases, such as CNV related to AMD, diabetic macular edema and macular edema caused by retinal vein occlusion. The intravitreal injection of 1.25 mg of bevacizumab has been shown to be comparable in efficacy to the officially approved treatment with ranibizumab. The CATT Study (The Comparison of Age-Related Macular Degeneration Treatment Trials) and IVAN Study (Inhibit VEGF in Age Related Choroidal Neovascularization) compared the relative safety and effectiveness of bevacizumab and ranibizumab in treatment [7, 8]. Patients with newly diagnosed exudative AMD were followed up for 2 years, and the results showed similar outcomes using either drug, with bevacizumab showing non-inferiority to ranibizumab.

The half-live in vitreous humour is similar with both compounds - 7 days for bevacizumab and 9 days for ranibizumab, resulting in similar treatment intervals [10, 11]. However, the systemic half-life is different, i.e. 20 days for bevacizumab vs. 6 hours for ranibizumab, and the maximum systemic exposure with 1.25mg bevacizumab is 20-690 ng/ml and with 0.5mg ranibizumab 0.8-2.9 ng/ml [12]. Since the systemic exposure is lower with ranibizumab, it may be associated with lower systemic toxicity [13]. In fact, it has been shown that intravitreal injections of bevacizumab decrease VEGF levels in blood by up to 117-fold within 1 day and by 4-fold up to 1 month later. This decrease is similar to that achieved with intravenous therapy, and it is possible that intravitreal bevacizumab may suppress baseline physiologic VEGF activity [14, 15]. Furthermore, bevacizumab has been detected and associated with regression of contralateral proliferative diabetic retinopathy and iris neovascularization in rabbits after intravitreal injection in contralateral eyes [16].

![Western blotting from the ARPE-19 cells exposed solely to 0.25 mg/ml bevacizumab or 1 µM MG-132 proteasome inhibitor or combination of bevacizumab and MG-132 for 1 hour, 4 hours, 1 day, 3 days or 7 days. α-tubulin was used as a loading control.](image)

*Fig. 2.* Western blotting from the ARPE-19 cells exposed solely to 0.25 mg/ml bevacizumab or 1 µM MG-132 proteasome inhibitor or combination of bevacizumab and MG-132 for 1 hour, 4 hours, 1 day, 3 days or 7 days. α-tubulin was used as a loading control.
Table 1. Bevacizumab exposure, short. Autophagosomes in ARPE-19 cells exposed to bevacizumab 0.25 mg/ml for 1 and 4 hours. Control cells were cultured in normal medium. Autophagosomes were calculated from pictures taken with a transmission electron microscope. There was no statistically significant difference between any of the groups studied. MA = mean value, SD = standard deviation, N = samples analyzed.

<table>
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<th>SD</th>
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<tr>
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<tr>
<td>1 hour</td>
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<tr>
<td>4 hours</td>
<td>10</td>
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There is no statistical difference between the groups (p>0.1).

Table 2. Bafilomycin exposure, short. Autophagosomes in ARPE-19 cells exposed to bafilomycin 50 nM for 1 hour and 4 hours. Control cells were cultured in normal medium. Autophagosomes were calculated from pictures taken with a transmission electron microscope. There was a clear statistically significant difference between control and bafilomycin groups studied. MA = mean value, SD = standard deviation, N = samples analyzed.

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<tr>
<td>Control</td>
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<tr>
<td>Bafilomycin, 1 hour</td>
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<td>7.36</td>
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<tr>
<td>Bafilomycin, 4 hours</td>
<td>9</td>
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P-value between the control and both bafilomycin groups is p<0.001.

Table 3. Bevacizumab exposure, long. Autophagosomes in ARPE-19 cells exposed to bevacizumab 0.25 mg/ml for 1 day, 5 days and 7 days. Control cells were cultured in normal medium. Autophagosomes were calculated from pictures taken with a transmission electron microscope. There was no statistically significant difference between any of the groups studied. MA = mean value, SD = standard deviation, N = samples analyzed.

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<tr>
<td>1 day</td>
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<td>7 days</td>
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<td>6.46</td>
<td>2.83</td>
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There is no statistical difference between the groups (p>0.1).

Table 4. Bafilomycin exposure, long. Autophagosomes in ARPE-19 cells exposed to bafilomycin 50 nM for 24 hours. Control cells were cultured in normal medium. Autophagosomes were calculated from pictures taken with a transmission electron microscope. There was high statistically significant difference between the groups studied. MA = mean value, SD = standard deviation, N = samples analyzed.

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<th>N</th>
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<th>SD</th>
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<tbody>
<tr>
<td>Control</td>
<td>41</td>
<td>6.34</td>
<td>3.47</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>28</td>
<td>18.29</td>
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P-value between the two groups studied is p<0.0001.
In preclinical safety studies with bevacizumab, the compound was shown to be safe in general. In several studies, bevacizumab showed no direct cytotoxicity in various cultured ocular cell lines or in cultured retinas [17, 18]. Similarly, in short-term in vivo rabbit models, intravitreal bevacizumab did not cause any serious ocular toxicity [19, 20]. However, in one study mitochondrial disruption was observed in the photoreceptor inner segments, and proteins associated with apoptosis were detected in the outer plexiform, outer nuclear, and photoreceptor layers [21]. In addition, bevacizumab, but not ranibizumab, has been shown to accumulate in the RPE cells, which may reduce heterophagic ability in these cells [22, 23].

AMD is a neurodegenerative aggregation disease that shares its cellular pathology with Alzheimer’s disease [24]. Recently, it has been observed that one crucial mechanism behind AMD is impaired proteasomal and lysosomal proteolysis that associates with the accumulation of lipofuscin and drusen [1, 25, 26]. Autophagy is a basic lysosomal catabolic mechanism which "self eats" cellular components which are unnecessary or dysfunctional for the cell [1, 26, 27]. Autophagy comprises three intracellular pathways in eukaryotic cells, which are macroautophagy (hereafter referred to as autophagy), microautophagy, and chaperone-mediated autophagy. Autophagy has a key role in normal cellular homeostasis, but it is also triggered as an adaptive response during AMD associated stress conditions [3, 4]. Autophagy process begins with the formation of isolation membranes called phagophores; these later then become elongated and surround portions of cytoplasm containing oligomeric protein complexes and organelles to form mature double membrane autophagosomes. The autophagosomes fuse with lysosomes and their content is then degraded by lysosomal enzymes. Failure of autophagy in aged postmitotic cells, including RPE cells, can result in accumulation of aggregate-prone proteins, cellular degeneration and finally cell death that secondarily leads to photoreceptor damage and visual loss [26, 28]. SQSTM1/p62 (Sequestosome 1) is the best-characterized and ubiquitously expressed autophagy receptor that connects proteasomal clearance with lysosomes [28-32]. Alleviation of autophagy is usually accompanied by an accumulation of SQSTM1/p62 mostly in large perinuclear aggregates or inclusion bodies which are also positive for ubiquitin. In this study, we show that bevacizumab does not affect autophagy clearance in ARPE-19 cell during proteasome inhibition and protein aggregation.

Materials and methods

Cell culture

ARPE-19 human RPE cells were obtained from the American Type Culture Collection (ATCC). The cells were grown to confluence in a humidified 10% CO2 atmosphere at 37°C in Dulbecco’s MEM/Nut MIX F-12 (1:1) medium (Life Technologies, 21331) containing 10% inactivated fetal bovine serum (Hyclone, SV30160-03), 100 units/ml penicillin and 100 mg/ml streptomycin (Lonza, DE17-602E) and 2 mM L-glutamine (Lonza, BE17-605E). The cells were exposed solely to 0.25 mg/ml bevacizumab (Pharmacy of Kuopio University Hospital) or 1 µM MG-132 proteasome inhibitor (Calbiochem, 474790) or combination of bevacizumab and MG-132 for 0.5 hours, 3 hours, 12 hours and 24 hours. Moreover, the cells were exposed solely to 0.25 mg/ml bevacizumab for 1 hour, 4 hours, 1 day, 3 days or 7 days. To prevent autophagy flux, 50 nM bafilomycin A1 (Sigma, B1793) for 1 hour and 4 hours was used.

Western blotting

The whole cell extracts were prepared in M-PER (Mammalian Protein Extraction Reagent, Thermo Scientific, 78501) according to the manufacturer’s protocol. Proteins were diluted in 3X sodium dodecyl sulphate (SDS) protein gel loading solution, boiled for 5 minutes, separated on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and processed following standard procedures. The rabbit polyclonal ubiquitin antibody (DakoCytomation, Z0458) was diluted in 3% milk powder, 0.3% Tween 20/PBS at 1:500. Secondary antibody was diluted in 3% milk powder, 0.3% Tween 20/PBS at 1:20 000. The mouse monoclonal anti-SQSTM1/p62 (Santa Cruz Biotech, sc-28359) was diluted in 0.5% bovine serum albumin, 0.3% Tween 20/PBS at 1:1000. Secondary antibody was diluted in 3% milk powder, 0.3% Tween 20/PBS at 1:20 000. The mouse monoclonal anti-α-tubulin (Sigma, T5168) was diluted in 1% milk powder, 0.05% Tween 20/PBS at 1:8000. Secondary antibody was diluted in 1% milk powder, 0.05% Tween 20/PBS at 1:10 000. The rabbit monoclonal anti-MAP1LC3A/LC3 (anti-LC3, microtubule-associated protein light chain 3A) (Cell Signaling, 3868) was diluted in 5% bovine serum albumin, 0.1% Tween 20/PBS at 1:1000. Secondary antibody was diluted in 3% milk powder, 0.1% Tween 20/PBS at 1:2000. The nitrocellulose membranes signals were detected by chemiluminescence. Experiments were performed in duplicate for each cell preparation and α-tubulin was used to normalize the data. Statistical analysis of western blot data was performed on the densitometric values obtained with the NIH image software 1.61 (downloadable at http://rsb.info.nih.gov/nihimage) and Quantity OneH software.

Transmission electron microscopy
ARPE-19 cell culture samples were prefixed with 3% glutaraldehyde in PBS at 4°C overnight. After 3x10 min washing in PBS, the samples were post-fixed in 2% osmium tetroxide for 60 min, washed with phosphate buffer for 15 min prior to standard ethanol dehydration, and embedded in Araldite®. Ultrathin (60 nm) sections were cut and examined with a Zeiss 902 electron microscope. Autophagic vesicles were manually counted. Eight cells were randomly selected from each group for counting autophagic vesicles. Statistical analysis was performed using SPSS (v. 19; IBM, SPSS Inc). The significance of differences between control and treated groups was analyzed with Mann-Whitney U-test. A p-value of 0.05 or less was considered significant.

**Fig. 3.** Transmission electron microscope pictures from RPE cells predisposed to 0.25 mg/ml bevacizumab for 1 and 4 hours. (A). RPE cells cultured in normal medium were used as a control. To provide a positive control the cells were either non-stressed or exposed to 50 nM bafilomycin for 1 hour or 4 hours (B).
Results

First, the effect of proteasome inhibition on expression of ubiquitin protein conjugates, SQSTM1/p62 and MAP1LC3A/LC3 were studied in human ARPE-19 RPE cells. The cells were either non-stressed or exposed to bevacizumab 0.25mg/ml or 1 μM MG-132, or simultaneously exposed to both of the drugs for 30 min, 3 hours, 6 hours and 24 hours and analyzed by western blotting. A robust increase in the ubiquitin protein conjugates were observed in all the studied time points during proteasome inhibition (Figure 1). Increased SQSTM1/p62 levels were seen at the 6 and 24 hours time points in response to proteasome inhibition. Increased lipidation of the MAP1LC3A/LC3 was detected at the 24 hours time point during proteasome inhibition revealing autophagy activation. No changes of protein levels were found in samples treated solely with bevacizumab which indicates a good tolerability.

Fig. 4. Transmission electron microscope pictures from RPE cells predisposed to 0.25 mg/ml bevacizumab for 1, 5 and 7 days. RPE cells cultured in normal medium were used as a control. To provide a positive control the cells were either non-stressed or exposed to 50 nM bafilomycin for 24 hours (B).
To study the long-term effect of bevacizumab (0.25 mg/ml) on the expression of ubiquitin protein conjugates, SQSTM1/p62 and MAP1LC3A/LC3, the cells were either non-stressed or exposed to bevacizumab for 1 hour, 4 hours, 1 day and 7 days and analyzed by western blotting. Bevacizumab did not change the expression levels of any of the proteins studied (Figure 2).

The ultrastructure and amount of autophagosomes were examined using transmission electron microscopy. Neither in control or in 0.25 mg/ml bevacizumab cells treated for 1 hour or 4 hours, cellular abnormalities or changes in autophagosomes were observed (Figure 3A, Table 1). Bafilomycin is known to be an autophagy inhibitor preventing the fusion of autophagosomes and lysosomes, but robustly increasing the amount of autophagosomes [26, 33]. To provide a positive control, the cells were either non-stressed or exposed to 50 nM bafilomycin for 1 hour or 4 hours. As expected, there was a clear statistically significant difference between control and bafilomycin groups studied; p>0.01 (Figure 3B, Table 2). We did not observe cellular abnormalities or changes in autophagosomes for a long time exposure up to seven days with bevacizumab (Figure 4A, Table 3). However, when cells were exposed to 50 nM bafilomycin, already at 24 hours a clear statistically significant difference between control and bafilomycin groups studied could be observed; p>0.001 (Figure 4B, Table 4).

Discussion

We show that bevacizumab does not affect autophagy clearance in ARPE-19 cells during accumulation of intracellular protein aggregates and proteasome inhibition. Moreover, in contrast to bafilomycin, bevacizumab does not affect autophagy during short or long time exposures. Our observations reveal that bevacizumab is well tolerated in RPE cells and does not disturb the crucial autophagy clearance system.

In human AMD donor samples both accumulations of autophagic markers and decreased lysosomal activity can be observed [34]. Furthermore, an effective autophagic clearance system has recently been documented in human RPE cells [26, 28, 35-37]. Indicating a declined proteolysis in RPE cells during aging, lipofuscin accumulates in lysosomes and drusen between Bruch’s membrane and RPE cell layer [1, 38]. Lipofuscin is a toxic compound in RPE cells which promotes the damage of mitochondria and misfolding of intracellular proteins, creating an additional oxidative stress in the RPE cells [39, 40]. When autophagy flux is inhibited by lysosome inhibitors, such as bafilomycin, it induces autophagosome formation but does not increase lysosomal proteolysis due to the decreased lysosomal enzyme activity. At both short and long term exposure, bafilomycin evoked an intense autophagosome formation, in parallel with autophagy receptor SQSTM1/p62 accumulation [26]. Bevacizumab did not change SQSTM1/p62 expression levels confirming that there is no detrimental effect on autophagy clearance.

Ranibizumab, aflibercept and bevacizumab have been shown to be well-tolerated and they have equal efficacy in the treatment outcomes for wet AMD [7-9, 41]. These molecules differ in molecular size, structure, binding activity and binding targets which may result in different effects on the cellular level [6]. At concentrations used in clinical practice, there are no toxic effects on retinal pigment epithelial cells, neural retina, or endothelial cells in vitro [18, 42-45]. However, bevacizumab exposure has been documented to decrease in mitochondrial activity in both the proliferating and non-proliferating endothelial cells in vitro [44]. This suggests a selective action of bevacizumab on endothelial cells. Moreover, bevacizumab may accumulate in RPE cells, decrease heterophagy and exert pro-fibrotic effects on human RPE cells [1, 22, 23, 46]. These effects of bevacizumab can be considered as complications that may affect long-term bevacizumab treatment results. All those detrimental effects may be anticipated to affect autophagy, a key proteostasis regulator, in normal cellular homeostasis, but also an adaptive response during AMD associated stress conditions [3, 4]. In our study, we were not able to show any effects of bevacizumab on autophagy regulation. In that aspect, bevacizumab might be considered as a safe drug in the regulation of cellular proteostasis. Comparable studies with ranibizumab and aflibercept on autophagy regulation would be interesting to carry out.

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Conflict of interests

The authors declare no conflict of interest.

References


