Moreno-Páramo et al., 1:2
http://dx.doi.org/10.4172/scientificreports.166

Open Access Scientific Reports

Research Article Open Access

Changes in the Retinal Structure and in the Gene Expression Levels of VEGF, After Rapamycin and Rapamycin Plus Bevacizumab Intravitreal Injection

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Abstract

Purpose: To evaluate the anatomical changes and gene expression levels of VEGF, after rapamycin and rapamycin plus bevacizumab intravitreal injection on an animal model of choroidal neovascularization.

Methods: We included the eyes of twelve Landrace pigs with laser-induced choroidal neovascularization. The animals were divided in four groups. Group A received a control dose of intravitreal BSS. Group B received an intravitreal dose of rapamycin (1mg/ml), group C received intravitreal bevacizumab (2.25mg/ml) and group D received a combination of both drugs with the same doses. Twenty one days after injection, the eyes were enucleated and retina tissue was harvested for mRNA extraction, reverse transcription and RT-PCR. The gene expression was estimated with a linear regression model. A Friedman/Wilcoxon *W* test and Mann Whitney *U* test was used to compare VEGF expression levels between groups

Results: The level of VEGF expression in group A was: 240pg/ml, in group B: 186pg/ml, in group C: 100pg/ml and in group D: 70pg/ml. There was a statistical difference between the control and group C and D ($p<0.05$ and 0.01). The histological analysis yielded no structural changes in any group.

Conclusions: Intravitreal injection of rapamycin and rapamycin plus bevacizumab appears to have no effect on the anatomical structure of the retina. The VEGF expression level was reduced in all study groups. The combination of both drugs appears to have an additive effect but the mechanism is not yet clear.

Keywords: Animal Model; Bevacizumab; Choroidal neovascularization; Intravitreal; Rapamycin

Introduction

The hallmark of proliferative eye diseases is the formation of abnormal new vessels from preexisting capillaries in a phenomenon known as angiogenesis [1-3]. In advanced stages of the disease, the patients may experience loss of vision, usually secondary but not limited, to tractional retinal detachment [1,4]. The formation and contraction of the membranes and glial tissue that is accompanying the new vessels is the major risk factor [4]. Other causes of visual loss in patients with proliferative retinopathies are: vitreous hemorrhages, subhyaloid hemorrhages involving the macula, subretinal hemorrhage, macular ischemia and cystoid macular edema among others [4-6].

Angiogenesis is one of the most studied processes in the human eye. It is a complex mechanism that involves stimulation, proliferation and migration of vascular endothelial cells, as well as proteolytic breakdown and degradation of the capillary basement membrane and extracellular matrix [7]. Growth factors have a regulatory activity over angiogenesis. Depending on the type, they can stimulate or inhibit the phenomenon and can be found in all the retinal layers, vitreous and choroid of patients with proliferative diseases [2,8,9]. Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein overexpressed under hypoxic conditions.[10] It is one of the most potent angiogenic growth factors as well as one of the most studied. It is a mitogen glycoprotein of 46 kDa with various subtypes and has been identified as a key factor in the retina angiogenesis process [10-12].

Age-related macular degeneration (AMD) is the leading cause of vision loss among individuals of 50 years or older with an incidence that reaches 40 to 50% at 60 years of age [13,14]. Choroidal neovascularization (CNV) accounts for roughly 10% of the cases, but is responsible for 90% or so of the cases with severe visual loss [15]. Increased levels of VEGF have been found in patients with active CNV and treatments directed to blocking its biological activity have reported promising results [16-18].

Bevacizumab (Avastin®, Genetech Inc, San Francisco CA.) is a recombinant monoclonal antibody that binds all forms of VEGF. It is approved by the FDA for the treatment of metastatic colorectal cancer, metastatic breast cancer and non-small cell pulmonary cancer [19]. In recent years, intravitreal injection of bevacizumab has been used as an off-label treatment for CNV of different etiologies with good safety profile and efficacy in a small series of patients; however, a large prospective multicentre study is still yet to come [16,18,20].

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Received November 28, 2012; **Published** July 30, 2012

Citation: Moreno-Páramo D, Velez-Montoya R, Flores J, Sepúlveda-Vásquez H, Rodriguez-Reyes A, et al. (2012) Changes in the Retinal Structure and in the Gene Expression Levels of VEGF, After Rapamycin and Rapamycin Plus Bevacizumab Intravitreal Injection. 1: 166. doi:10.4172/scientificreports.166

Rapamycin is a potent broad-spectrum antibiotic which has demonstrated antineoplastic and antiangiogenic activity [21,22]. It is presumed that the antiangiogenic activity is due to its property to downregulate VEGF [21,22] Therefore the aim of this study is to evaluate the safety and efficacy of intravitreal rapamycin and the combination of rapamycin and bevacizumab versus bevacizumab alone as well as the variation on the VEGF expression levels in each group on an animal model of CNV.

Material & Methods

The study was reviewed and approved by the hospital internal review board and the Institutional Animal Care and Use Committee. All the procedures were performed according to the statement for the use of animals in ophthalmic and visual research from the Association of Research in Vision and Ophthalmology (ARVO).

We included twelve eyes from twelve Landrace pigs. We divided the population into four different groups of three animals each (Group A, B, C and D). Pharmacological mydriasis was induced on all right eyes with 3 drops of tropicamide (0.8%) and phenylephrine (5%) every 10 minutes, three times. Choroidal neovascularization was induced in all animals' right eyes, with the use of a double frequency Nd: YAG laser (Ophthalas 532 Eye Lite, Alcon Labs, Dallas Forth Worth, Tx) using 30 burns of 1.5 W of power, a spot diameter of 500 μm and a burn duration of 300 ms. The burns were placed above and temporal to the *area centralis*, outside the main vascular arcade. CNV was successfully induced in all eyes, and was evident 5 days after laser administration in fundus examination and fluorescein angiography (data not shown).

After checking for the successful induction of CNV, all animals were sedated with ketamine hydrochloride (0.1%) at a dose of 1 μg/Kg and locally anesthetized with topical drops of lidocaine hydrochloride (4%). After surgical preparation and drape (only the right eye), instillation of drops of povidone iodine (10%) in conjunctival fornices and the placement of an eyelid speculum, animals on group A received an intravitreal injection of 0.1ml balanced salt solution and served as a control group; group B received an intravitreal injection of rapamycin (1mg/ml) 0.1ml; group C received 0.1ml intravitreal injection of bevacizumab (2.25mg/ml) and group D received 0.2ml intravitreal injection of rapamycin and bevacizumab (same doses). Intraocular pressure was assessed after injection and 5 minutes later. If there was a high intraocular pressure, an anterior chamber paracentesis was done for pressure control.

Twenty one days after intravitreal injections, animals were euthanized and enucleated. Immediately, a piece of the retina (0.5mm x 0.5ml) was harvested and placed on a sterile eppendorf tube (Eppendorf AG, Hamburg, Germany) for mRNA extraction, using a commercially available extraction kit (total RNA microprep kit, Stratagene, La Jolla, CA) according to the manufacturer's instructions. The rest of the eye was fixed on a solution of 4% paraformaldehyde and preserved in paraffin for histological analysis.

Reverse transcription was carried out using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). Real-time Polymerase Chain Reaction (RT-PCR) was performed with a set of primers and probes, specially designed to detect VEGF mRNA of all isoforms. The expression levels of VEGF were determined by using a linear regression model (SPSS software, version 16.0 Sigma Stat; Systat Software, Inc., San Jose, CA, USA).

The tissue preserved in paraffin was cut on sections of 10μm and placed on glass slides. The slides were placed in a solution of Triton (0.05%) and PBS for 40 minutes and stained with hematoxylin and eosin dyes.

The statistical analysis was performed using SPSS software, version 16.0. Data are presented as median and standard deviation. A Friedman/Wilcoxon *W* test and Mann Whitney *U* test was used to compare VEGF expression levels between groups. A *P* value of less than 0.05 was considered to be statistically significant.

Results

The examination with light microscopy of the 10μm sections yielded no evidence of thinning of the retinal layers, loss of photoreceptors or disturbances in the retinal architecture in any of the study eyes (Figure 1) as well as no evidence of inflammatory infiltration.

According to the linear regression model, the highest level of VEGF expression was seen in group A (BSS), with 240±2.25 pg/ml. Group B (rapamycin), exhibited an expression level of 186±3.52 pg/ml. Group C (bevacizumab), expressed 100±5.47 pg/ml of VEGF. The lowest level of VEGF expression was seen in the last group (group D, Bevacizumab + Rapamycin) with 70±8.13 pg/ml. There was a statistical difference in the expression levels of VEGF between groups A and D (P=0.01) and between groups A and C (P=0.05). There were no statistical differences between groups A and B or between groups B and C.

Conclusion

In the present study, light microscopy demonstrates that intravitreal injection of rapamycin and the injection of the combined doses of rapamycin and bevacizumab have no effect on the structural anatomy of the retinal layers. There was no evidence of an inflammatory response in any of the study animals either. The expression level of VEGF was significantly reduced with the combination of rapamycin and bevacizumab when compared to the control group (BSS). A similar result was observed after the injection of rapamycin alone, but the reduction was not statistically significant.

As life expectancy in the western world increases, AMD incidence has risen to become the leading cause of vision loss in aged people and by the year 2025, it may cause up to 7 million cases of severely impaired vision in the United States alone [15]. Currently, the AMD-related disabilities and the poor quality of life associated with AMD have become heavy socio-economic burdens in industrialized countries [23,24].

Substantial efforts have been aimed at investigating a number of different treatments for AMD. The ones with the most promising results, currently the gold standard in AMD treatment, is the use of human antibodies against VEGF [17,25]. Their safety and efficacy is demonstrated in a number of studies worldwide. Nevertheless, only

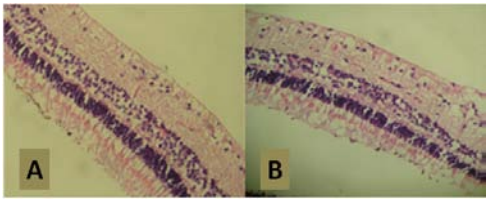

Figure 1: Hematoxylin and eosin preparation of the retinal tissue from a pig belonging to group D at 10 x magnifications. A) Mid Periphery. B) Area Centralis. Note that in both slides, the normal retina architecture is conserved despite the intravitreal injection.

one (ranibizumab) is currently approved by the FDA as intravitreal treatment for the neovascular form of AMD [26].

In recent studies, the use of macrolides agents, such as rapamycin, have been described. Guba et al, demonstrated that rapamycin was able to inhibit the growth of primary and metastatic tumors, by reducing the biological action of VEGF [21]. A similar appreciation is seen in our study, but the reduction was not significant; moreover, the reduction of VEGF expression was greater in the bevacizumab group than in the rapamycin group alone. It is not clear to us why the expression levels were reduced in this group (group C), when bevacizumab has no known effect over the gene expression of VEGF. We speculate that a possible explanation of this result could be that the model of neovascularization used was not homogeneous. While we used a well established and validated model of choroidal neovascularization [27], the expression of VEGF in this model relies on a transient ischemia induced by the mechanical (rupture) and thermal injury of the Bruch's membrane [28]. VEGF expression is ischemia dependent; therefore, higher levels of ischemia are generally associated with higher expression and production of VEGF. In our study, although the induction of CNV was successful in all cases, not all laser burns ended in a clinically evident CNV. Retinal tissue in pigs in group C may have had lesser stimulus than in the other groups. This account for one of the limitations of our study and this assumption may have an effect in all our observations as well.

The failure of rapamycin alone in reducing the expression levels of VEGF may be explained by a lack of penetration of the retinal tissue by the drug or the use of an insufficient dose of the drug. Further studies are required in order to find the maximum tolerance of the retinal tissue to rapamycin exposure as well as to investigate its toxicity.

Finally, the use of the combined dose of bevacizumab and rapamycin yielded the most dramatic reduction in VEGF expression level. The mammalian target of rapamycin (mTOR) is increasingly recognized as a master regulator of fundamental cellular functions [29]. A central role of mTOR is the regulation of the HIF-1α-mediated VEGF production and it has been found to inhibit VEGF-driven endothelial proliferation and morphogenesis *in vitro* [29] The exact mechanism of the additive factor between both drugs is not clear, but a negative feedback signal of the VEGF receptor might be involved. Further studies are needed in order to rule out this possibility.

Acknowledgments

The authors thank Melanie Soberon for her help in the editing process.

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