Cytotoxic Effects of FGF2–Saporin on Bovine Epithelial Lens Cells In Vitro

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Purpose. To test the ability of two preparations of FGF2–saporin, either FGF2 chemically conjugated to saporin (FGF2–SAP) or genetically engineered FGF2–saporin (rFGF2–SAP) to inhibit the growth of bovine epithelial lens (BEL) cells in vitro when in solution and when immobilized on heparin surface-modified (HSM) polymethylmethacrylate (PMMA) intraocular lenses (IOLs).

Method. Bovine epithelial lens cells were incubated with various concentrations of FGF2–saporin for as long as 4 days. The number of surviving cells was determined by counting the number of nuclei. Because FGF2 binds to heparin, FGF2–saporin was incubated with HSM PMMA IOLs; excess toxin was washed off, and the BEL cells were grown on the FGF2–saporin-treated IOLs (HSM and non-HSM) for 4 days. Cell density was determined by image analysis.

Results. Both FGF2–SAP and rFGF2–SAP were highly cytotoxic (nM range), with rFGF2–SAP 10 times less active than FGF2–SAP. FGF2–saporin bound to the surface of HSM IOLs and eluted by 2M NaCl retained its activity. Toxin bound to HSM IOLs killed more than 90% of the BEL cells placed on the IOL surface within 4 days. The ability of FGF2–saporin to prevent the growth of cells on the IOL surface was strictly dependent on the presence of heparin on the IOL.


In 18% to 50% of patients who undergo surgery for lens extracapsular extraction (ECE), posterior capsule opacification develops within the next 2 to 5 years.1,2 Opacification occurs sooner in younger patients, probably because of the greater proliferative potential of the epithelial cells in young persons.3 Laser Nd:YAG capsulotomy can be performed to restore visual acuity after surgery.4,5 Opening the posterior capsule, however, can lead to severe complications, such as retinal detachment, macular edema and a transient, or persistent, refractory increase in intraocular pressure.6–8 Capsule opacification in humans is caused by the proliferation of residual cells over the equator of the lens and their migration onto the posterior lens capsule.

There they undergo transdifferentiation into fibroblast-like cells that retain the features of epithelial cells.9–13 An abortive attempt at lens fiber formation in the human eye is described clinically as the formation of Elschnig's pearls.14 Chemicals, mostly antimetotics, have been used to inhibit the proliferation and migration of these cells in vitro,15 but few reports have demonstrated clearly the potential benefit of these drugs for human eyes. Simply inhibiting cell proliferation is probably not enough to prevent further cell growth irreversibly.

We have demonstrated that basic fibroblast growth factor (FGF2) can be coupled to saporin (extracted from the plant Saponaria officinalis) and that this disulfide-linked FGF2–saporin (FGF2–SAP) is toxic to bovine epithelial lens (BEL) cells grown in culture.16 The FGF2–SAP is toxic to these cells because they bear receptors for FGF2, and the saporin blocks protein synthesis.17,18

Therefore, we examined the possibility that FGF2–SAP or recombinant FGF2–saporin (rFGF2–
SAP) could bind to the posterior lens capsule and so inhibit the proliferation of cells onto the capsule. Sodium hyaluronate is commonly used in intraocular surgery and is a potential vector for inserting FGF2–saporin into the capsular bag at the end of ECE of the lens. The effect of sodium hyaluronate (Healonid, Kabi–Pharmacia, Paris, France) on the toxicity of FGF2–saporin for BEL cells was tested in vitro.

We also investigated the capacity of heparin surface-modified (HSM) polymethylmethacrylate (PMMA) intraocular lenses (IOL) coated with FGF2–saporin to prevent the proliferation of epithelial lens cells over the surface of IOLs. Cytotoxic assays were performed on BEL cells cultured in multiwell plates, on bovine posterior capsules, and on PMMA IOLs.

MATERIALS AND METHODS

Toxic Agents

Saporin and disulfide-linked FGF2–saporin (FGF2–SAP) were prepared as previously described.17–19 Saporin was modified with SPDP and then reacted with FGF2 to form FGF2–SAP. The protein conjugate was purified by chromatography and heparin affinity chromatography. Recombinant FGF2–saporin (rFGF2–SAP) was constructed using the complete sequences of saporin and human FGF2, with a two-residue linker (ala-met) separating the two parent proteins. The saporin moiety was attached to the carboxyl terminus of the FGF2, and the inhibitory activity of the recombinant protein was similar to that of unconjugated saporin. rFGF2–SAP has a molecular mass of 46,900 kDa and contains 410 amino acids. Details of its construction will be published elsewhere.

Cell Culture

All cell culture reagents were purchased from Gibco BRL (Paisley, UK). Bovine epithelial lens cells were prepared as previously described20 and grown in Eagle’s minimum essential medium (MEM) supplemented with 6% fetal calf serum (FCS), 50 μg/ml gentamicin, 25 μg/ml amphotericin B, and 10 mg/ml glutamine at 37°C in a 5% CO2–95% air atmosphere. The cells were subcultured at a ratio of 1:2.

Effects of FGF2–SAP and rFGF2–SAP on Bovine Epithelial Lens Cells

Bovine epithelial lens cells (passages 13 and 14) were suspended in medium (MEM 6% FCS) and seeded in 24-well plates (Costar, Cambridge, MA) at 10 to 30 × 10^5 cells/well, and the cells were cultured for 24 hours. All determinations were performed in triplicate. Then, FGF2–SAP, rFGF2–SAP, FGF2 + saporin, or saporin alone were added to the medium (final concentrations: 0.01, 0.1, 1, 10, 100, or 1000 nM), and incubation was continued for 1, 2, 3, or 4 days. The number of living cells was counted after treating the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO) or after staining the nuclei with crystal violet.

Cultures to be stained with crystal violet were drained, rinsed with phosphate-buffered saline (PBS) to remove cell debris and dead cells, and incubated with 250 μl crystal violet per well for 30 minutes at 37°C. The stained nuclei were counted in a hemocytometer. For the MTT colorimetric test, the medium was removed and 250 μl MTT (0.2 mg/ml in MEM 6% FCS) was added to each well for 8 hours at 37°C. Two hundred fifty microliters of 0.07 N HCl in isopropanol was placed in each well, and the plates were shaken gently in the dark at room temperature for 20 minutes to dissolve the formazan crystals. The optical density (OD; 570 nm) was then read within 1 hour. A new standard curve was prepared for each experiment because of possible variations in enzymatic activity.

Effect of Exposure Time to FGF2–SAP and rFGF2–SAP

Bovine epithelial lens cells in MEM 6% FCS were grown in multiwell plates (3 × 10^5 cells/well) for 24 hours. The medium was replaced with medium containing FGF2–SAP (1 nM or 10 nM), and cells were incubated in this medium for 15, 30, or 60 minutes or 24 hours at 37°C. The cells were then washed three times with PBS and covered with fresh medium. They were grown in this medium for 4 days. The number of living cells was counted after staining nuclei and by the MTT test.

Proliferation of Bovine Epithelial Lens Cells on Bovine Posterior Lens Capsules Incubated With FGF2–SAP

The effect of FGF2–SAP bound to posterior lens capsules on the proliferation of BEL cells was tested. Bovine posterior lens capsules were excised and washed in double-distilled water and in 0.025 NaOH to remove any residual cells. The posterior capsules were incubated in 10 nM, 100 nM, or 1 μM FGF2–SAP in culture medium for 1 hour at 37°C. Control capsules were incubated in culture medium alone. The lens capsules were washed in PBS (3 × 10 minutes). Then, 10^5 BEL cells, suspended in culture medium, were placed on each capsule in a microwell plate, and the plates were incubated at 37°C for 4 days. The cells on the capsules were fixed in 4% paraformaldehyde, stained with hemalum–eosin, examined under the microscope, and photographed.

Effect of FGF2–SAP on Bovine Epithelial Lens Cells Cultured on Bovine Posterior Lens Capsules

The effect of FGF2–SAP on BEL cells cultured on untreated capsules was determined using excised,
washed, posterior lens capsules as above. After that, 10<sup>5</sup> BEL cells were suspended in 1 ml medium, placed on each capsule, and cultured for 24 hours. The medium was replaced with medium containing 10 nM FGF2-SAP, and the cultures were continued for 4 days. The cells were fixed, stained, examined under the microscope, and photographed.

**Effect of Hyaluronate on FGF2-Saporin Toxicity for Bovine Epithelial Lens Cells**

Bovine epithelial lens cells were distributed in the wells of multiwell plates (10<sup>4</sup> cells/well) in 1 ml medium and cultured for 24 hours. The cells were incubated for 4 days in medium containing 10 nM FGF2-SAP, rFGF2-SAP, 10nM FGF2-SAP, or rFGF2-SAP plus 250 µl/ml Healonid (sodium hyaluronate). Control cells were cultured in medium containing culturing 250 µl Healonid. Live cells was counted after nuclear staining with crystal violet.

**Proliferation of Bovine Epithelial Lens Cells on PMMA IOL (HSM and non-HSM) Coated With FGF2-SAP**

Sterile PMMA IOLs, both HSM and non-HSM (Pharmacia), were incubated in 1000 nM saporin or in 100 nM or 1000 nM FGF2-SAP (in PBS) and in PBS alone (controls) for 30 minutes at 20°C (nine IOLs per treatment). The IOLs were washed 3 times in PBS, placed 1/well in multiwell plates, and 10<sup>5</sup> BEL cells in 1 ml medium were added to and incubated with each lens for 24 hours. The lenses were incubated in fresh toxin-free medium for 4 days, fixed in 4% paraformaldehyde, stained with hemalum-eosin, and photographed. The cell density was quantified by digital image analysis (Historag*, Biocom 200, version 1.4 software). Results are expressed as the cumulated mean OD over the whole surface of the lens.

**Effect of FGF2-SAP Eluted From Heparin-Treated Lenses on Bovine Epithelial Lens Cell Culture**

Cytotoxin bound to a single heparin-treated IOL treated with FGF2-SAP or rFGF2-SAP was determined by eluting the bound toxin with a high ionic strength solution and assaying the eluted activity with BEL cells. HSM PMMA IOLs (three per treatment) were incubated in 10, 100, or 1000 nM FGF2-SAP or rFGF2-SAP in PBS for 10 minutes at room temperature, rinsed three times in PBS, and then incubated in 150 µl 2M NaCl for 3 minutes at room temperature to elute any bound FGF2-SAP. Bovine epithelial lens cells (10<sup>6</sup> in 1.5 ml medium/well) were grown for 24 hours in 24-well plates. Aliquots (150 µl/well) of high-salt eluate were added to these cells, and the plates were incubated for 4 days. Controls were incubated with 150 µl 2M NaCl. Surviving cells were counted after staining the nuclei. A toxin dose–response curve was prepared in parallel using BEL cells incubated with FGF2-SAP or rFGF2-SAP. This was used as the standard curve for estimating eluted toxin.

**RESULTS**

**Toxic Effects of FGF2-SAP on Bovine Epithelial Lens Cells In Vitro**

FGF2-SAP was toxic for BEL cells over the range of 1 to 100 nM. Low concentrations of FGF2-SAP (0.01 to 1 nM) induced proliferation of BEL cells after incubation for 24 hours. Cells showed signs of suffering, such as nuclear swelling and cytoplasmic vacuolation, from the second day, but there was no decrease in the number of cells per well incubated with 0.1, 1, and 10 nM FGF2-SAP (one-way analysis of variance and Bonferroni multiple comparison test; P > 0.05). There was a decrease in the number of cells per well incubated with 100 nM FGF2-SAP for 2 days (P < 0.001). Considerable cell death, with detachment from the support, occurred after 4 days in culture with FGF2-SAP concentrations above 1 nM (P < 0.001); few cells remained alive. The same results were obtained when cells were counted after MTT or nuclear staining (Figs. 1,2). Cells incubated with FGF2, FGF2 + saporin, or saporin alone showed no toxic effects.

**Toxic Effect of rFGF2-SAP on Bovine Epithelial Lens Cells in Culture**

rFGF2-SAP also had a dose-dependent toxic effect on BEL cells in vitro, but the lowest toxic concentration

**FIGURE 2.** Effect of FGF2-SAP, FGF2 + saporin, and saporin alone on bovine epithelial lens (BEL) cells in culture. BEL cells incubated for 4 days with FGF2-SAP showed a dosedependent cytotoxic response (1 to 100 nM). Cells were counted after nuclear staining, but the MTT test gave the same results. (*P < 0.001, analysis of variance and Bonferroni post hoc test). FGF2 + saporin or saporin alone have no effect.

was 10 nM. Hence, rFGF2-SAP was approximately 10-fold less toxic than FGF2-SAP (Fig. 3). Once again, FGF2 + saporin and saporin alone had no toxic effects.

**Effect of Incubation Time on Cytotoxicity**

The shortest contact time between cells and FGF2-SAP that resulted in cell death was determined by incubating cells with FGF2-SAP for varying periods, washing off excess drug, and measuring cell viability 4 days later. Bovine epithelial lens cells were killed by incubation with FGF2-SAP (1 or 10 nM) for 15 minutes at 37°C; 50% of the cells were dead 4 days later. The time courses of rFGF2-SAP and FGF2-SAP toxicity had the same profiles (Fig. 4).

**FIGURE 3.** Dose–response relationships for the toxicity of FGF2-SAP and rFGF2-SAP. The toxicities of FGF2-SAP and rFGF2-SAP for bovine epithelial lens (BEL) cells was measured after incubation for 4 days. The nuclei were stained and counted. The lowest toxic concentration of FGF2-SAP was 10 nM; it was 1 nM for FGF2-SAP. rFGF2-SAP toxicity was dose dependent over the range 10 to 100 nM.

**FIGURE 4.** Effect of incubation time on FGF2-SAP toxicity for bovine epithelial lens (BEL) cells. BEL cells were incubated with FGF2-SAP (1 nM and 10 nM) for as long as 24 hrs. The medium was then changed, and cells were incubated for 4 additional days. The living cells were quantified by MTT assay. Similar results were obtained with rFGF2-SAP.

**Inhibition of Bovine Epithelial Lens Cells Cultured on Bovine Posterior Lens Capsules Incubated With FGF2-SAP**

Bovine epithelial lens cells grown for 2 days on capsules treated with FGF2-SAP showed signs of toxicity, and there were vacuoles in the cytoplasm and nucleus (Figs. 5A to 5D). The cells died after 4 to 5 days in culture. Toxicity increased with the FGF2-SAP concentration in which the capsules had been incubated. Capsules incubated in 10 nM FGF2-SAP caused severe cell damage, but not cell death, after 4 days, whereas capsules incubated in 100 nM FGF2-SAP caused cell death and detachment after 4 days (Figs. 5E to 5H).

**Toxicity of FGF2-SAP for Bovine Epithelial Lens Cells Growing on Bovine Posterior Lens Capsules**

The majority of the cells grown on untreated capsules were killed by incubation with 10 nM FGF2-SAP. Few cells remained alive on the surface of the capsule after incubation for 4 days (Fig. 6).

**Interaction Between Hyaluronate and FGF2-SAP or rFGF2-SAP**

The influence of hyaluronate on the toxic action of FGF2-saporin was examined by assaying FGF2-SAP and rFGF2-SAP toxicity in the presence and absence of hyaluronate. Both FGF2-SAP and rFGF2-SAP had the same toxic effect on BEL cells when diluted in hyaluronate or PBS (Fig. 7). Thus, sodium hyaluronate did not interfere with the toxic activity of FGF2-saporin.

**Inhibition of Proliferation of Bovine Epithelial Lens Cells Cultured on PMMA IOLs**

HSM PMMA IOL and ordinary PMMA IOL were incubated with FGF2-SAP. Bovine epithelial lens cells
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FIGURE 5. Effect of treating capsules with FGF2-SAP on bovine epithelial lens (BEL) cell survival. Excised bovine posterior lens capsules were preincubated in 10 nM, 100 nM, or 1000 nM FGF2-SAP for 1 hour at 37°C and rinsed, and BEL cells were seeded onto the capsules and cultured for 4 days. Few BEL cells remained on the HSM PMMA IOLs that had been incubated in 10 or 100 nM FGF2-SAP, and there were no cells on the heparin-treated IOLs that had been incubated in 1000 nM FGF2-SAP. Daily observation of BEL cells on HSM-FGF2-SAP-coated IOLs showed that they became vacuolated in a way similar to cells in culture. This did not occur with non-HSM PMMA IOLs (Fig. 8a). Bovine epithelial lens cells proliferated to the same extent on the surfaces of control HSM-IOL and control non-HSM-IOL. The mean OD, which is proportional to the cell density on the surface of the lens, for control HSM and non-HSM PMMA IOL was similar. The OD on the surfaces of HSM-FGF2-SAP-coated IOLs were much lower, and the drops were dose-related (Fig. 8B). The OD of non-HSM FGF2-SAP-coated IOLs was the same as that of the control IOLs. Incubation of HSM-IOLs with 1000 nM saporin had the same effect as incubation with 100 nM FGF2-SAP (Fig. 8B). Treatment of HSM PMMA IOL with saporin alone seemed to prevent the adhesion of BEL cells rather than kill them. The few cells that survived had small cytoplasmic attachments and remained rounded up (results not shown).

Elution of FGF2-SAP and rFGF2-SAP From Heparin-Coated Lenses

The amount of active protein bound to a single IOL was estimated by assaying the toxin eluted from a single IOL. The FGF2-SAP or rFGF2-SAP eluted from the heparin-coated IOLs was toxic to BEL cells in a dose-dependent manner (Fig. 9). The eluate from IOLs incubated with 10 nM FGF2-SAP or 10 nM rFGF2-SAP gave two thirds of the maximal toxic effect, indicating that the heparin sites on the sur-
face of the lenses were saturated at this concentration.

**DISCUSSION**

The recombinant protein rFGF2–SAP is highly cytotoxic for BEL cells in vitro, as is the conjugated protein. However, it has approximately one tenth the activity of the disulfide-linked toxin for these cells. The difference is probably caused by the way in which the two proteins are processed within the cell (Tetzke and Parandoosh, unpublished data). The current results show that there is no significant difference between the chemically and genetically prepared proteins except for this slight difference in activity. The genetically engineered protein has the advantage of being a homogenous product readily manufactured and tested clinically.

It is particularly significant for future in vivo applications that the contact time between target cells and toxin need be as little as 15 minutes to produce efficient cell killing. Similar results have been obtained with conjugates of antibodies and other ribosome-inhibiting enzymes. Low concentrations of toxin (1 nM) have approximately the same toxicity after a 24-hour exposure and after a 30-minute exposure. The toxicity of high concentrations of toxin (10 nM) increases with exposure time. There appears to be no significant difference between effects of FGF2–SAP and rFGF2–SAP after short contact times. The extracellular matrix contains large concentrations of heparan sulfate, which can bind FGF2 and both the FGF2–SAP conjugates. As a result, the toxin may be stored in the extracellular matrix as a low-affinity complex between it and the heparan sulfate. The toxin may then be delivered to free high-affinity, cell-membrane FGF2 receptors in subsequent steps at a later time.

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The adhesion of cells to capsules treated with 100 nM toxin was normal, but the cells were killed on schedule. This suggests that FGF2–SAP is sequestered from high-affinity receptors on low heparan sulfate sites on the capsule extracellular matrix. Such sequestration would explain why a large excess of the toxin must be added to the capsule before adding cells. As might be expected, FGF2–SAP also efficiently killed BEL cells already growing on a posterior capsule. The concentration of FGF2–SAP needed to clear the capsule of all epithelial cells is the same as that found in the in vitro cytotoxic assays (10 nM) because of the higher affinity of the toxin for specific FGF2 receptors directly accessible when epithelial cells are already growing on the capsules. FGF2–SAP binds to the posterior lens capsule through low-affinity, heparan-like molecules and to cells through their high-affinity FGF2 receptors. Binding to the cell receptors results in internalization of the FGF2–SAP, blockage of protein synthesis by saporin, and cell death.

Healonid and other hyaluronic acid-containing preparations are used routinely in lens extraction procedures. If rFGF2–SAP is to be used to prevent lens opacification, hyaluronate may be a suitable vector for delivery to the capsular bag if ECE is performed without IOL implantation. The current results indicate that hyaluronate does not alter the toxic effects of FGF2–SAP or rFGF2–SAP on BEL cells in vitro. Hence, the use of Healonid for intraocular surgery is compatible with the use of rFGF2–SAP and should not interfere with the biologic action of the drug.

Immobilization of the drug on heparin-coated lenses could be an excellent way to deliver the drug at the time of IOL implantation. Preincubation of heparin-coated lenses with FGF2–SAP causes the death of BEL cells that have proliferated on the surface of the lenses. The residual number of living cells on the lens surface is minimal after preincubation with 100 nM or 1000 nM FGF2–SAP. But there was no cytotoxic effect with non-HSM IOLs. Drug bound to heparin sites on the lens surface seems to be released and subsequently bound to specific FGF2 receptors on epithelial cells. Further study is needed to find ways of improving and controlling the binding of FGF2–SAP to heparin sites on IOL. The in vitro toxicity of FGF2–SAP-coated HSM IOL for the proliferation of BEL cells could be of considerable practical significance because a surface-modified IOL could function as the drug release system for a specific cytotoxic. The hydrophilic nature of HSM-PMMA lenses reduces the adhesion of platelets, monocytes, and fibroblasts, as well as the appearance of long-term deposits on the lens surface.24,25 HSM IOLs are useful in complicated surgery (combined glaucoma and cataract surgery), in inflamed eyes (uveitis), and in non-white people.28 To our knowledge, HSM PMMA IOLs have never been shown to reduce epithelial lens cell proliferation in vitro or to prevent secondary cataract in vivo. Treating heparin surface-modified PMMA IOL with FGF2–SAP may allow this. Its action is caused not by the increased hydrophilicity of the material, but to the activity of

FIGURE 8. Effect Of Heparin surface-modification on the binding of FGF2-SAP to IOLs. Heparin surface-modified (HI) PMMA IOLs and normal PMMA IOLs (I) were preincubated with 100 nM or 1000 nM FGF2-SAP (FS100, FS1000) or 1000 nM saporin (S1000). Bovine epithelial lens (BEL) cells were seeded on the lens surface and grown for 4 days in basic medium. The cells were fixed with 4% paraformaldehyde and stained with hemalum–eosin. (A) Photomicrographs of fixed, stained BEL cells grown on treated PMMA IOLs. (B) Digital image analysis of the cell density on the corresponding PMMA IOLs. The cumulative optical density measured by Biorag (Biocom*) was proportional to the cell density on the surface of the lens (nine lenses per treatment).

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the toxin on the cells bound to the lens. The results obtained with FGF2–SAP-coated HSM IOL could reduce the deposition of cells on the IOL surface and prevent the proliferation of epithelial lens cells when the treated IOL is implanted in the capsule bag.

The treatment of HSM-PMMA lenses with saporin seems to reduce the adhesion of BEL cells to the surface of the lens. The few cells observed on the IOL surface remained round up but contained no cytoplasmic vacuoles. Perhaps HSM PMMA IOL could be treated with saporin to potentiate the anti-adhesive properties of HSM IOL. The fact that the toxic activity of FGF2–SAP eluted from IOLs preincubated with FGF2–SAP does not depend on the concentration of FGF2–SAP suggests that the heparin sites on the HSM IOLs were saturated.

In conclusion, both FGF2–SAP and rFGF2–SAP are toxic for BEL cells in vitro and when they are bound to the surface of the posterior lens capsule or HSM IOLs. This suggests that FGF2–saporin can be used to inhibit the proliferation of epithelial lens cells in vivo, thereby preventing posterior capsule opacification after extracapsular extraction of the lens.

**Key Words**
epithelial lens cells, fibroblast growth factor 2–saporin, heparin-coated intraocular lens, posterior lens capsule, toxicity

**References**


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