Korean J Ophthalmol
Vol. 18:121-131, 2004

Effect of Basic Fibroblast Growth Factor-Saporin (bFGF-SAP) Conjugate on Bovine Choriocapillary Endothelial Cells

Yeong Hoon Kim, MD, Hwa Seon Kim*, Jin Seong Yoo, MD**

Department of Ophthalmology, St. Paul's Hospital, Catholic University Medical College,
**Department of Ophthalmology, Kangnam St. Mary's Hospital,*
Catholic University Medical College,
***Open St. Mary Eye Center, Seoul, Korea*

We evaluated the effect of a basic fibroblast growth factor (bFGF) and saporin conjugate (bFGF-SAP) on proliferation, migration and tubule formation in bovine choriocapillary endothelial cells (BCECs). Cell proliferation and MTS assays were done with 0.01, 0.1, 1, 10, and 100 nM bFGF-SAP, and an equimolar concentration of bFGF and saporin. TUNEL assay was performed to confirm apoptosis. Cells were treated with 1, 10, and 100 nM bFGF-SAP and migration assay and tubule formation assay were done. Results were evaluated with image analysis. All experiments were performed in triplicate and repeated three times. Viable cells (ID_{50} = 0.62) and cell proliferation by MTS assay (ID_{50} = 0.75 nM) were inhibited. Saporin caused cytotoxicity and inhibition of proliferation at high concentration. DNA fragmentation was identified by TUNEL assay. Migration and tubule formation were also inhibited. All mechanisms responsible for neovascularization were inhibited, and this could be applied in the management of subretinal choroidal neovascularization (SRN).

Key words: basic FGF, saporin, choriocapillary endothelial cell, immunotoxin, choroidal neovascularization

INTRODUCTION

Life expectancy in the general population has improved progressively and the importance of age-related macular degeneration in ophthalmology has increased proportionally.[1-4] Subretinal neovascularization (SRN) may be caused by pathologic myopia and trauma in the younger population. Choroidal neovascular membrane, characterized by subretinal growth of choriocapillary endothelial cells, is the common determinant in these disorders.

Laser photoablation of SRN for prevention of future visual loss has been reported to be the most effective therapy for the management of SRN[5], but its own complications and limitations make it inappropriate in many cases.

Prevention based on environmental and nutritional factors related to the development of SRN are under investigation,[6-8] but methods that act directly on preformed neovascular membranes have not been reported so far. At present, medicine is being used indirectly as an adjuvants in dye laser photocoagulation.[9-11]

Reprint requests to Yeong Hoon Kim, MD, Department of Ophthalmology, St. Paul's Hospital, Catholic University Medical College, 620-56 Jeonnong-dong, Dongdaemoon-ku, Seoul 130-709, Korea.

This study was presented in part at the Korean Ophthalmological Society Annual Meeting, 2003, Pusan, Korea.

The similarity of choroidal neovascularization to tumors has led researchers to anticancer therapy modalities. Immunotoxins are being used widely against various tumors, and have also been shown to have a significant inhibitory effect against choroidal neovascularization.[12,13] Immunotoxins are chimeric molecules in which toxins are conjugated to carriers. The carriers bind to specific receptors, and the toxins in the conjugate destroy the particular cell.[14,15]

Conjugates of the carrier basic fibroblast growth factor (bFGF) and toxin saporin act specifically on bFGF receptors that exist in choroidal neovascular membranes, resulting in high selectivity and relatively low retinal toxicity.[16-18]

With this background, we used bovine choriocapillary endothelial cells (BCECs) to study the effect of bFGF-SAP on *in vitro* neovascularization.

MATERIALS AND METHODS

1. Culture of bovine choriocapillary endothelial cells (BCECs)

BCECs and recombinant bFGF-SAP conjugate were kindly provided by Dr. Stephen J. Ryan at Doheny Eye Institute. BCECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM: Gibco) supplemented with 10% fetal bovine serum (FBS: Gibco, MD, USA). BCECs were confirmed to be vascular endothelial cells by positive immunostaining for von Willebrand factor (Santa Cruz Biotechnology, CA, USA) and binding of di1-acetylated-low density lipoprotein (LDL).[19] Fourth to seventh passaged cell were used.

2. Cell proliferation assay

BCECs (2×10^4) were seeded on 6-well culture plates and incubated for 24 hours in DMEM supplemented with 10% FBS until a confluency of 70-80% was observed. Various concentrations of bFGF-SAP (0.01, 0.1, 1, 10, 100 nM), an equimolar concentration of bFGF (R&D Systems Inc, MN, USA) or an equimolar concentration of saporin (Sigma, MO, USA) were then added and the mixture was further cultured for 72 hours. Viable cell numbers were finally counted by trypan blue exclusion. Every experiment was done in triplicate and repeated 3 times.

3. MTS assay

BCEC suspensions were seeded (5×10^3 cells/well) on 96-well culture plates, and then each well was further filled to a volume of 100 μl with 10% FBS supplemented with DMEM. The cells were incubated for 24-48 hours until a confluency of 70-80% was observed. Various concentrations of bFGF-SAP (0.01, 0.1, 1, 10, 100 nM), an equimolar concentration of bFGF (R&D Systems Inc, MN, USA) or an equimolar concentration of saporin (Sigma, MO, USA) were then added and the mixture was further incubated for 72 hours. Cell Titer 96 Aqueous 1 solution (Promega, WI, USA) was added (20 μl/well) at 37°C for 2-4 hours. Absorption at 490 nm was measured in a 96-well plate reader. Every experiment was done in triplicate and repeated 3 times.

The effect of serum bFGF on bFGF-SAP was also evaluated by MTS assay. Cells showing a confluency of 70-80% were incubated under FBS free and 10% FBS conditions, and then various concentrations of bFGF-SAP (0.01, 0.1, 1, 10, 100 nM) were added. MTS assay was done as described above.

Every experiment was done in triplicate and repeated 3 times.

4. Identification of apoptosis by TUNEL fluorostaining

BCECs (2×10^4) were seeded on 6-well culture plates and incubated for 24 hours in DMEM supplemented with 10% FBS until a confluency of 70 - 80% was observed. Then 10 nM bFGF-SAP was added before further incubation for 72 hours. TUNEL fluorostaining was performed using the ApopTag Fluorescein Kit (Intergen, NY, USA) as described in the user's manual. Briefly, cells were dried at room temperature and then fixed with 4% paraformaldehyde solution (4% in phosphate buffered saline: PBS, pH 7.4) at room temperature for 10 minutes. After washing with PBS, the samples were left in ice (4°C) for 2 minutes with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). The samples were dried and 50 μl of TUNEL reaction mixture was added before reaction

proceeded at 37°C for 1 hour in a humidified chamber. Photographs were taken after washing 3 times with PBS.

Every experiment was done in triplicate and repeated 3 times.

5. Cell migration assay

BCECs (5×10^5) were seeded on 35 mm dishes and incubated until confluence. A spatula was used to denude half the area of each dish, and the margin was marked under each dish. Various concentrations of bFGF-SAP (1, 10, 100 nM) were added and incubated for 72 hours. Microphotographs were taken with an Axiovert S100 phase contrast microscope (Carl Zeiss, NY, USA).

Photoshop V6.0 was used to open the micrographs, and migration assay was performed using a method derived from a technique reported by Lehr et al.[20]

A premarked margin was used as a reference line, and the area in which cells were present was selected. The dimension of this area was measured using the Histogram function of Photoshop. The total area of the whole photograph was also taken as a reference and the relative migration area was calculated as the percentage ratio of the cellular area to this reference value. Percentage migration was quantified by calculating the ratio of the experimental migration area to the control migration area.

Every experiment was done in triplicate and repeated 3 times.

6. Microtubule formation assay

Three-dimension collagen gel mixture [7 volumes of rat tail type I collagen (Roche, Germany), 2 volumes of 5 times concentrated DMEM and 1 volume of 200 mM HEPES (Gibco); pH fixed at 7.0 with 1 N NaOH] was added to 24-well plates (0.4 ml/well) and incubated at 37°C for 3 hours. After polymerization of the gels, BCECs (2×10^4) were seeded on each gel and incubated until a confluency of about 70-80% was observed. Various concentrations of bFGF-SAP (1, 10, 100 nM) were added and incubated for 24 hours. Microphotographs of the tubule-like formations were taken with a phase contrast microscope. Modified image analysis methods used in the evaluation of tubules were applied.[21-23]

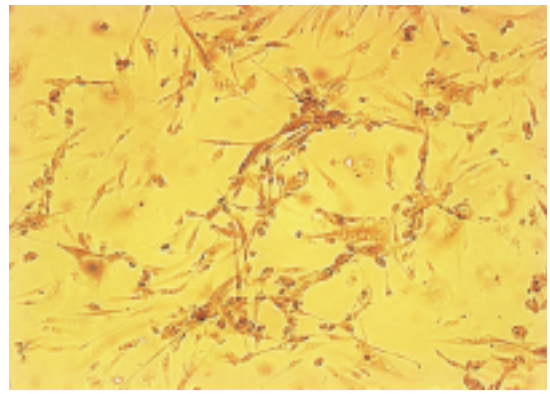

Fig. 1. Characterization of bovine choriocapillary endothelial cells. Bovine choriocapillary endothelial cells showed dark brown colored positive immunostaining for von Willebrand factor. (magnification, ×10).

Photoshop was used to calculate the total dimension of the tubule-like structures as in the previous migration assay. Microtubule formation was quantified by calculating the percentage ratio of the experimental tubule formation area to the control tubule formation area.

Every experiment was done in triplicate and repeated 3 times.

7. Statistical analysis

Paired two-tailed t test (Sigmaplot, SPSS Inc., IL, USA) was used to statistically analyze the differences between the experimental and control groups. Nonlinear regression (SPSS, SPSS Inc.) was used to analyze cell proliferation and MTS assay.

RESULTS

1. The effect of bFGF-SAP on BCEC proliferation

The bFGF-SAP conjugate inhibited the cellular proliferation of BCEC in a dose-dependent manner. Nonlinear regression revealed a typical reverse S standard curve of inhibition. The ID_{50} was 0.62 nM (Fig. 1-A).

In contrast, bFGF alone stimulated cell proliferation ($P < 0.01$). At low concentration saporin had no significant effect ($P = 0.58$ at 0.05 nM), but showed

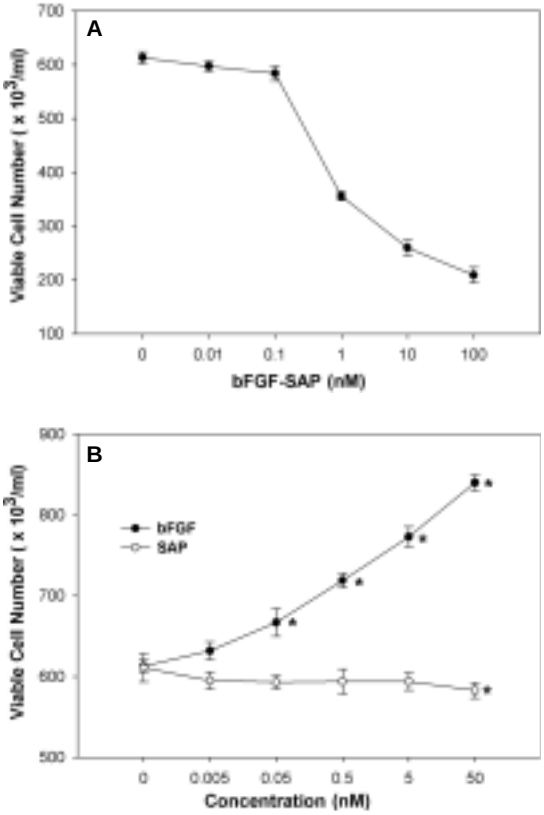

Fig. 2. Effect of bFGF-SAP, bFGF and saporin on bovine choriocapillary endothelial cells growth. A: bFGF-SAP showed a dose-dependent cytotoxicity to choriocapillary endothelial cells. Analysis by nonlinear regression using Sigmaplot V7.0 revealed a typical reverse sigmoid standard curve. ID_{50} was 0.62 nM. B: bFGF induced the proliferation of bovine choriocapillary endothelial cells dose-dependently. Saporin had no significant effect at lower concentrations, but showed cytotoxicity at a high concentration of 50 nM. Results are given as a mean S.D. * P < 0.01 compared with untreated control.

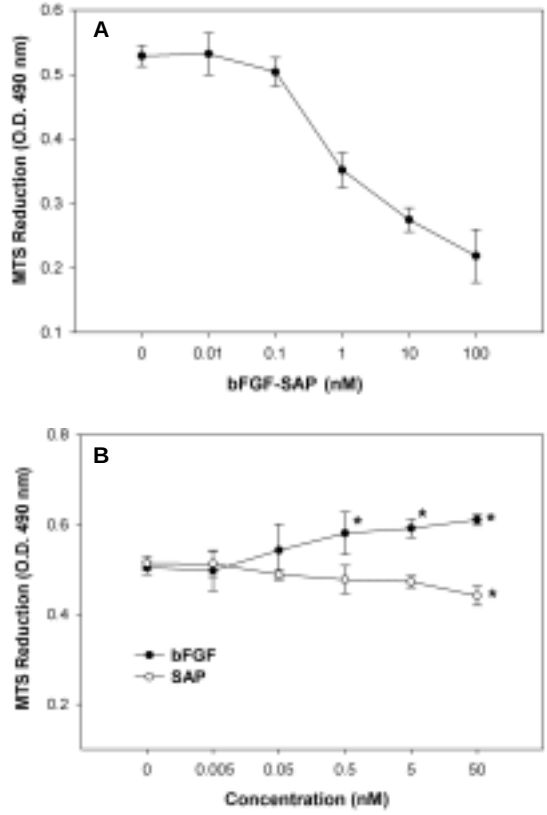

Fig. 3. Effect of bFGF-SAP, bFGF and saporin on bovine choriocapillary endothelial cells proliferation. A: bFGF-SAP showed dose-dependent inhibition of proliferation. Analysis by nonlinear regression using Sigmaplot V7.0 revealed a typical reverse sigmoid standard curve. ID_{50} was 0.75 nM. B: bFGF induced proliferation dose-dependently. Saporin showed inhibition of proliferation at a high concentration of 50 nM. Results are given as a mean S.D. * P < 0.01 compared with untreated control.

significant inhibition at high concentration (P < 0.01 at 50 nM) (Fig. 1-B).

2. MTS assay

The bFGF-SAP conjugate inhibited the proliferation and metabolism of BCEC in a dose-dependent manner. Nonlinear regression revealed a typical reverse S standard curve of inhibition (Fig. 2-A). The ID_{50} was 0.75 nM (Fig. 2-A).

In contrast, bFGF alone stimulated proliferation and metabolism (P < 0.01) at higher concentrations. Saporin showed significant inhibition at high concentration (P < 0.01 at 50 nM) (Fig. 2-B).

Meanwhile, bFGF-SAP showed significantly higher inhibition in the FBS-free group compared to the 10% FBS group. However, both groups showed dose-dependent inhibition (Fig. 3).

3. Apoptosis identification by TUNEL assay

Apoptosis was confirmed by TUNEL assay with the appearance of positive bright green fluorescence corresponding to DNA fragments (Fig. 4).

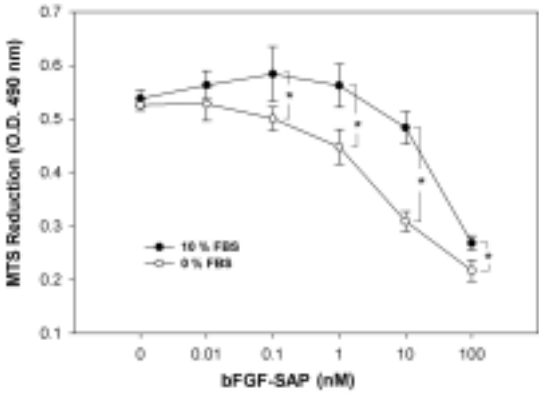

Fig. 4. Effect of serum on bFGF-SAP-induced inhibition of bovine choriocapillary endothelial cells. In comparison to the 10% FBS group, cell proliferation was decreased in the serum-free group at a given concentration of bFGF-SAP, but still showed a dose-dependent inhibition. The effect of serum was significant. Results are given as a mean S.D. * P < 0.01 compared between serum-free and 10% FBS at a given concentration of bFGF-SAP.

4. Migration assay

The cellular area of the control group (bFGF-SAP free) was 85.48 ± 2.74%, and that with bFGF-SAP at 1, 10, and 100 nM was 73.66 ± 4.09, 61.68 ± 5.07 and 56.21 ± 4.79%, respectively.

This corresponds to a significant dose-dependent inhibition of 13.8, 27.7 and 34.3% with bFGF-SAP at 1, 10 and 100 nM, respectively ($P < 0.01$) (Figs. 5, 6).

5. Microtubule formation assay

The control group (bFGF-SAP free) showed microtubule formation of 27.75 ± 3.37%. Microtubule formation of 24.46 ± 2.18, 10.38 ± 0.84 and 5.15 ± 0.56% was observed with bFGF-SAP at 1, 10, and 100 nM, respectively.

The bFGF-SAP conjugate at 1 nM inhibited microtubule formation by 11.85%, but was not significant. At 10 and 100 nM, significant inhibition of 62.58 and 81.42% was observed (P < 0.01) (Figs. 7, 8)

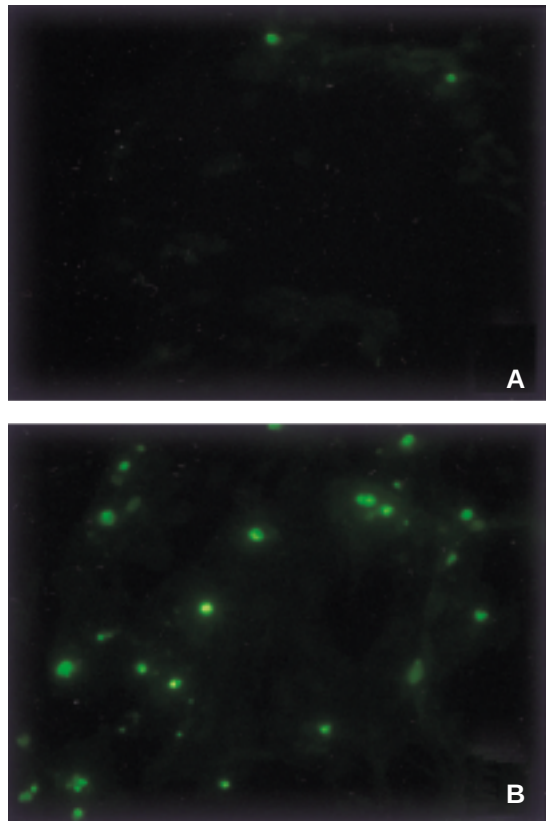

Fig. 5. bFGF-SAP-induced apoptosis of bovine choriocapillary endothelial cells. A: Control (bFGF-SAP-free). B: bFGF-SAP (10 nM). Cells positive for the TUNEL apoptosis marker were seen as green fluorescein labeled nuclei. (magnification, ×20).

DISCUSSION

Choriocapillary neovascularization into the subretinal space, or SRN, is one of the most important causes of visual loss, and the main lesion in exudative age related macular degeneration (ARMD). It can also develop in pathologic myopia and after trauma.[24-27]

The ideal treatment modality requires a complete destruction of the preformed neovascular membranes and prevention of its recurrence. Unfortunately, no definite treatment has been reported so far.

Direct excision of the neovascular membrane and radiologic therapies have been tried, but complica-

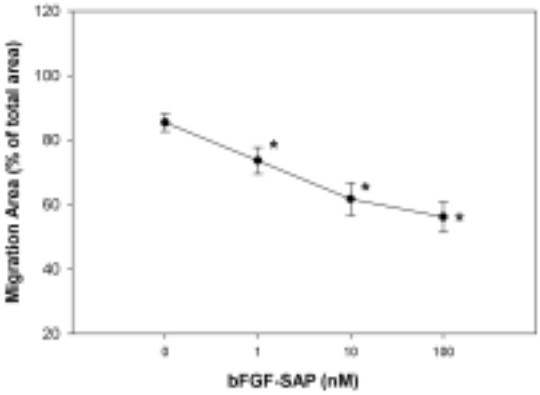

Fig. 6. Migration assay of bovine choriocapillary endothelial cells. Migration area represents cellular migration indirectly. The control group showed a migration area of 85.48%. bFGF-SAP showed a migration area of 73.66 ± 4.09, 61.68 ± 5.07 and 56.21 ± 4.79% at the concentration of 1, 10 and 100 nM, respectively. Inhibition rate was dose-dependently increased, showing 13.8, 27.7 and 34.3% inhibition at 1, 10 and 100 nM of bFGF-SAP, respectively. Results are given as a mean S.D. * $P < 0.01$ compared with untreated control.

tions are severe and their efficacy remains questionable.[28-31] Direct photoablation using laser is accepted as the most efficient treatment,[5] but its own complications and limitation of application impose a strict limit to cases that can be treated.

Preventive methods based on environmental and nutritional factors related to the development of SRN are under investigation,[6-8] but agents that act directly on preformed neovascular membranes have not yet been reported. At present, medicine is being used indirectly in the form of adjuvants in dye laser photoablation.[9-11]

SRN is similar to tumors due to its high rate of recurrence and rich vascularity. In this context, the application of antitumor agents is feasible. Several treatment modalities exist for the control of tumors, and with its emphasis on safety, efficacy and availability, the widely used immunotoxin has been considered to be the best candidate.[12,13,32-37]

Immunotoxins are chimeric molecules in which toxins derived from bacteria and plants are conjugated to carriers such as antibodies, hormones, and growth hormones. The carriers facilitate transport of the toxins, and also enhance efficacy and reduce

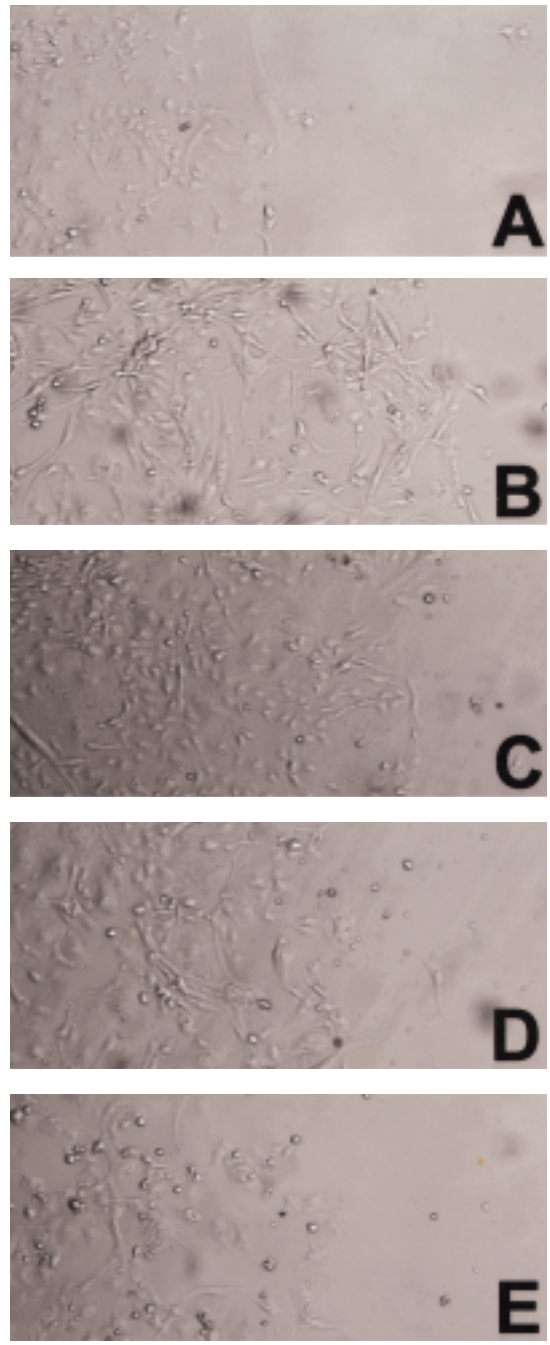

Fig. 7. Phase contrast photomicrograph of bovine choriocapillary endothelial cell migration. Migration area was dose-dependently decreased by bFGF-SAP. A: Initial control (bFGF-SAP-free) at day 0. B: Control after 72 hours culture. C: bFGF-SAP (1 nM). D: bFGF-SAP (10 nM). E: bFGF-SAP (100 nM). (magnification, ×10).

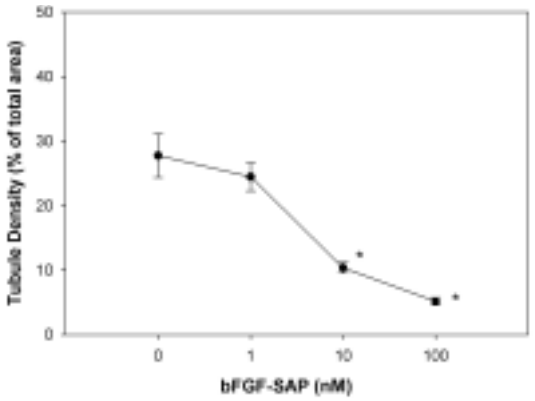

Fig. 8. Tubule formation assay of bovine choriocapillary endothelial cells. Tubule density represents tubule formation indirectly. The control group showed a tubule area of 27.75 ± 3.37%. bFGF-SAP showed a tubule density of 24.46 ± 2.18, 10.38 ± 0.84 and 5.15 ± 0.56% at the concentration of 1, 10 and 100 nM, respectively. Inhibition rate was dose-dependently increased, showing 11.84, 62.58 and 81.42% inhibition at 1, 10 and 100 nM of bFGF-SAP, respectively. Results are given as a mean S.D. * $P < 0.01$ compared with untreated control.

adverse effects by binding to specific receptors. After binding to these receptors, the toxins in the conjugate destroy the particular cell.[15]

At present immunotoxins are being used in various forms, and results are being continuously published. As with other treatment modalities, complications have also been reported.[38-40] However, these have appeared under systemic application, whereas local application would suffice in SRN. Clinical application would be easier.

In the field of ophthalmology, immunotoxins have been mostly studied for preventing the development of posterior capsular opacity in aftercataract.[41-43]

Studies involving neovascularization have usually used vessels that can be easily obtained. Although it is helpful in studying the pathogenesis or pathology of neovascularization, there is the problem of endothelial cells in different tissues showing complete phenotypic and antigenic heterogeneity.[44-48] This could lead to completely different results from similar experiments. In this respect Hoffmann et al have firmly suggested using choroidal endothelial cells in the study of choroidal diseases.[48]

It has been reported that bFGF is a potent mitogen and chemoattractant of various cells, and stimulates microtubule formation of endothelial cells in three-dimension collagen gel.[49,50]

Saporin is a type I ribosome inactivating protein extracted from the seeds of *Saponaria officinalis* that induces apoptosis by hydrolyzing rRNA.[15,40,51] It is composed of a single chain so its internalization is limited, but at high concentrations (10^{-8}-10^{-6} M) it shows some toxicity.[51-53] Highly resistant to degeneration and hydrolyzation, it can safely be conjugated to other carriers, facilitating its application.

The bFGF-SAP chimera is a conjugate of the carrier bFGF and the toxin saporin. It is highly specific to bFGF receptors and is also a potent competitor of bFGF. Apoptosis is responsible for cytotoxicity, thereby enabling the destruction of the pathologic tissue with minimal damage to adjacent normal tissues. At its effective inhibitory dose, it exerts minimal toxicity to normal tissues that do not express bFGF receptors.

A large number of bFGF receptors exist in choroidal neovascular membranes, so the bFGF-SAP conjugate acts specifically on these receptors, resulting in high selectivity and relatively low retinal toxicity.[13,54]

The cytotoxicity of bFGF-SAP was dose-dependent and its ID_{50} was 0.62 nM. The ability of bFGF alone to stimulate proliferation confirmed the existence of bFGF in BCEC. Single use of saporin showed significant inhibition only at high concentrations (50 nM). This agrees with other studies in which saporin alone was used, as internalization of the toxin is difficult.

MTS assay also showed a dose-dependent inhibition (ID_{50} = 0.75). Serum decreased the inhibitory effect of bFGF-SAP. Growth factors other than bFGF could have been responsible for this effect, but as bFGF-SAP acts specifically on bFGF receptors, it is highly probable that this inhibition results from competition with bFGF in the serum.[16] Regardless of the presence of serum, bFGF-SAP showed consistent dose-dependent inhibition.

The cytotoxicity of saporin is reported to be caused by apoptosis,[55] and this was confirmed by TUNEL assay.

Migration assay of endothelial cells requires a

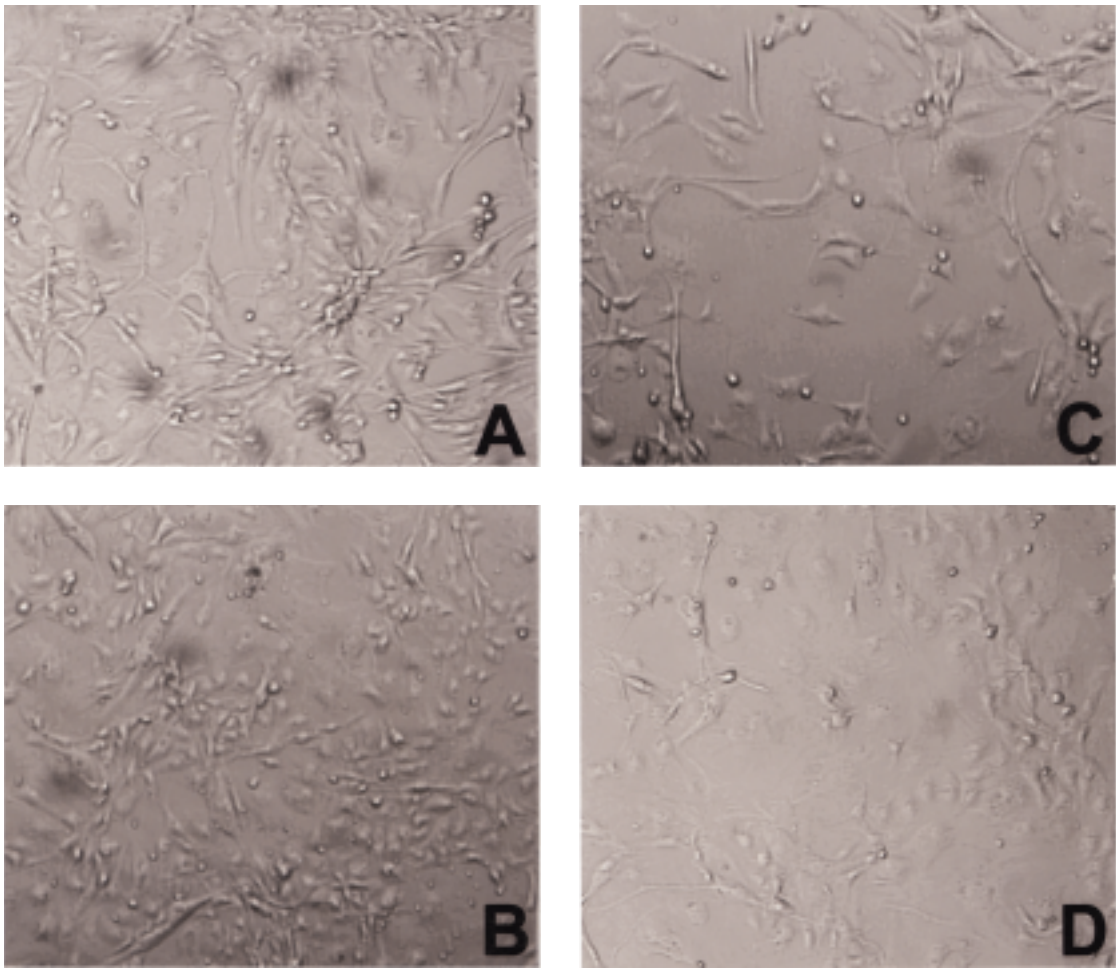

Fig. 9. Phase contrast photomicrograph of tubule formation. bFGF-SAP shows a dose-dependent inhibition of tubule formation. A: Control (bFGF-SAP-free). B: bFGF-SAP (1 nM). C: bFGF-SAP (10 nM). D: bFGF-SAP (100 nM). (magnification, ×15).

specific angiogenic factor, but BCECs exert contact inhibition resulting in spontaneous migration.[56] As no physical barrier was used, migration was dose-dependently inhibited through bFGF-SAP. In this study we evaluated migration inhibition using a modified technique with photographs and a graphics program (Photoshop).[20,57]

In contrast to other cultures, BCECs require a three-dimensional gel to form tubule-like structures. As with migration, tubule formation is spontaneous when a three-dimensional gel is used.[56,58] This may be the reason that some have reported contradictory results with similar experiments using different endothelial cells. We used an appropriate gel matrix, so the results were satisfactory and inhibition could be identified.

Image analysis programs or graphics programs such as Photoshop are widely used in the analyses of tubule-like structures.[20-23] The same technique as in the migration assay was used to evaluate the inhibition of tubule formation. Tubule formation was also inhibited in a dose-dependent manner.

Studies have revealed that SRN requires endothelial cell proliferation, migration and differentiation. In this experiment we confirmed that bFGF-SAP inhibits all these aspects responsible for the development of choriocapillary neovascularization.

In conclusion, bFGF-SAP is highly specific to

bFGF receptors and is a potent competitor to bFGF. Apoptosis is responsible for the cytotoxicity, thereby enabling the destruction of the pathologic tissue with minimal damage to adjacent normal tissues.

As previously mentioned, saporin is very stable, so carriers in the immunotoxin can be easily modified. This means that various receptors responsible for the development and growth of SRN can be targeted. As ARMD is suspected to have a multifactorial cause, various receptors may have to be simultaneously blocked. At present, VEFG and hGF (scatter factor) look very promising. A cocktail therapy with various carriers and saporin could be very effective.

Allowing these considerations, at this stage the true role of bFGF-SAP alone in the actual management of SRN has not yet been substantiated. However, we have confirmed that it plays an important background role in immunotoxin-related treatment modalities.

REFERENCES

1. Sarks SH. New vessel formation beneath the retinal pigment epithelium in senile eye. *Br J Ophthalmol*. 1973;57:951-965.
2. Teeters VW, Bird AC. A clinical study of the vascularity of senile disciform macular degeneration. *Am J Ophthalmol.* 1973;75:53-65.
3. Ryan SJ, Mittl RN, Maumenee AE. The disciform response: an historical perspective. *Graefe's Arch Clin Exp Ophthalmol*. 1980;215:1-20.
4. Green WR, Wilson DJ. Choroidal neovascularization. *Ophthalmology*. 1986;93:1169-1176.
5. Macular Photocoagulation Study Group. Argon laser photocoagulation for neovascular maculopathy after five years. *Arch Ophthalmol*. 1991;109:1109-1114.
6. Frank RN, Amin RH, Puklin JE. Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration. *Am J Ophthalmol.* 1999;127:694-709.
7. Jampol LM. Antioxidants, zinc, and age-related macular degeneration: results and recommendations. *Arch Ophthalmol.* 2001;119:1533-1534.
8. Jampol LM, Ferris FL 3rd. Antioxidants and zinc to prevent progression of age-related macular degeneration. *JAMA*. 2001;286:2466-2468.
9. Schmidt-Erfurth U, Miller J, Sickenberg M, Bunse A, Laqua H, Gragoudas E, Zografos L, Birngruber R, van den Bergh H, Strong A, Manjuris U, Fsadni M, Lane AM, Piguet B, Bressler NM. Photodynamic therapy of subfoveal choroidal neovascularization: clinical and angiographic examples. *Graefe's Arch Clin Exp Ophthalmol*. 1998;236:365-374.
10. Ladd BS, Solomon SD, Bressler NM, Bressler SB. Photodynamic therapy with verteporfin for choroidal neovascularization in patients with diabetic retinopathy. *Am J Ophthalmol.* 2001;132:659-667.
11. Bressler NM. Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: two-year results of a randomized clinical trial including lesions with occult with no classic choroidal neovascularization-verteporfin in photodynamic therapy report 2. *Am J Ophthalmol*. 2002;133: 168-169.
12. Pastan I, Willingham MC, Fitzgerald DJP. Immunotoxins. *Cell.* 1986;47:641-648.
13. Beitz JG, Davol P, Clark JW, Kato J, Medina M, Frackelton AR, Lappi DA, Baird A, Calabresi P. Antitumor activity of basic fibroblast growth factor-saporin mitotoxin in vitro and vivo. *Cancer Res*. 1992;52:227-230.
14. Siena S, Lappi DA, Bregni M, Formosa A, Villa S, Soria M, Bonadonna G, Gianni AM. Synthesis and characterization of an antihuman T-lymphocyte saporin immunotoxin (OKT1-SAP) with in vivo stability into nonhuman primates. *Blood*. 1988;72:756-765.
15. Santanche S, Belleli A, Brunori M. The unusual stability of saporin, a candidate for the synthesis of immunotoxins. *Biochem Biophys Res Commun*. 1997;234:129-132.
16. Lappi DA, Martineau D, Baird A. Biological and chemical characterization of basic FGF-saporin mitotoxin. *Biochem Biophys Res Commun*. 1989;160:917-923.
17. Lappi DA, Martineau DG, Nakamura S, Buscaglia M, Baird A. Basic fibroblast growth factor-saporin mitotoxin: an endothelial cell growth inhibitor. *J Cell Biochem.* 1990;14E (Suppl.):222.
18. Lappi DA, Maher PA, Martineau D, Baird A. The basic fibroblast growth factor mitotoxin acts through the basic fibroblast growth factor. *J Cell Physiol.* 1991;147:17-26.
19. Axel DI, Riessen R, Runge H, Viebahn R, Karsch KR. Effects of cerivastatin on human arterial smooth muscle cell proliferation and migration in transfilter cocultures. *J Cardiovasc Pharmacol.* 2000;35:619-629.
20. Lehr HA, van der Loos CM, Teeling P, Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-

based image analysis. *J Histochem Cytochem*. 1999; 47:119-126.
21. Matsumura T, Wolff K, Petzelbauer P. Endothelial cell tube formation depends on cadherin 5 and CD31 interactions with filamentous actin. *J Immunol*. 1997;158:3408-3416.
22. Murata T, He S, Hangai M, Ishibashi T, Xi XP, Kim S, Hsueh WA, Ryan SJ, Law RE, Hinton DR. Peroxisome proliferator-activated receptor-gamma ligands inhibit choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2000;41:2309-2317.
23. Yamakawa K, Bhutto IA, Lu Z, Watanabe Y, Amemiya T. Retinal vascular changes in rats with inherited hypercholesterolemia - corrosion cast demonstration. *Curr Eye Res.* 2001;22:258-265.
24. Kobayashi H, Kobayashi K. Radiotherapy for subfoveal neovascularisation associated with pathological myopia: a pilot study. *Br J Ophthalmol.* 2000;84 :761-766.
25. Cho J, Jun BK, Lee YJ, Uhm KB. Factors associated with the poor final visual outcome after traumatic hyphema. *Korean J Ophthalmol*. 1998;12:122-129.
26. Lim JI. Iatrogenic choroidal neovascularization. *Surv Ophthalmol*. 1999;44:95-111.
27. Hargrave S, Weakley D, Wilson C. Complications of ocular paintball injuries in children. *J Pediatr Ophthalmol Strabismus*. 2000;37:338-343.
28. Eckstein M, Wells JA, Aylward B, Gregor Z. Surgical removal of non-age-related subfoveal choroidal neovascular membranes. *Eye*. 1998;12:775-780.
29. Wolf S, Lappas A, Weinberger AW, Kirchhof B. Macular translocation for surgical management of subfoveal choroidal neovascularizations in patients with AMD: first results. *Graefe's Arch Clin Exp Ophthalmol.* 1999;237:51-57.
30. Jaakkola A, Heikkonen J, Tarkkanen A, Immonen I. Visual function after strontium-90 plaque irradiation in patients with age-related subfoveal choroidal neovascularization. *Acta Ophthalmol Scand.* 1999;77:57-61.
31. Flaxel CJ, Friedrichsen EJ, Smith JO, Oeinck SC, Blacharski PA, Garcia CA, Chu HH. Proton beam irradiation of subfoveal choroidal neovascularisation in age-related macular degeneration. *Eye*. 2000;14: 155-164.
32. Davol P, Beitz JG, Mohler M, Ying W, Cook J, Lappi DA, Frackelton AR. Saporin toxins directed to basic fibroblast growth factor receptors effectively target human ovarian teratocarcinoma in an animal model. *Cancer*. 1995;76:79-85.
33. Davol P, Frackelton AR. The mitotoxin, basic fibroblast growth factor-saporin, effectively targets human prostatic carcinoma in an animal model. *J Urol*. 1996;156:1174-1179.
34. Chandler LA, Sosnowski BA, McDonald JR, Price JE, Aukerman SL, Baird A, Pierce GF, Houston LL. Targeting tumor cells via EGF receptors: selective toxicity of an HBEGF-toxin fusion protein. *Int J Cancer*. 1998;78:106-111.
35. Sforzini S, de Totero D, Gaggero A, Ippoliti R, Glennie MJ, Canevari S, Stein H, Ferrini S. Targeting of saporin to Hodgkin's lymphoma cells by anti-CD30 and anti-CD25 bispecific antibodies. *Br J Haematol*. 1998;102:1061-1068.
36. Pastan I, Kreitman RJ. Immunotoxins for targeted cancer therapy. *Adv Drug Deliv Rev*. 1998;31:53-88.
37. Davol PA, Frackelton AR. Targeting human prostatic carcinoma through basic fibroblast growth factor receptors in an animal model: characterizing and circumventing mechanisms of tumor resistance. *Prostate*. 1999;40:178-191.
38. Scott CF, Lambert JM, Goldmacher VS, Blattler WA, Sobel R, Schlossman SF, Benacerraf B. The pharmacokinetics and toxicity of murine monoclonal antibodies and of gelonin conjugates of these antibodies. *Int J Immunopharmacol.* 1987;9:211-225.
39. Ghetie V, Vitetta E. Immunotoxins in the therapy of cancer: from bench to clinic. *Pharmac Ther*. 1994; 63:209-234.
40. Bolognesi A, Tazzari PL, Olivieri F, Polito L, Falini B, Stirpe F. Induction of apoptosis by ribosome-inactivating proteins and related immunotoxins. *Int J Cancer*. 1996;68:349-355.
41. Behar-Cohen FF, David T, D'Hermies F, Pouliquen YM, Buechler Y, Nova MP, Houston LL, Courtois Y. In vivo inhibition of lens regrowth by fibroblast growth factor 2-saporin. *Invest Ophthalmol Vis Sci.* 1995;36:2434-2448.
42. Wee WR, Parandoosh Z, Sakamoto T, Caton M, Nova M, McDonnell PJ. Antiproliferative effect of basic fibroblast growth factor-saporin mitotoxin on keratocytes in culture. *Korean J Ophthalmol*. 1996;10:1-7.
43. Bretton RH, Swearingen A, Kash RL, Cooley R. Use of a polylysine-saporin conjugate to prevent posterior capsule opacification. *J Cataract Refract Surg*. 1999;25:921-929.
44. Jaffe EA. Cell biology of endothelial cells. *Hum Pathol*. 1987;18:234-239.
45. Kumar S, West DC, Ager A. Heterogeneity in endothelial cells from large vessels and microvessels. *Differentiation*. 1987;36:57-70.
46. Fajardo LF. The complexity of endothelial cells: a

review. *Am J Clin Pathol.* 1989;92:241-250.
47. Page C, Rose M, Yacoub M, Pigott R. Antigenic heterogeneity of vascular endothelium. *Am J Pathol*. 1992;141:673-683.
48. Hoffmann S, Spee C, Murata T, Cui JZ, Ryan SJ, Hinton DR. Rapid isolation of choriocapillary endothelial cells by Lycopersicon esculentum-coated Dynabeads. *Graefe's Arch Clin Exp Ophthalmol*. 1998;236:779-784.
49. Baird A. Fibroblast Growth Factors: What's in a name? *Endocrinology*. 1993;132:487-488.
50. Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. *Lab Invest*. 1993;69:508-517.
51. Stirpe F, Bailey S, Miller SP, Bodley JW. Modification of ribosomal RNA by ribosome-inactivating proteins from plants. *Nucleic Acids Res*. 1988;16:1349-1357.
52. Perkins AC, Pimm MV, Baldwin RW. Demonstration of the hepatic uptake of radiolabelled immunotoxins using gamma scintigraphy. *Eur J Cancer Clin Oncol.* 1987;23:1225-1227.
53. Bergamaschi G, Cazzola M, Dezza L, Savino E, Consonni L, Lappi D. Killing of K562 cells with conjugates between human transferrin and a ribosome-inactivating protein (SO6). *Br J Haematol*. 1988;68:379-384.
54. Lappi DA, Baird A. Mitotoxins: Growth factor-targeted cytotoxic molecules. *Progress in Growth Factor Research*. 1990;2:223-236.
55. Bergamaschi G, Perfetti V, Tonon L, Novella A, Lucotti C, Danova M, Glennie MJ, Merlini G, Cazzola M. Saporin, a ribosome-inactivating protein used to prepare immunotoxins, induces cell death via apoptosis. *Br J Haematol.* 1996;93:789-794.
56. Morse LS, Sidikaro Y. Isolation and characterization of bovine choroidal microvessel endothelium and pericytes in culture. *Curr Eye Res.* 1990;9:631-642.
57. Chapman N, Witt N, Gao X, Bharath AA, Stanton AV, Thom SA, Hughes AD. Computer algorithms for the automated measurement of retinal arteriolar diameters. *Br J Ophthalmol.* 2001;85:74-79.
58. Sakamoto T, Sakamoto H, Murphy TL, Spee Christine, Soriano D, Ishibashi T, Hinton DR, Ryan SJ. Vessel formation by choroidal endothelial cells in vitro is modulated by retinal pigment epithelial cells. *Arch Ophthalmol.* 1995;113:512-520.