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Effect of the cytostatic agent idarubicin on fibroblasts of the human Tenon's capsule compared with mitomycin C

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Abstract

***Background/aims*—To investigate the in vitro effect of a short time exposure to the anthracycline idarubicin on proliferation, protein synthesis, and motility of human Tenon's capsule fibroblasts in comparison with the antitumour antibiotic mitomycin C.**

***Methods*—After determination of effective concentrations of idarubicin, fibroblasts of the human Tenon's capsule were exposed to idarubicin or mitomycin C at concentrations ranging from 0.1 μg/ml to 1 μg/ml or from 2.5 μg/ml to 250 μg/ml, respectively, for 0.5, 2, or 5 minutes and cultured for 60 days. Cell death by apoptosis caused by idarubicin treatment was confirmed by Hoechst 33258 staining. Further proliferation was explored by cell counting and by ^{3}H-thymidine uptake. Protein synthesis was measured by ^{3}H-proline uptake and motility was assessed by agarose droplet motility assay.**

***Results*—Idarubicin is able to exert toxicity and to induce apoptosis during a short time exposure of 0.5 minutes at concentrations of 0.3–1 μg/ml resulting in a significant reduction in cell number compared with the control after 60 days. For mitomycin C, higher concentrations and longer expositions were necessary. Even after treatment with 1 μg/ml idarubicin or 250 μg/ml mitomycin C a few cells were able to incorporate ^{3}H-thymidine. ^{3}H-proline uptake up to 10 days after exposure to 0.3 μg/ml idarubicin was found not to be decreased. Cell motility was reduced after treatment with 1 μg/ml idarubicin for 5 minutes or with 250 μg/ml mitomycin C for 2 or 5 minutes. For low mitomycin C concentrations, an increase in motility was found during the first 10 days.**

***Conclusion*—Idarubicin reduces proliferation of human Tenons's capsule fibroblasts after incubation for 0.5 minutes at concentrations as low as 0.3–1 μg/ml. In comparison, mitomycin C requires longer exposure times and higher doses for equal results. Therefore, idarubicin may be useful in the prevention of glaucoma filtering surgery failure.**

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The goal of filtration surgery for glaucoma is to create an additional outflow channel for aqueous humour, producing a filtering bleb in the subconjunctival space. One important reason for failure is the encapsulation of the filtering bleb. Various risk factors are known including youth[1] and long term antiglaucomatous drop therapy.[1–3] This scarring process is due to proliferation of fibroblasts at the site of the fistula and has been found to take place in the first 1–2 weeks after surgery.[4] However, later bleb failure is also possible.[5]

Treatment with cytostatics has been established to reduce the proliferation of connective tissue around the bleb. Currently, mitomycin C, an antitumour antibiotic, is usually applied on the sclera under the conjunctival flap before the sclerostomy is performed. It has been shown to induce apoptosis in human Tenon's capsule fibroblasts[6] and bovine trabecular meshwork cells.[7] In filtration surgery it is used in concentrations between 200 and 500 μg/ml during an exposure of 2.5–5 minutes.[8–11] Although the procedure means important progress in glaucoma surgery, severe complications such as bleb leakage,[8] hypotonic maculopathy,[9] and impaired function of the corneal epithelium barrier[12] have been reported.

With this in mind, we looked for a different cytostatic agent, which would allow a shorter intraoperative treatment of the sclera in order to reduce diffusion into neighbouring regions, where an inhibition of proliferation should be avoided—for example, the sclerocorneal limbus and the outer parts of conjunctiva. Additionally, shorter exposure times increase the comfort for patient and surgeon. We chose the anthracycline idarubicin because of its rapid entry into cells.[13 14] It accumulates in the nucleus. In haematopoetic cells, 200–300-fold differences in concentration between the nucleus and the extracellular fluid were found.[14] Idarubicin binds to DNA and induces cell cycle arrest and DNA breakdown leading to apoptosis.[15] In ophthalmology, idarubicin has been used for the experimental inhibition of corneal neovascularisation after alkaline burning in rabbits.[16]

The aim of the present study was to investigate the long term effect of idarubicin on fibroblasts of the human Tenon's capsule after short time exposure in comparison with mitomycin C. We exposed the cells to different concentrations of the drugs for 0.5–5 minutes and assessed proliferation and motility at intervals up to 60 days. Apoptosis due to idarubicin treatment was confirmed by Hoechst 33258 staining for the initial phase of the experiment.

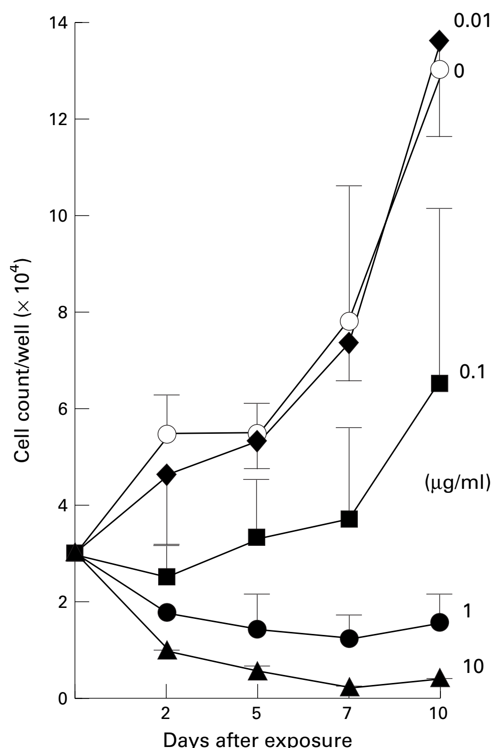

Figure 1 Effect of the idarubicin concentration on proliferation of fibroblasts of the human Tenon's capsule. To determine effective concentrations, cells of two different patients were treated in triplicate with different concentrations ranging from 0.01 µg/ml to 10 µg/ml for 5 minutes. Afterwards fibroblasts were cultured for 10 days and counted (six samples per point) at day 2, 5, 7, and 10 after exposure to idarubicin. Additional concentrations are shown in Table 1.

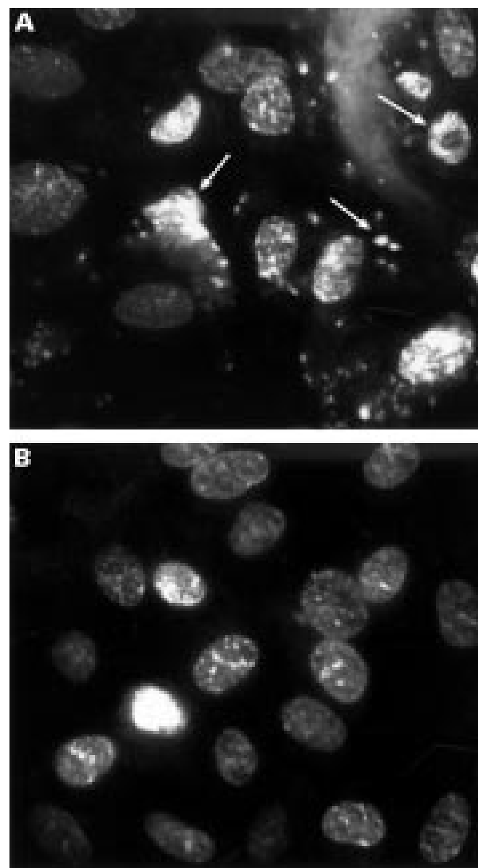

Figure 2 Assessment of apoptosis. To confirm cell death by apoptosis in the initial phase of the experiment Tenon's capsule fibroblasts were exposed to 1 µg/ml idarubicin for 0.5 minutes (A) or to buffer (B) and stained with Hoechst 33258. The cells treated with the cytostatic agent show the typical morphological feature of apoptosis with chromatine condensation, DNA fragmentation, and release of apoptotic bodies (arrows).

Additionally, we investigated the ^{3}H-proline uptake 5 and 10 days after exposure to idarubicin.

Materials and methods

CELL CULTURE

Specimens of the Tenon's capsule were taken from two healthy patients during strabismus surgery. The specimens were incubated in Dulbecco's Modified Eagle's Medium (Gibco Life Technologies, Paisley) supplemented with 10% fetal calf serum (Gibco), L glutamine (200 mM, Gibco), tylosine (100 µg/ml, Sigma Aldrich Chemie Deisenhofen, Germany), penicillin (100 U/ml, Boehringer Mannheim, Germany), streptomycin (100 µg/ml, Boehringer), and glucose (30 mM, Serumwerk Bernburg, Germany) ("medium") and processed at the same day. The pieces were chopped up, placed under coverslips in 50 mm petri dishes and supplied with medium. The medium was changed regularly three times a week. The antimycotics minocycline (10 µl/ml, Sigma Aldrich) and tiamuline (12.5 µg/ml, Serva Feinbiochemica Heidelberg, Germany) were added alternately. The cells were incubated at 37°C with 5% carbon dioxide. After reaching confluence, the cells were detached from the petri dishes with trypsin-EDTA (Gibco), centrifuged, and passaged into culture flasks. Further passages were done without minocycline or tiamuline.

Table 1 Effect of idarubicin concentration on proliferation of human Tenon's capsule fibroblasts. Cells of two different patients were treated in triplicate with idarubicin at concentrations ranging from 0.01 µg/ml to 20 µg/ml for 5 minutes and counted at day 2, 5, 7, and 10 after exposure. Data represent the cell count per well ×10⁴ (mean of 6 samples (SD))

Idarubicin concentration (µg/ml)	*Days after exposure*			
	2	*5*	*7*	*10*
0	5.5 (0.8)	5.5 (0.6)	7.8 (2.8)	13.0 (0.6)
0.01	4.7 (2.3)	5.4 (0.6)	7.4 (0.8)	13.6 (1.9)
0.03	4.1 (1.1)	5.1 (1.0)	7.9 (2.5)	10.8 (1.0)
0.1	2.5 (0.7)	3.3 (1.2)	3.7 (1.9)	6.6 (3.6)
0.3	1.8 (0.3)	2.0 (0.2)	1.9 (0.5)	2.6 (2.0)
1	1.8 (0)	1.4 (0.7)	1.2 (0.5)	1.6 (0.6)
2.5	2.1 (0.9)	0.9 (0)	0.8 (0.1)	0.7 (0.2)
5	1.6 (0.2)	0.6 (0.4)	0.9 (0)	0.6 (0)
10	1.0 (0)	0.6 (0.1)	0.2 (0)	0.4 (0)
20	0.6 (0.2)	0.4 (0.2)	0.3 (0.3)	0.4 (0.2)

DRUG TREATMENT

A total of 3×10^4 cells per well were placed into 24 well tissue culture plates and allowed to settle for 24 hours. Stock solutions of idarubicin (Farmitalia Milan, Italy) and mitomycin C (Sigma Aldrich) were dissolved in Hank's balanced salt solution (HBSS, pH 7.4, 310 mOsm; Gibco). After washing with HBSS, the cells were exposed to different concentrations of the cytostatics for 0.5, 2, or 5 minutes while being mildly shaken. Control groups were treated with salt solution alone. Afterwards cells were washed carefully twice with HBSS

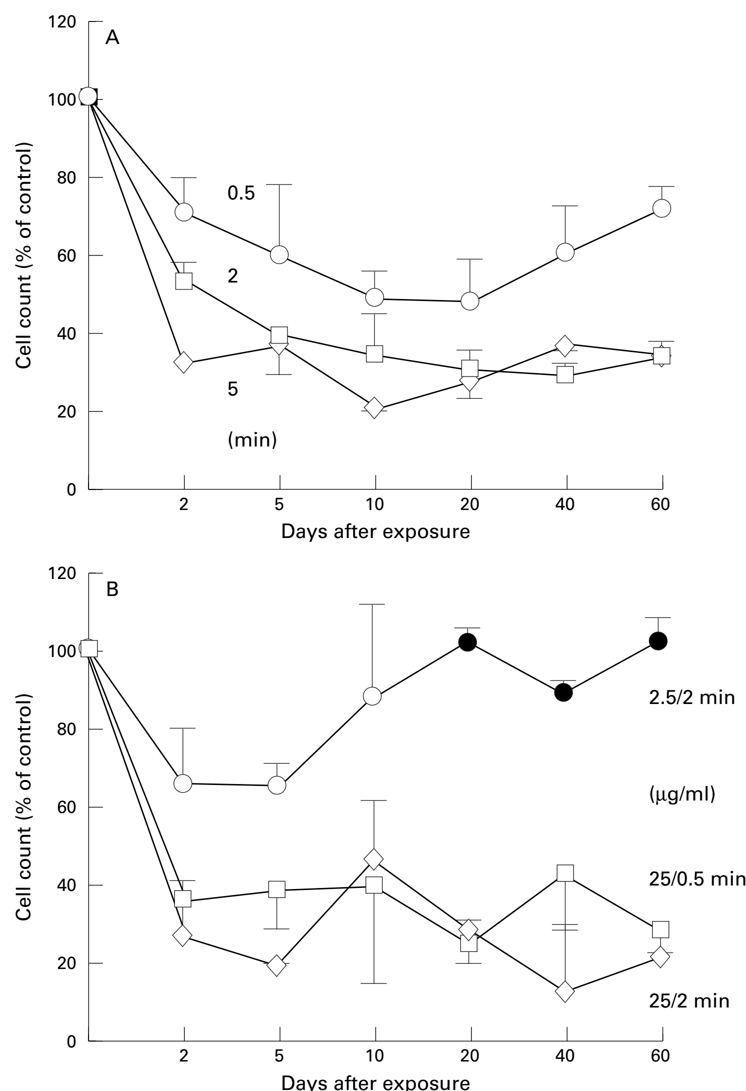

Figure 3 Comparison of the effect of idarubicin and mitomycin C treatment on the proliferation of fibroblasts during long term culturing. (A) Cells were exposed to 0.3 µg/ml idarubicin for 0.5, 2, and 5 minutes. (B) Cells were exposed to mitomycin C at a concentration of 2.5 µg/ml mitomycin C for 2 minutes or 25 µg/ml for 0.5 and 2 minutes. The data shown reflect the mean value of fibroblast preparations of two different patients (16 samples per point). Filled symbols, cell number not significantly reduced compared with the control (p >0.05).

and supplied with 1 ml of medium. The medium was changed three times a week. The assays described below were done with cells after the indicated time of culturing.

HOECHST 33258 STAINING

Cells were harvested by trypsination, washed with HBSS, and resuspended in 250 µM Hoechst 33258 (Sigma Aldrich), and dissolved in HBSS. After keeping 10 minutes on ice in the dark, the cells were centrifuged, washed, resuspended in HBSS, and photographed under a fluorescence microscope. The dye permeates membranes, stains DNA, and enables the visualisation of the nucleic morphology.

CELL COUNTING

Cells were washed with HBSS, detached by trypsin-EDTA, and counted manually using a Neubauer counting chamber (Feinoptik Blankenburg, Germany). Counting was done from each well used for one of the assays.

^{3}H-THYMIDINE AND ^{3}H-PROLINE INCORPORATION ASSAYS

Cell proliferation was determined by methyl-^{3}H-thymidine incorporation (DuPont NEN Products, Boston, MA, USA) and protein synthesis by L-2,3-^{3}H-proline uptake (DuPont) according to standard protocols. Fibroblasts were incubated with medium supplemented with 0.06 µCi ^{3}H-thymidine (1.6 mM, specific activity 10 mCi/mmol) or 1 µCi ^{3}H-proline (17 mM, specific activity 2 mCi/mmol) for 24 hours at 37°C. The incorporation of the radioisotopes was terminated by removing the medium and carefully washing with HBSS. Cells were trypsined and a small aliquot was used for cell counting. The cells were treated with 10% sodium desoxycholate and 10% trichloracetic acid. After centrifugation, cells were washed with acetone and put in 2% SDS. One ml scintillation cocktail was added, followed by counting with a TRI-CARB TR 1600 liquid scintillation analyser (Packard) for 6 minutes. The assays were done in triplicate for fibroblast preparations of two different patients. ^{3}H-thymidine uptake was measured for all concentrations and exposure times, ^{3}H-proline uptake assay was performed 5 and 10 days after treatment with 0.3 µg/ml idarubicin for 0.5, 2, or 5 minutes and controls.

AGAROSE MOTILITY ASSAY

Cells were washed with HBSS, trypsined, and a small aliquot was counted. The content of each well was centrifuged. The pellets were dissolved in 5 µl of 0.3% melted sea plaque agarose (Sigma Aldrich) and the droplets were transferred to the centres of wells of a 24 well tissue culture plate. In order to receive a gelatinised precoat of agarose, the plate was refrigerated at 4°C for 5 minutes. Droplets were covered with cooled DMEM without additions and incubated for 24 hours as stated above. Finally, the horizontal and vertical diameters of the cell patches were measured under light microscope equipped with an ocular scale. For each concentration and treatment time the experiment was performed twice for fibroblast preparations of two different patients.

For statistical comparisons, the U test of the STAT-VIEW software (SAS Institute, Cary, NC, USA) was used. $p<0.05$ was considered significant.

Results

EFFECTIVE CONCENTRATION OF IDARUBICIN

To determine effective concentrations of idarubicin, the dose dependent action of idarubicin on cell proliferation was measured for 10 days. The cells were incubated in triplicate for fibroblast preparations of two different patients with idarubicin at concentrations ranging from 0.01 µg/ml up to 20 µg/ml for 5 minutes. Cells were counted at days 2, 5, 7, and 10 after exposure. The cell count was significantly diminished at concentrations of 0.3 µg/ml and higher ($p<0.05$) 10 days after exposure (Fig 1, Table 1). The further long term experiments were done with idarubicin at concentrations of 0.1, 0.3, and 1 µg/ml or with mitomycin C at

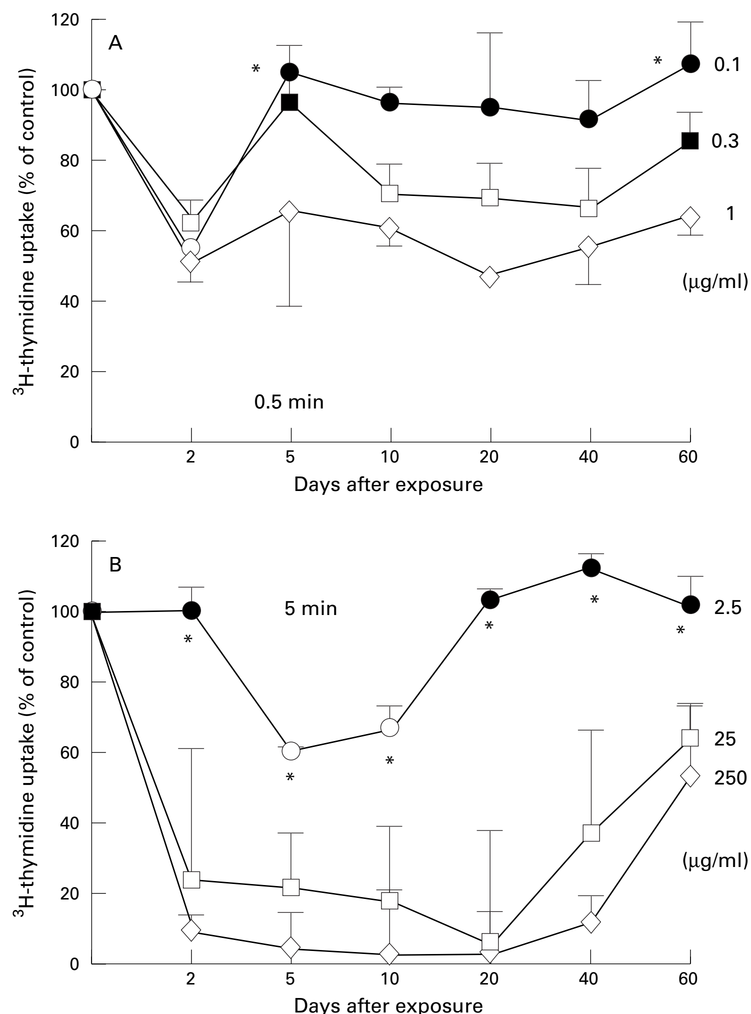

Figure 4 Effect of idarubicin and mitomycin C on DNA replication. The incorporation of ^{3}H-thymidine was measured for fibroblasts treated with idarubicin at concentrations of 0.1–1 µg/ml for 0.5 minutes (A) or with mitomycin C at concentrations of 2.5–250 µg/ml for 5 minutes (B) during long term culture. The experiment was done in triplicate for fibroblast preparations of two different patients. Filled symbols, ^{3}H-thymidine uptake not significantly reduced compared with the control (p>0.05). Asterisks, cell number not significantly reduced compared with the control (p>0.05).

concentrations of 2.5, 25, and 250 µg/ml. The cells were exposed to the cytostatics for 0.5, 2, or 5 minutes and kept in culture for 60 days.

ASSESSMENT OF APOPTOSIS

To confirm the initial decrease in cell number is due to apoptosis caused by idarubicin treatment we exposed cells to 1 µg/ml idarubicin for 0.5 minutes. Cells were incubated for 48 hours and stained with the DNA dye Hoechst 33258. The cells treated with the cytostatic agent show the typical morphological feature of apoptosis with chromatin condensation, DNA fragmentation, and apoptotic bodies (Fig 2A), whereas the nuclei of control cells appear normal (Fig 2B).

TIME COURSE OF INHIBITION

Idarubicin exerts significant toxicity on fibroblasts at concentrations of 1 µg/ml applied for 0.5 minutes as well as 0.3 µg/ml (Fig 3A) and 2 or 5 minutes' exposure. These cells were able to slow reproliferation 20 days after exposure. However, proliferation stayed below that of the control ($p < 0.001$ 60 days after exposure). Incubation with 1 µg/ml during 2 or 5 minutes resulted in a breakdown of cell proliferation below 20% of control cell number 10–40 days and 10–60 days after treatment, respectively. Exposure to 0.3 µg/ml idarubicin for 0.5 minutes ($p < 0.005$; Fig 3A) or to 0.1 µg/ml for 5 minutes ($p < 0.005$) or 2 minutes ($p < 0.0001$) also resulted in a significant reduction of cell count compared with the control after 60 days, but the cell number stayed above 50% of the control throughout the culture period. Idarubicin at a concentration of 0.1 µg/ml for 0.5 minutes did not affect proliferation in a significant extent (data not shown).

Mitomycin C inhibited proliferation almost completely at a concentration of 25 and 250 µg/ml at all exposure times investigated ($p < 0.0001$). At a concentration of 2.5 µg/ml, no remarkable effect was found in comparison with the control incubation (Fig 3B).

^{3}H-THYMIDINE INCORPORATION

The ^{3}H-thymidine incorporation represents the ability of cells to proliferate. The control cells reached confluent growth in the wells of the culture plate between fifth and tenth day of culturing. Cells treated with idarubicin or mitomycin C even at the highest concentrations are able to incorporate ^{3}H-thymidine after a short delay, but incubation with 1 µg/ml idarubicin for 0.5 minutes or with 25 or 250 µg/ml mitomycin C for 2 or 5 minutes resulted in a significant decrease of ^{3}H-thymidine incorporation up to 60 days after exposure (Fig 4A and B).

^{3}H-PROLINE INCORPORATION

^{3}H-proline incorporation was measured in fibroblasts treated with idarubicin at a concentration of 0.3 µg/ml. No significant decrease of uptake per cell compared with the control was found for idarubicin treated cells at days 5 and 10 after exposure to the cytostatic (data not shown).

MOTILITY ASSAY

Idarubicin decreases cell motility only at a concentration of 1 µg/ml applied for 5 minutes after long term culturing. Other concentrations and exposure times did not influence cell motility as assessed by agarose motility droplet assay (Fig 5A).

There is evidence that mitomycin C at low concentrations (2.5 µg/ml for 0.5 or 2 minutes and 25 µg/ml for 0.5 minutes) increases cell motility throughout the first 10 days after exposure. High concentrations (250 µg/ml for 2 or 5 minutes) are able to diminish cell motility during long term culturing (Fig 5B).

Discussion

The reason for failed fistulation surgery is the proliferation of connective tissue, which is triggered by inflammation following surgical trauma. This is especially important for patients, who have received long term topical antiglaucomatous therapy and are reported to have increased numbers of inflammatory cells

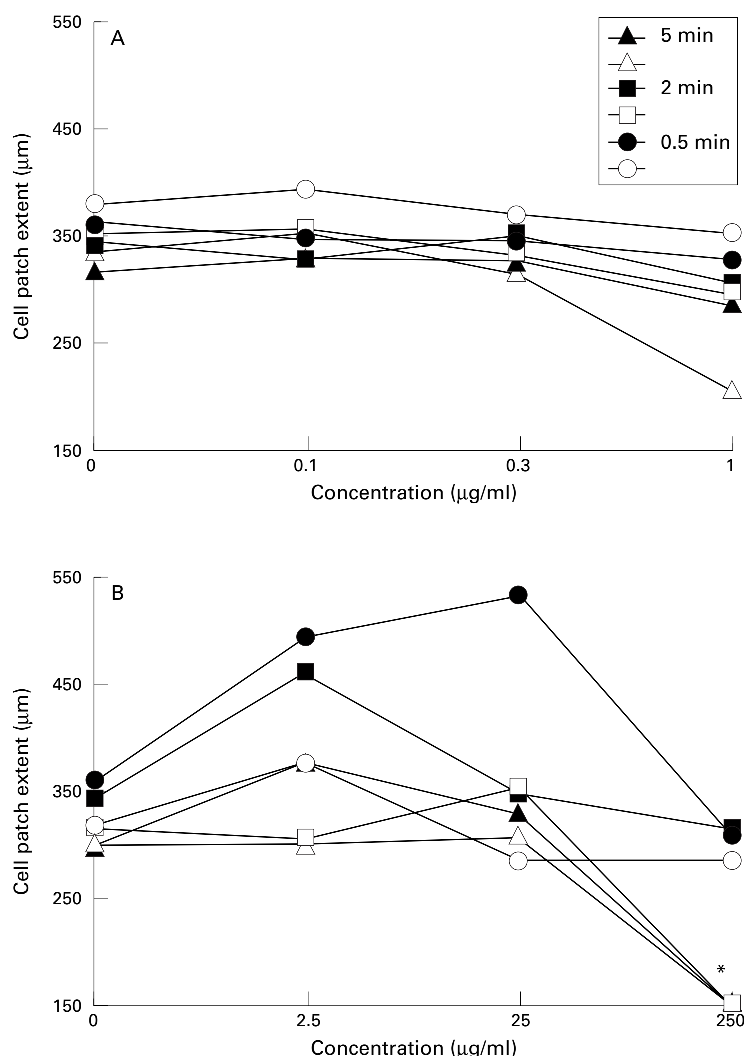

Figure 5 Effect of idarubicin and mitomycin C on cell motility. Fibroblasts were exposed to idarubicin at concentrations of 0.1–1 μg/ml for 0.5–5 minutes (A) or to mitomycin C at concentrations of 2.5–250 μg/ml for 0.5–5 minutes (B). Motility was assessed by agarase droplet motility assay 10 and 60 days after exposure twice for fibroblast preparations of two different patients. Standard deviations ranged from 2.65 μm to 117.2 μm, mean 44.8 μm for the idarubicin experiments and from 0 μm to 143.2 μm, mean 61.5 μm for the mitomycin C experiments. Filled symbols, 10 days after exposure; open symbols, 60 days after exposure. Asterisk, no cell patch.

in conjunctiva and Tenon's capsule.[2] Treatment with cytostatics has been established to prevent this proliferation of the connective tissue.

The aim of our study was to define the conditions for long time suppression of fibroblast proliferation by the cytostatic idarubicin after a short application period compared with mitomycin C. For that purpose, cell cultures derived from human Tenon's capsule were exposed to idarubicin or mitomycin C at various conditions. Idarubicin is able to exert toxicity on fibroblasts during a short exposure of 0.5 minutes at concentrations of 0.3–1 μg/ml resulting in a significant reduction in cell number compared with the control after 60 days. Exposure to 0.1 μg/ml idarubicin for 5 or 2 minutes also resulted in a significant reduction in cell count throughout long term culturing. Idarubicin at a concentration of 0.1 μg/ml for 0.5 minutes did not affect proliferation to a significant extent.

Mitomycin C inhibited proliferation almost completely at concentrations of 25 and 250 μg/ml at all exposure times investigated. At a concentration of 2.5 μg/ml, no remarkable effect was found in comparison with the control incubation. Our results for mitomycin C are similar to those of Khaw *et al.*[17] In this study, mitomycin C was applied for 5 minutes on fibroblasts of human Tenon's capsule in vitro. A significant inhibition of proliferation compared with untreated cells was found for concentrations from 10 μg/ml up to 1 mg/ml for 36 days.

Recently, apoptosis due to mitomycin C treatment of human Tenon's capsule fibroblasts has been shown by Crowston *et al.*[6] We confirmed cell death by apoptosis in the initial phase after exposure to idarubicin by Hoechst 33258 staining. The drug treated cells showed the typical feature with chromatin condensation, DNA fragmentation, and release of apoptotic bodies.

The ability of the cells to proliferate after exposure to idarubicin or mitomycin C was measured by ^{3}H-thymidine uptake. Even after treatment with 1 μg/ml idarubicin or 250 μg/ml mitomycin C a few cells were able to incorporate ^{3}H-thymidine and to proliferate. A possible explanation is that there are cells which are insensitive to the applied cytostatic agent and are able to proliferate further. Whether these cells belong to specific clones was not detectable by our experiment; but the surviving cells were scattered on the surface of the well. Madhavan *et al*[18] compared surgical results and sensitivity to mitomycin C for fibroblasts of the same patients. They postulated mitomycin C resistant cell lines, which needed higher doses of the drug compared with sensitive cell lines. The surgical outcome correlated with the evaluation of the patient's cells. Tumour cell lines not susceptible to idarubicin are also reported, but resistance to idarubicin is thought to occur less often compared with other anthracyclines.[19]

It is questionable whether a complete long term cut off of proliferation is an advantage considering the fine equilibrium between wound healing and avoidance of scarification of the filtering bleb.

In a study of Occleston *et al*,[20] it was shown that exposure to mitomycin C results in an initial increase in the production of collagen type III and decreased levels of collagen type I and fibronectin throughout a 48 day culture period. McGuigan *et al*[21] showed that daunorubicin, the parent substance of idarubicin, affects intracellular ^{3}H-proline incorporation only at a very high dose, which inhibits proliferation almost completely. Extracellular incorporation remained unchanged compared with the control. Cells were incubated with daunorubicin at a concentration of 25 μg/ml for 48 hours. We therefore assessed the ^{3}H-proline uptake 5 and 10 days after exposure to 0.3 μg/ml idarubicin, when the cell number was diminished to about 20% to 50% of the control, and found it had not decreased. According to these results, the remaining cells are able to produce collagens

and the effect of the cytostatic is due to inhibition of cell proliferation and not of protein synthesis.

In our study, there was an inhibition of motility 60 days after treatment with 1 µg/ml idarubicin for 5 minutes or with 250 µg/ml mitomycin C for 2 or 5 minutes. For low mitomycin C concentrations, we even found an increase in motility in the first 10 days after application. Yamamoto *et al*[22] showed that mitomycin C at a concentration of 10 µg/ml does not affect the motility of fibroblasts significantly. Again it has to be taken into consideration that the effect of the cytostatics is probably due to the reduced amount of cells.

Idarubicin diminishes the long term proliferation of fibroblasts in the human Tenon's capsule at concentrations as low as 0.3–1 µg/ml applied for 0.5 minutes. In comparison, mitomycin C requires longer applications and higher doses for equal results. Owing to the high toxicity of idarubicin, with rapid entry into the cells and little resistance, it would require only a short exposure time intraoperatively. Therefore, it could help to reduce complications resulting from a longer application of cytostatics with diffusion into neighbouring areas and to increase the comfort for patient and surgeon, and this could be useful in prevention of glaucoma filtering surgery failure.

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