Eur J Ophthalmol 2011; 21 (Suppl. 6): S75-S79 DOI: 10.5301/EJO.2010.6060

ORIGINAL ARTICLE

The effect of triamcinolone acetonide, sodium hyaluronate, and chondroitin sulfate on human endothelial cells: an in vitro study

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Purpose. *To report the in vitro effect of triamcinolone acetonide (TA), sodium hyaluronate (SH), chondroitin sulfate (CS), and their combination on human endothelial cells.*
Methods. *The antiangiogenic proprieties of 2 formulations of TA (TA-1 and TA-2), 2 formulations of glycosaminoglycan-containing viscoelastic agents (V-1 and V-2), and the association of TA-1 and V-1 were investigated. Their effect on angiogenesis was tested using human endothelial cells cocultured with human fibroblasts and myoblasts. After fixation and staining for CD31, a colorimetric output was obtained. A BCIP/NBT-buffered substrate allowed image analysis of tubule formation.*
Results. *Both formulations of TA significantly reduced tubule formation as compared with controls ($p<0.01$). Moreover, the antiangiogenic effect of TA-1 was maintained when combined with V-1 ($p<0.01$).*
Conclusions. *Triamcinolone acetonide alone or in combination with hyaluronate and chondroitin sulfate is able to significantly reduce human endothelial cell proliferation in an in vitro model.*

Key Words. *Angiogenesis, Chondroitin sulfate, Endothelial cells, Sodium hyaluronate, Triamcinolone acetonide*

Accepted: October 26, 2010

INTRODUCTION

Several lines of evidence suggest that many retinal pathologies are related to angiogenic processes and vascular permeability disorders. Clear examples of these are represented by choroidal neovascularization due to age-related macular degeneration and diabetic macular edema, which are the 2 most common causes of legal blindness in the developed world (1, 2). Vascular endothelial growth factor (VEGF) is an angiogenic peptide, which is prominently increased in response to hypoxia in retinal cells (3). Vascular endothelial growth factor is also known to be a potent vasopermeability enhancer and plays a pivotal role in the pathogenesis of macular edema and ocular neovascularization (4, 5). Inhibition of the pathogenic cascades triggered by VEGF has been the subject of many studies in recent years.

Both steroids and glycosaminoglycans (GAGs) such as hyaluronate have been shown to interfere with the angiogenic and hyperpermeability processes related to VEGF overexpression (6-9). Triamcinolone acetonide (9α-fluoro-16α-hydroxyprednisolone) (TA) is an intermediate-acting corticosteroid, which has been used periocularly or intravitreally for the treatment of choroidal neovascularization (CNV) and cystoid macular edema resulting from diabetic retinopathy (10-13). Recently, the anti-angiogenic properties of hyaluronate have been investigated in different in vitro and in vivo models (9,14). Local inhibition of angio-

genesis has been achieved in the preovulatory rat follicle by secretion of hyaluronic acid (8). Another study showed that intraarticular 1% sodium hyaluronate (SH) injection inhibited VEGF receptor-2 expression (14). Less is known about anti-VEGF proprieties of other GAGs, like chondroitin sulphate (CS). In a recent clinical study, we reported that a suspension of TA, CS, and SH was effective in the treatment of diabetic macular edema refractory to laser photocoagulation (12).

The aim of the present study was to verify the inhibiting effect of different formulations of TA, SH, and CS and their combination on human endothelial-vascular proliferation in an in vitro model.

MATERIALS AND METHODS

The effects on angiogenesis of 2 formulations of TA—benzyl alcohol preserved TA (TA-1) and TA preservative-free suspension (TA-2)—and 2 different viscoelastics containing SH and CS (V-1) and SH (V-2) were assessed using the Angiokit (TCS-CellWorks Ltd, Buckingham, UK) (15). This system uses human endothelial cells (HUVEC) cocultured with human fibroblasts and myoblasts in a 24-well plate containing optimized media supplied by the manufacturer. Endothelial cells, which initially form small islands in the culture matrix, subsequently begin to proliferate and then migrate through the matrix to form tubular structures. By the end of 2 weeks, they merge to form a network of anastomosing tubules closely resembling a capillary bed. Human endothelial cells were cultured in a 24-well plate within a matrix of human diploid fibroblasts of dermal origin in optimized medium supplied by the company. The cocultured cells were incubated throughout the experiment at 37°C under 5% CO_2 in a humidified incubator. On days 1, 4, 7, and 9, the culture medium was replaced with 1) fresh medium containing TA-1 or TA-2 (2.66 mg/mL) or 2) V-1 (SH 1 mg/mL and sodium CS 1.33 mg/mL) and V-2 (SH 1 mg/mL) or 3) the association between TA-1 and V-1. Suramin (20 µM) and VEGF (2 ng/mL) were used respectively as an antiangiogenic and proangiogenic control. Each compound was tested in triplicate. On day 11, cells were washed (phosphate-buffered saline [PBS]) and 70% ethanol (1 mL) was added to each well for 30 minutes to fix the cells. After fixation, the cells were washed with blocking buffer (1 mL, PBS plus 1% BSA; Sigma, UK) and stained for CD31 (a specific human cell surface antigen also known as PECAM-1) according to the manufacturer's instructions (TCS-CellWorks). Using a primary mouse antihuman CD31 monoclonal antibody linked to a soluble chromogenic substrate (secondary antibody: goat antimouse Ig and alkaline phosphatase-conjugated), Angiokit permitted, in the first stage, the generation of a rapid colorimetric output that allowed the visualization of tubule formation: color development was proportional to the degree of tubule formation. Then supernatant was removed from the wells and transferred to a labeled 96-well microplate for absorbance reading (405 nm) in a standard microplate reader. Because color development used a nondestructive substrate (p-NPPY buffered substrate), in the second phase of the assay, Angiokit wells were washed and stained using the second substrate (BCIP/NBT-buffered substrate) that stained the tubules in situ for subsequent image analysis. The extent of tubule formation was evaluated using a Leitz Orthoplan light microscope and quantified using computerized image analysis (Angiosys; TCS-CellWorks). Images were acquired with a Leica DC300 F camera and processed by dedicated Windows software (Leica IM 4, Venice, Italy).

RESULTS

The Angiokit enabled both a quantitative lecture of the formation of vessels and their visualization (Fig. 1). As shown in Figure 2, the treatment of HUVEC cells with both formulations of TA (TA-1 or TA-2) significantly reduced tubule formation as compared with untreated controls ($p<0.01$). In addition, the antiangiogenic effect of TA-1 was maintained when combined with V-1 ($p<0.01$). The observed reductions were comparable to that caused by 20 µM Suramin, used as a positive antiangiogenic standard. No significant angiogenesis reduction was caused by V-1 or V-2.

DISCUSSION

A large body of evidence has demonstrated that many retinal conditions are related to abnormal endothelial cell behavior. Triamcinolone acetonide is known to inhibit vascular proliferation and improve blood-retinal barrier both in in vitro and in vivo models (6, 7). In the present study, we have shown in an in vitro model of human endothelial cells that both preserved TA (TA-1) and TA preservative-free suspension (TA-2) effectively reduced tubule formation. The 2

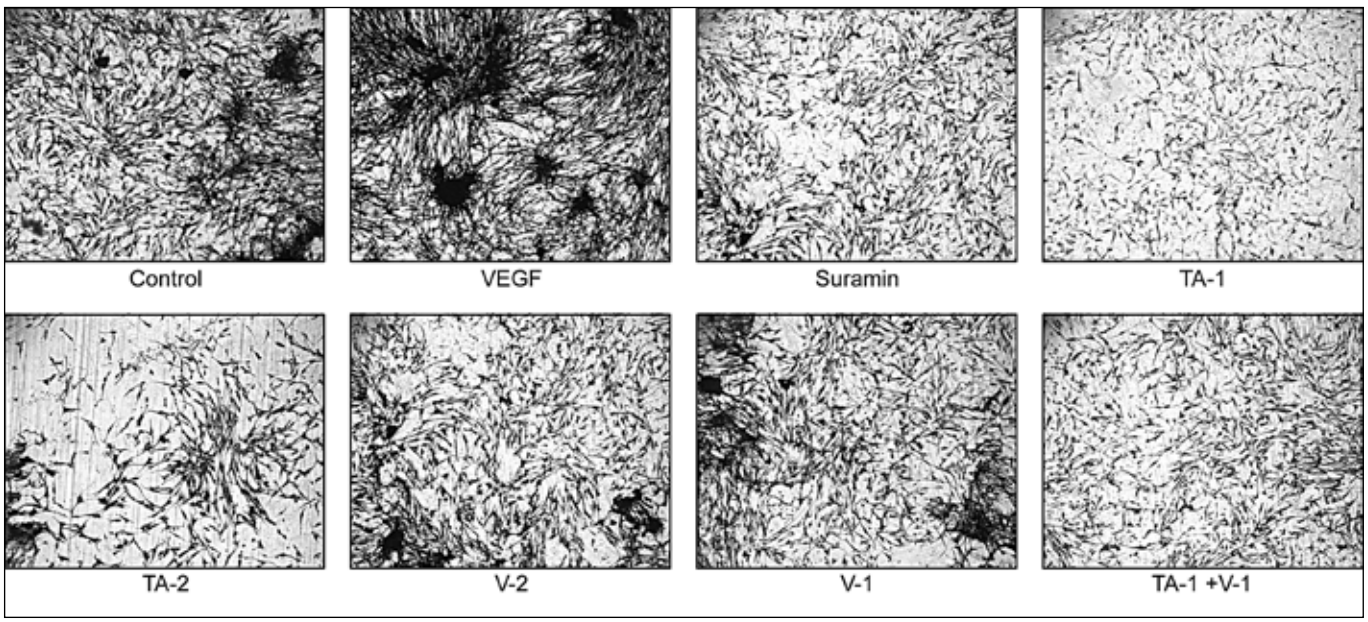

Fig. 1 - *The in vitro effect of the tested formulations on human endothelial cells. Pictures were obtained at 12.5 × 0.04 (magnification/aperture). TA = triamcinolone acetonide; V = viscoelastic; VEGF = vascular endothelial growth factor.*

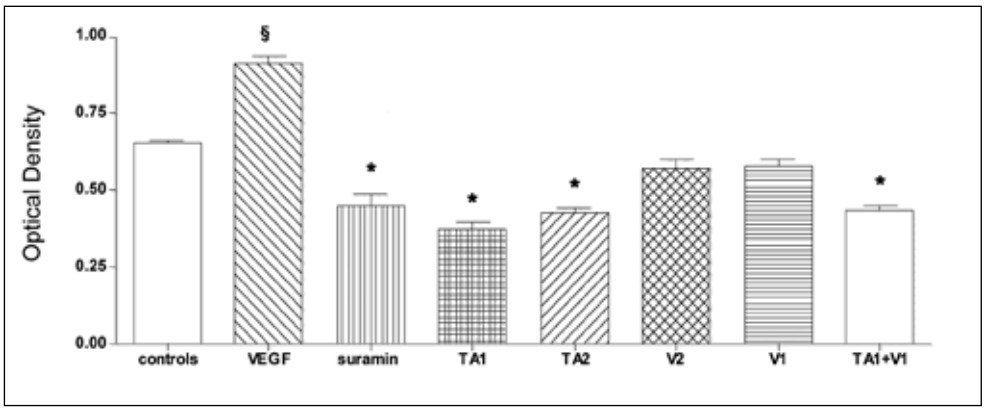

Fig. 2 - *Sample's optical density measured as absorbance reading at 405 nm. *Statistically significant difference between treated and untreated cells (p<0.01), whereas a mean value marked with § was significantly different from all conditions (p<0.01) (analysis of variance, Tukey-Kramer test). TA = triamcinolone acetonide; V = viscoelastic; VEGF = vascular endothelial growth factor.*

TA formulations have different physical properties. A previous phase-contrast microscopy study showed a notable heterogeneity of crystal size in TA-1 formulation, with very large and irregular crystals occasionally found, whereas the crystals of TA-2 appeared to be relatively uniform in size (16). These morphologic aspects may have a significant impact on the half-life of the drug both in vivo and in vitro. This hypothesis is based on the fact that smaller crystals have a superior surface area to volume ratio, allowing them to be dissolved more rapidly. The formulation containing crystals that widely vary in size, and thus including larger crystals, may theoretically grant a wider time drug-concentration curve because of their slower dissolution. In the present study, we found that both TA-1 and TA-2 had a statistically significant effect in reducing the endothelial cell proliferation. Although the anti-angiogenic effect of the two TA formulations tested was similar, a notable difference in the distribution of the endothelial cells was found when looking at the light microscope images of TA-1 and TA-2 samples. The first sample showed a uniform reduction of endothelial cells, whereas TA-2 exhibited a less homogeneous decrease of those cells. This may be

attributable to a better diffusion of the TA-1 formulation throughout the medium. Also, we found that the presence of benzyl alcohol as a preservative in TA-1 did not show any sign of toxicity on the cultured cells. Other authors have previously shown volume-related electroretinogram and structural damage of TA vehicles in rabbit retina when injected intravitreally (17-19).

Hyaluronate is a nonsulfated glycosaminoglycan composed of repeated D-glucuronic acid and D-N-acetylglucosamine disaccharides. Polymers of SH can range in size from 5 kDa to 20,000 kDa in vivo. SH has been shown to manifest biological activities apparently mediated through interaction with cell surface receptors such as CD44 and the receptor for hyaluronate-mediated motility (RHAMM), resulting in activation of intracellular signaling pathways (20, 21). As regarding its activity on endothelial cell proliferation, SH is thought to be dependent on the molecular mass. High molecular weight SH has shown anti-angiogenic proprieties, whereas small hyaluronate polymers induce angiogenesis both in vitro and in vivo (22, 23). Chondroitin sulfate is a sulfated glycosaminoglycan composed of a polymer of alternating monosaccharides (D-glucuronic acid and N-acetyl-D-galactosamine). Little is known about its biological effect on endothelial cell behavior. In our study, we tested the effect of 2 formulations of high molecular weight (MW) (>500 kDa) viscoelastics on endothelial cell proliferation. V-1 formulation was composed of SH (MW 500 kDa) and CS (MW 22.5 kDa). V-2 formulation consisted of SH with a MW of 3,000 kDa. Neither viscoelastic had significant antiangiogenic activity in vitro, although both V-1 and V-2 showed a reduction in endothelial cell density when compared to the control. One additional test was carried out employing the same suspension that we used, as juxtascleral depot, in a previous clinical study (12). The combination of TA-1 and V-1 showed a statistically significant inhibitory effect on endothelial cell proliferation in vitro. In our clinical experience, this formulation in a juxtascleral route demonstrated very favorable clinical and anatomic outcomes in the treatment of diabetic macular edema resistant to laser photocoagulation (12). Whether SH viscoelastics used via juxtascleral administration may have a direct antiangiogenic effect or rather act as a vehicle which increases the TA-sclera contact time in the retromacular space due to the improved viscosity needs to be evaluated further.

In conclusion, our study shows that TA alone or in combination with HA and CS is able to significantly reduce human endothelial cell proliferation in an in vitro model.

The authors report no proprietary interest or financial support.

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REFERENCES

1. Javitt JC, Zhou Z, Maguire MG, Fine SL, Willke RJ. Incidence of exudative age-related macular degeneration among elderly Americans. Ophthalmology 2003; 110: 1534-9.
2. Blindness caused by diabetes: Massachusetts, 1987–1994. MMWR Morb Mortal Wkly Rep 1996; 45: 937-41.
3. Miller JW, Adamis AP, Shima DT, et al. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 1994; 145: 574-84.
4. Bressler SB. Introduction: understanding the role of angiogenesis and antiangiogenic agents in age-related macular degeneration. Ophthalmology 2009; 116: S1-7.
5. Nicholson BP, Schachat AP. A review of clinical trials of anti-VEGF agents for diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol 2010; 248: 915-30.
6. Valamanesh F, Berdugo M, Sennlaub F, et al. Effects of triamcinolone acetonide on vessels of the posterior segment of the eye. Mol Vis 2009; 15: 2634-48.
7. Zhang X, Bao S, Lai D, Rapkins RW, Gillies MC. Intravitreal triamcinolone acetonide inhibits breakdown of the blood-retinal barrier through differential regulation of VEGF-A and its receptors in early diabetic rat retinas. Diabetes 2008; 57: 1026-33.
8. Ebrahem Q, Minamoto A, Hoppe G, Anand-Apte B, Sears

JE. Triamcinolone acetonide inhibits IL-6 and VEGF-induced angiogenesis downstream of the IL-6 and VEGF receptors. Invest Ophthalmol Vis Sci 2006; 47: 4935-41.
9. Tempel C, Gilead A, Neeman M. Hyaluronic acid as an anti-angiogenic shield in the preovulatory rat follicle. Biol Reprod 2000; 63: 134-40.
10. Katome T, Naito T, Nagasawa T, Shiota H. Efficacy of combined photodynamic therapy and sub-Tenon's capsule injection of triamcinolone acetonide for age-related macular degeneration. J Med Invest 2009; 56: 116-9.
11. Maberley D. Photodynamic therapy and intravitreal triamcinolone for neovascular age-related macular degeneration: a randomized clinical trial. Ophthalmology 2009; 116: 2149-57.
12. Veritti D, Lanzetta P, Perissin L, Bandello F. Posterior juxtascleral infusion of modified triamcinolone acetonide formulation for refractory diabetic macular edema: one-year follow-up. Invest Ophthalmol Vis Sci 2009; 50: 2391-7.
13. Beck RW, Edwards AR, Aiello LP, et al. Three-year follow-up of a randomized trial comparing focal/grid photocoagulation and intravitreal triamcinolone for diabetic macular edema. Arch Ophthalmol 2009; 127: 245-51.
14. Zhou JL, Liu SQ, Qiu B, Hu QJ, Ming JH, Peng H. Effects of hyaluronan on vascular endothelial growth factor and receptor-2 expression in a rabbit osteoarthritis model. J Orthop Sci 2009; 14: 313-9.
15. Kumar S, Witzig TE, Timm M, et al. Bone marrow angiogenic ability and expression of angiogenic cytokines in myeloma: evidence favouring loss of marrow angiogenesis inhibitory. Blood 2004; 104: 1159-65.
16. Chang LK, Gomes NL, Zhou J, Chang S. Physical properties of commercially available formulations of triamcinolone acetonide. Br J Ophthalmol 2009; 93: 1265-6.
17. Li Q, Wang J, Yang L, et al. A morphologic study of retinal toxicity induced by triamcinolone acetonide vehicles in rabbit eyes. Retina 2008; 28: 504-10.
18. Morrison VL, Koh HJ, Cheng L, et al. Intravitreal toxicity of the Kenalog vehicle (benzyl alcohol) in rabbits. Retina 2006; 26: 339-44.
19. Macky TA, Helmy D, El Shazly N. Retinal toxicity of triamcinolone's vehicle (benzyl alcohol): an electrophysiologic and electron microscopic study. Graefes Arch Clin Exp Ophthalmol 2007; 245: 817-24.
20. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 1990; 61: 1303-13.
21. Ghosh P, Guidolin D. Potential mechanism of action of intra-articular hyaluronan therapy in osteoarthritis: are the effects molecular weight dependent? Semin Arthritis Rheum 2002; 32: 10-37.
22. Slevin M, West D, Kumar P, Rooney P, Kumar S. Hyaluronan, angiogenesis and malignant disease. Int J Cancer 2004; 109: 793-4.
23. Slevin M, Kumar S, Gaffney J. Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. J Biol Chem 2002; 277: 41046-59.