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Suppression of inflammatory and fibrotic responses in allergic inflammation by the amniotic membrane stromal matrix

A. Solomon*, M. Wajngarten†, F. Alviano†, I. Anteby*, U. Elchalal‡, J. Pe'er* and F. Levi-Schaffer†

*Department of Ophthalmology, Hadassah University Hospital, The Hebrew University – Hadassah Faculty of Medicine, Jerusalem, Israel, †Department of Pharmacology, School of Pharmacy, The Hebrew University – Hadassah Faculty of Medicine, Jerusalem, Israel and ‡Obstetrics and Gynecology, Hadassah University Hospital, The Hebrew University – Hadassah Faculty of Medicine, Jerusalem, Israel

Summary

Background The amniotic membrane (AM), which is the innermost layer of the placenta, was shown to possess anti-inflammatory and anti-fibrotic properties in various *in vitro* and clinical studies.

Purpose To evaluate the anti-fibrotic and anti-inflammatory effects of the AM matrix (AMM) on human conjunctival and lung fibroblasts in an *in vitro* system that tests fibrotic and inflammatory responses at the effector stages of allergic inflammation.

Methods Human conjunctival or lung fibroblasts were seeded on plastic or on the stromal aspect of the AM, which was mounted on plastic inserts. Sonicates of human peripheral blood eosinophils activated with lipopolysacharide (LPS), or human mast cell (HMC-1) leukaemia cell sonicates, were added to sub-confluent fibroblast monolayers. Proliferation of the sub-confluent fibroblasts was assessed using the [^{3}H]-thymidine incorporation assay. The production of transforming growth factor (TGF)-β_1, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-8 in conjunctival or lung fibroblasts was measured in conditioned media from these cultures by ELISA.

Results After 4 days in culture, the [^{3}H]-thymidine incorporation assay indicated a reduced proliferation of activated conjunctival and lung fibroblasts when cultured directly on the AMM. The production of both TGF-β_1 and IL-8 was significantly suppressed in activated conjunctival fibroblasts cultured on the AMM compared with those cultured on plastic, while the production of both TGF-β_1 and GM-CSF was decreased in human lung fibroblast cultured on the AMM.

Conclusions The AMM is capable of suppressing fibrotic responses in an *in vitro* system of effector stages of ocular allergic inflammation. These data may provide a basis for exploring matrix components in the AM for the treatment of allergic eye disease.

Keywords allergy, amniotic membrane, eosinophils, fibroblasts, mast cells

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Introduction

The amniotic membrane (AM) has been used over the last decade as a surgical tool in ocular surface reconstruction for a variety of indications. The AM is the innermost layer of the placenta, and consists of a thick basement membrane and a subjacent avascular stroma. As its introduction to ophthalmic surgery [1], many studies have described its use for managing various ocular surface disorders including chemical or thermal burns [1–3], pterygium [4], persistent corneal ulcers of different aetiologies [5–7], symptomatic bullous keratopathy [8], and other causes leading to limbal stem cell deficiency [2, 9, 10]. Recently, the use of the AM was described in a few cases of severe vernal keratoconjunctivitis [11, 12]. In most of these studies, the AM was noted to possess significant anti-inflammatory and anti-fibrotic properties. These effects were observed both clinically and in several *in vitro* studies.

Allergic inflammation may target several tissues such as the lungs, the skin and the eye. Allergic responses of the ocular surface are encountered in relatively benign conditions such as seasonal and perennial allergic conjunctivitis and in severe chronic disorders such as vernal keratoconjunctivitis, giant papillary conjunctivitis and atopic keratoconjunctivitis. Chronic allergic inflammation of the ocular surface may lead to tissue damage associated with inflammation and fibrosis [13, 14], including recurrent shield ulcers and corneal plaques [15], giant papillae of the tarsal conjunctiva and in severe cases – partial stem cell deficiency [16, 17]. The major players in allergic inflammation are mast cells and eosinophils, which perpetuate the allergic inflammatory cascade. Recent data indicate that fibroblasts are also important effector cells of the allergic inflammatory response, thanks to their ability to respond to inflammatory stimuli, and release mediators that influence mast cells and eosinophils [18]. Activated fibroblasts

Correspondence: Abraham Solomon, Department of Ophthalmology, Hadassah University Hospital, The Hebrew University – Hadassah Faculty of Medicine, PO Box 12000, Jerusalem 91120, Israel.
E-mail: avisol@md.huji.ac.il

may induce fibrosis, which is one of the results of allergic disorders [14]. Down-regulation of cytokines associated with inflammatory and fibrotic responses in allergic inflammation may prevent the long-term sequelae of these disorders.

The AM was found to down-regulate fibrotic and inflammatory responses in several *in vitro* models [19–21]. These studies had preceded the actual use of the AM in various ocular surface inflammatory disorders. As of today, the possible application of the AM in severe chronic allergic disorders of the ocular surface has not been investigated. In order to explore the possible utilization of the AM in severe allergic eye diseases, we decided to investigate the effects of the AM *in vitro* with players that participate in allergic inflammation and fibrosis. The purpose of this study was to test the effects of the AM matrix (AMM) in down-regulating inflammation and fibrosis in an *in vitro* system that tests fibrotic and inflammatory responses at the effector stages of allergic inflammation. This model utilizes mast cell or eosinophil sonicates to activate human conjunctival or lung fibroblasts, which are cultured either on the AM stromal matrix or on plastic. Here we provide evidence that the AMM has *in vitro* anti-fibrotic and anti-inflammatory effects, which are partially mediated through down-regulation of transforming growth factor (TGF)-β_1, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-8 production by the fibroblasts.

Materials and methods

Human fetal lung fibroblasts and conjunctival tenon fibroblasts

All procedures followed the tenets of the Helsinki declaration, and were approved by the Hadassah University Hospital Review Board.

Human fetal lung fibroblasts (HFLF) (FHS 738, HTB-157) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM with 10% heat-inactivated fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel), with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Biological Industries).

Human conjunctival tenon biopsies were taken during strabismus surgery, from subconjunctival tissue surrounding the medial rectus fascia, during the process of muscle separation. Tissue samples were used for explant cultures to generate third-generation conjunctival fibroblasts (CF). Each specimen was cut into explants of approximately 1×1 mm and placed onto 100 mm tissue culture dishes. Ten minutes later, each explant was covered with a drop of FCS, and placed overnight in an incubator at 37 °C with 95% humidity and 5% CO_2. Ten millilitres of medium containing DMEM enriched with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin was added the next day, and the medium was changed every 2 days thereafter. Fibroblasts were subcultured by treatment of the monolayers with 0.05% trypsin and 0.85 mM EDTA in a calcium-free MEM medium at 80–90% confluence with 1 : 3–4 split for three passages. Three cell lines of HFLF and three cell lines of human CF were used in this study.

Human mast cell-1 cells

The human mast cell (HMC-1) leukaemia cell line, HMC-1, was used as a source of mast cell mediators [22, 23]. HMC-1 cells were cultured in Iscove's medium (Biological Industries), supplemented with 10% FCS containing Fe [2], 50 U/mL penicillin, 0.05 μg/mL streptomycin and 160 μL/L (1.2 mM) monothioglycerol. Cells were passaged twice a week.

Human peripheral blood eosinophils

Eosinophils were purified from the peripheral blood as previously described [24]. Briefly, venous blood (100–150 mL) was taken from mildly atopic volunteers (eosinophilia $< 10\%$), not taking any oral treatment for their condition. Written informed consent was obtained from the volunteers according to the guidelines of the Hadassah University Hospital Review Board. Venous blood was collected in heparinized syringes, and left to sediment in 6% dextran. Leucocytes were centrifuged on Ficoll–Paque (density = 1.077) for 25 min at 700 ***g*** at 4 °C. Neutrophils in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 antibodies, lymphocytes to anti-CD3 antibodies and mononuclear cells to anti-CD14 antibodies (Miltenyi, Biotech Gmbh, Bergisch Gladbach, Germany). The tagged cells were then eliminated by passing them through a magnetic field (MACS). Eosinophils were collected at a purity of 98–100% as assessed by Kimura staining, and at a viability of $> 99\%$ as assessed by Trypan Blue staining.

Preparation of human amniotic membrane

The human AM was prepared following a previously described protocol [21, 25]. Briefly, human placenta was obtained from caesarean section delivery of healthy donors, from which maternal blood was negative to serological tests for human immunodeficiency virus type 1 and 2, and hepatitis B and hepatitis C viruses. The AM was separated from the chorion by blunt dissection, and then thoroughly rinsed with filter-sterilized phosphate-buffered saline (PBS) containing 50 μg/mL penicillin, 50 μg/mL streptomycin and 2.5 μg/mL amphotericin B, until all of the blood clots were completely removed from the membrane. Next, pieces of the AM were placed on a sterile nitrocellulose filter paper, with the stromal side facing the filter paper for orientation, and stored in medium containing a 1 : 1 v/v ratio of DMEM and glycerol, and stored at -80 °C for at least 1 week before use.

Before use, the vials containing the frozen AM were thawed in a 37 °C water bath and washed at least three times in sterile PBS, to remove all traces of medium and glycerol. The AM was then incubated for 2 h at 37 °C in 0.02% EDTA, to remove the epithelial cell layer. Thereafter, the AM was washed again three times to wash away the EDTA and spread out in a sterile dish wetted with sterile PBS, cut into pieces measuring approximately 2×2 cm and used in experiments.

Fibroblast adhesion and viability studies

In order to anchor the AM and to culture cells on its stromal side, a culture system using a plastic ring insert (Fig. 1) was used as previously described [26]. Briefly, a ring insert with an inner diameter of 10 mm (Millicell – CM culture plate inserts,

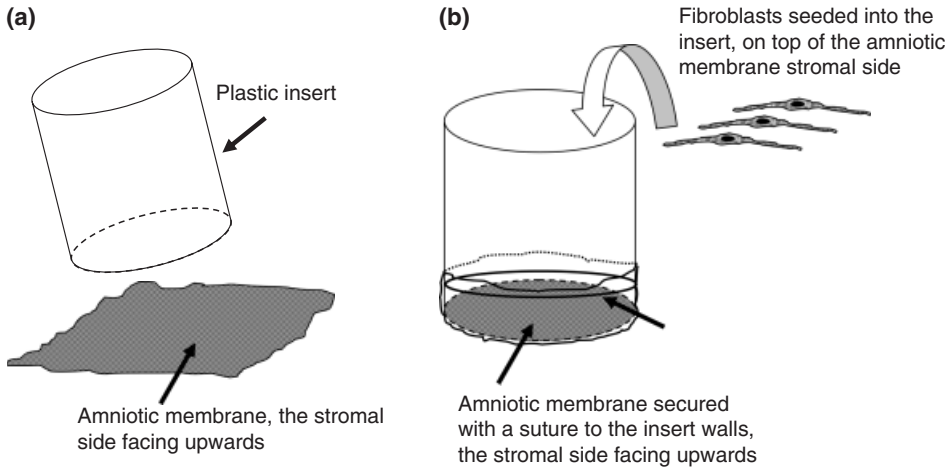

Fig. 1. Amniotic membrane (AM) culture system. A ring insert is placed over the stromal side of the AM (a). The AM is secured to the outer walls of the insert with a suture. Fibroblasts are seeded into the insert, on top of the AM stroma (b).

Millipore, Bedford, MA, USA) was used after removing its polycarbonate membrane. This ring insert was applied over a piece of the AM, with its stromal side facing up (Fig. 1a). The edges of the AM were secured to the outer circumference of the plastic ring with a 4-0 nylon suture, so that the AM would be stretched under the ring, and the stromal side, which faces the inner part of the ring, faces up (Fig. 1b). The insert, with the AM secured to its bottom side, was placed in a 24-well culture plate (NUNC^{TM}, Roskilde, Denmark) and immersed with medium.

Third-generation HFLF or normal CF were seeded inside the AM inserts, on the stromal side of the AM, at an initial density of 3×10^4 cells per insert. Cultures were maintained at 37 °C for up to 7 days. The AM was then gently removed from the insert, spread out on a small petri dish and stained with trypan blue (0.4% in saline) and toluidine blue (0.70 mg/mL in a 50% ethanolic solution). After staining, the cultures were washed with PBS for three times, and fixed by air drying. The adhesion, viability and morphology of the fibroblasts on the AMM were assessed under an inverted microscope (Olympus, Melville, NY, USA) at $\times 200$ and $\times 400$ magnifications.

In parallel cultures, the effect of the inserts alone (without AM) on fibroblasts proliferation was evaluated by placing the empty inserts in the plastic wells seeded by fibroblasts.

Preparation of sonicates from human mast cell-1 (HMC-1) - cells or eosinophils

HMC-1 cells were centrifuged (120 ***g***, 5 min) and resuspended in 2–5 mL of 2% FCS in DMEM. Cells were counted, and then bath sonicated (2 min, 0 °C; Heat Systems/Ultrasonic 380 W, duty cycle 5 s; output power, 50%). Sonicates were then microcentrifuged for 5 min at 4 °C, 120 ***g*** (Hettich Ultracentrifuge, Heitich Zentrifugen, Tuttlingen, Germany) to remove cell debris. The debris-free sonicate supernatant ('sonicate') was collected into aliquots and stored at − 80 °C.

Eosinophils, which were previously conditioned with lipopolysacharide (LPS) (10 μg/mL), were bath sonicated as above, and stored at − 80 °C.

Cell cultures on the amniotic membrane matrix

The AM was stretched flat on tissue culture inserts, as described above, and placed in a 24-well culture plate. The growth area of the culture inserts and the wells (in 24-well plates) was 0.7 and 1.75 cm^2, respectively. The well to insert growth area ratio was 2.5. According to this ratio, different cell densities were calculated for fibroblasts, HMC-1 sonicates and eosinophil sonicates (Table 1). Third-generation HFLF or CF were seeded in the inserts on top of the AMM or on plastic, in 24-well culture plates. Cell densities were 72 000 cells/mL for the AMM cultures, and 180 000 cells/mL for the plastic cultures (Table 1). These cell densities were proportional to the calculated area of the AM insert and the 24-well plastic surface, respectively. Cells were incubated at 37 °C, in a 5% CO_2 humidified incubator until confluence, then washed two times with DMEM and 2%FCS. HMC-1 sonicates were then added to the confluent fibroblast monolayers (HFLF or CF) cultured on the AMM, using sonicates from 1.2×10^5 cells/mL for the inserts and 3.0×10^5 cell/mL for the plastic wells. In parallel studies, eosinophil sonicates were added to confluent fibroblasts monolayers (CF), prepared from 2×10^6 cells/mL for the AMM insert cultures and from 5×10^6 cells/mL for the plastic cultures (Table 1).

Fibroblast proliferation assay

Proliferation of subconfluent fibroblasts was assessed using [^{3}H]-thymidine incorporation assay. Tissue culture inserts with AM were prepared as described above, and placed in a 24-well culture dish (NUNC^{TM}). Fibroblasts were seeded on the AMM inserts and in plastic 24 wells at densities as described above, and maintained in DMEM with 2% FCS. The plate was then incubated at 37 °C, in a 5% CO_2 humidified incubator. After 1 day the medium was removed and HMC-1 sonicates were added to the AMM and plastic cultures at the appropriate concentrations, all at a final volume of 0.5 mL/well.

After 48 h in culture, the inserts and wells were washed two times with DMEM supplemented with 2% FCS to remove all

Table 1. Cell densities prepared for the AM inserts and the plastic wells

	AM insert	Plastic well (24-well plate)
Growth area (cm^2)	0.7	1.75
Fibroblasts cell density (cells/mL)	0.72×10^5	1.8×10^5
HMC-1 cell density (cells/mL)	1.2×10^5	3.0×10^5
Eosinophil cell density (cells/mL)	2×10^6	5×10^6

The ratio between cell densities for wells/inserts was proportional to the growth areas of the well/inserts, respectively.
HMC-1, human mast cell-1; AM, amniotic membrane.

HMC-1 sonicates. A final 24 h pulse (2 or 5 μC/well added to inserts or plastic wells, respectively) of [^{3}H]-thymidine (DuPont NEN, Boston, MA, USA) was added to each well and insert, diluted in DMEM and 2% FCS to a final volume of 0.5 mL/well. At the end of the incubation cells were washed with cold PBS, fixed with methanol and precipitated with 5% trichloracetic acid (Merck, Darmstadt, Germany). The cells were lysed with 0.1% NaOH, transferred to scintillation vials and counted in a β-counter (LKB 1211 Rackbeta, Pharmacia, Uppsala, Sweden). These measurements were then turned into a proliferation index, where the values found for fibroblasts cultured on plastic were defined as 100%, and the other culture values were normalized relative to fibroblasts on plastic. All samples were carried out in triplicates and the same method was used for both HFLF and CF.

Evaluation of TGF-β$_1$, GM-CSF and IL-8 production

TGF-β_1 and GM-CSF were evaluated in the culture supernatants of lung fibroblasts, cultured on the AMM or on plastic, with or without previous activation with HMC-1 sonicates. For TGF-β_1, samples of 150 μL from the conditioned medium were taken at 8, 24 and 48 h after activation and kept frozen at −70 °C until ELISA was performed. For GM-CSF, samples of 150 μL from the conditioned medium were taken at 24 h, 4 and 7 days after activation and kept frozen at −70 °C until ELISA was performed. Samples from different time-points were taken from separate sets of cultures, and all samples were taken from triplicate cultures.

TGF-β_1 and IL-8 were evaluated in culture supernatants of CF, cultured on the AMM or on plastic, with or without previous activation with eosinophils sonicates. Samples of 150 μL from the conditioned medium were taken at 48 h for both cytokines, and frozen at −70 °C until ELISAs were performed.

The concentration of these cytokines was also measured in medium alone or in medium with HMC-1 sonicates or eosinophil sonicates, on the AMM or on plastic, and subtracted from the corresponding culture results where CF were present, to account for the background levels of cytokines which are present in the sonicates or in the culture medium.

TGF-β_1, GM-CSF and IL-8 were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

The total cellular protein content in the cell lysate was determined by the micro-BCA protein assay reagent kit (Pierce, Rockford, IL, USA) at the same time-points and the same culture conditions where cytokine concentrations were measured. The protein concentration of TGF-β_1, GM-CSF and IL-8 in the culture supernatant was adjusted by its corresponding total cellular protein content, to account for possible differences in cultured cell numbers. Thus, all ELISA results were expressed as picograms per micrograms of the total cellular protein, and represented the mean of three different experiments, performed in three different cell lines. All experiments were performed in triplicate.

Results

Fibroblast proliferation on the amniotic membrane matrix

Human lung and CF were found to attach, proliferate and maintain their viability and normal spindle-shaped morphology on the AMM.

Culturing human lung fibroblasts on the AMM was associated with a significant reduction in the proliferation index of these cells compared with cells cultured on plastic ($P = 0.018$, Fig. 2). HMC-1 cell sonicates increased the proliferation of human lung fibroblasts cultured on plastic. When HMC-1 cell sonicates were added to fibroblasts cultured on the AMM, a significantly reduced proliferation was demonstrated when compared with the proliferation of activated fibroblasts cultured on plastic (Fig. 2). The mean proliferation index for the cell lines of HFLF activated by HMC-1 sonicates was $122.38 \pm 14.88\%$. The proliferation index was $73.32 \pm 6.69\%$ when the HMC-1-activated fibroblasts were cultured on the AMM ($P = 0.007$).

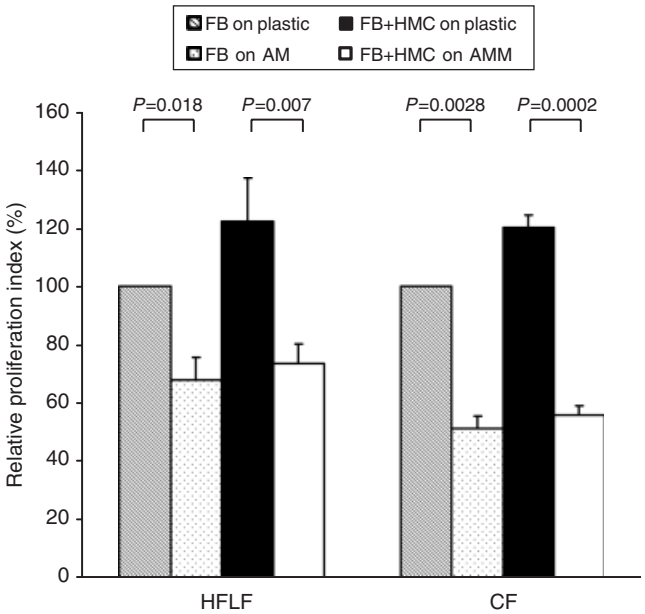

Fig. 2. Suppression of fibroblast proliferation by the AMM. The proliferation of HFLF or human CF activated by HMC-1 sonicates was suppressed when cultured on the AMM compared with plastic cultures. Bars represent the mean of three individual experiments, performed in three different cell lines. Proliferation index of 100% indicates normal proliferation of non-activated fibroblasts cultured on plastic. The CPM readings of the other conditions were normalized relative to the non-activated fibroblasts. HFLF, human fetal lung fibroblasts, CF, conjunctival fibroblasts, FB, fibroblasts, HMC, human mast cell-1 cell sonicates, AMM, amniotic membrane matrix.

Similar results were demonstrated in CF (Fig. 1). The proliferation of activated CF was significantly smaller when cultured on the AMM ($55.6 \pm 3.2\%$) compared with activated cells cultured on plastic ($120.3 \pm 4.4\%$, $P = 0.0002$).

No reduction in fibroblasts proliferation was demonstrated when the empty inserts were added to fibroblasts cultured in the plastic wells.

Down-regulation of TGF-β_1 and GM-CSF production in lung fibroblasts cultured on the AMM

TGF-β_1 secreted from HFLF was measured after 8, 24 and 48 h in culture on plastic or on the AMM, and following activation with HMC-1 cell sonicates on HFLF cultured on plastic or on the AMM.

No significant differences between plastic and AMM cultures in TGF-β_1 protein levels were demonstrated after 8 and 24 h in non-activated cultures (Fig. 3). Following activation with HMC-1 sonicates, significantly lower TGF-β_1 levels were evident at 24 h in AMM compared with plastic cultures (5.17 ± 0.25 pg/μg protein on plastic compared with 4.27 ± 0.20 pg/μg protein on AMM, $P = 0.0086$) (Fig. 3).

At 48 h, cultures on AMM produced smaller quantities of TGF-β_1 in non-activated as well as in HMC-1-activated cells (Fig. 3). In non-activated cultures, TGF-β_1 production was 6.8 ± 0.16 pg/μg protein in plastic cultures and 5.6 ± 0.16 pg/μg protein in AMM cultures ($P = 0.001$). In HMC-1-activated cultures, TGF-β_1 was 7.75 ± 0.25 pg/μg protein in plastic cultures and 4.83 ± 0.22 pg/μg protein in AMM cultures ($P = 0.0001$) (Fig. 3).

GM-CSF was measured in HFLF culture supernatants, cultured on plastic or on AMM, with or without HMC-1 activation. No significant changes were demonstrated in GM-CSF content in non-activated lung fibroblasts cultured on plastic or AMM (Fig. 4).

HFLF grown on the AMM and activated by HMC-1 sonicates showed significantly lower levels of GM-CSF production compared with activated fibroblast cultured on plastic. This effect was demonstrated assessing the cultures at 4 and 7 days (Fig. 3). On day 4, GM-CSF content in AMM cultures was 0.57 ± 0.095 pg/μg protein compared with 1.37 ± 0.12 pg/μg protein in plastic cultures ($P = 0.0008$). On day 7, the concentration of GM-CSF was 2.25 ± 0.09 pg/μg protein in plastic cultures and 1.03 ± 0.09 pg/μg protein in AMM cultures ($P = 0.0001$) (Fig. 4).

Down-regulation of TGF-β_1 and IL-8 in conjunctival fibroblasts by the AMM

TGF-β_1 was measured in culture supernatants of human CF cultured on the AMM or on plastic, with or without activation with LPS-activated peripheral blood eosinophils sonicates.

A significant down-regulation of TGF-β_1 protein secretion by the cultured CF on the AMM compared with plastic was demonstrated for both non-activated and activated cells (Fig. 5a). As in HFLF, this effect was more prominent in activated cultures. A 1.9-fold lower TGF-β_1 concentration was measured in AMM compared with plastic cultures in non-activated CF (0.73 ± 0.06 pg/μg protein in plastic, compared with 0.39 ± 0.08 pg/μg protein in AMM, $P = 0.0054$). A 20-fold lower level was measured in eosinophil-sonicates-activated cultures (0.84 ± 0.35 pg/mL in plastic, compared with 0.042 ± 0.026 pg/mL in AMM, $P = 0.0167$) (Fig. 5a).

A less prominent effect was measured for IL-8 secretion (Fig. 5b). A 1.2-fold lower level in IL-8 was measured in non-activated fibroblasts in AMM compared with plastic cultures ($P = 0.038$), and a 1.3-fold lower level was found in the activated cultures ($P = 0.028$).

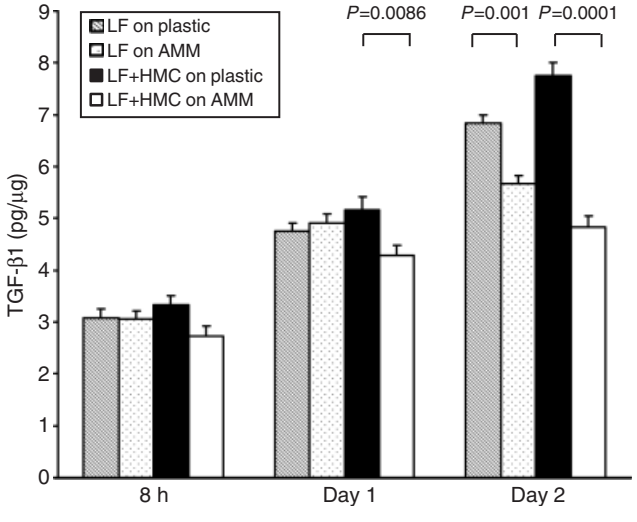

Fig. 3. Down-regulation of TGF-β_1 by the AMM. The secretion of TGF-β_1 from lung fibroblasts, with or without activation with HMC-1 sonicates, was down-regulated by the AMM compared with plastic cultures after 24 and 48 h. Bars represent the means of three different experiments performed in three different cell lines. Experiments were carried out in triplicate. The protein concentration of TGF-β_1 is expressed as picogram cytokine per microgram of total cellular protein. LF, human fetal lung fibroblasts, AMM, amniotic membrane matrix, HMC-1, human mast cell-1 cell sonicates; TGF, transforming growth factor.

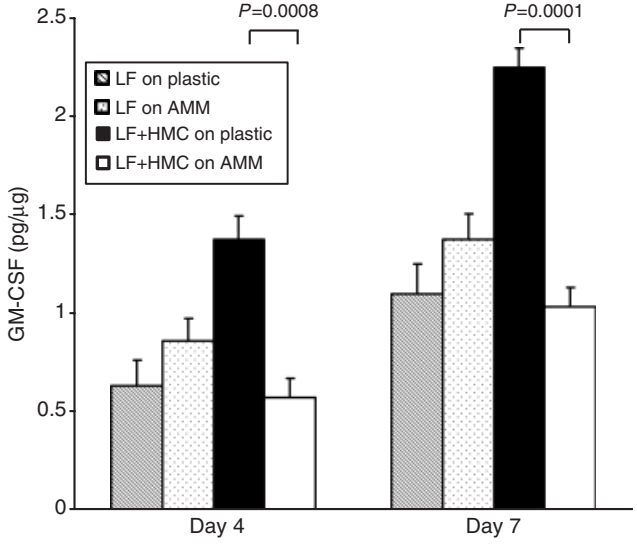

Fig. 4. Down-regulation of granulocyte–macrophage colony-stimulating factor (GM-CSF) by the AMM. The secretion of GM-CSF from lung fibroblasts, activated with HMC-1 sonicates, was down-regulated by the AMM compared with plastic cultures. Bars represent the means of three different experiments performed in three different cell lines. Experiments were carried out in triplicate. Protein concentration of GM-CSF is expressed as picograms of cytokine per micrograms of total cellular protein. LF, human fetal lung fibroblasts, AMM, amniotic membrane matrix, HMC-1, human mast cell-1 cell sonicates.

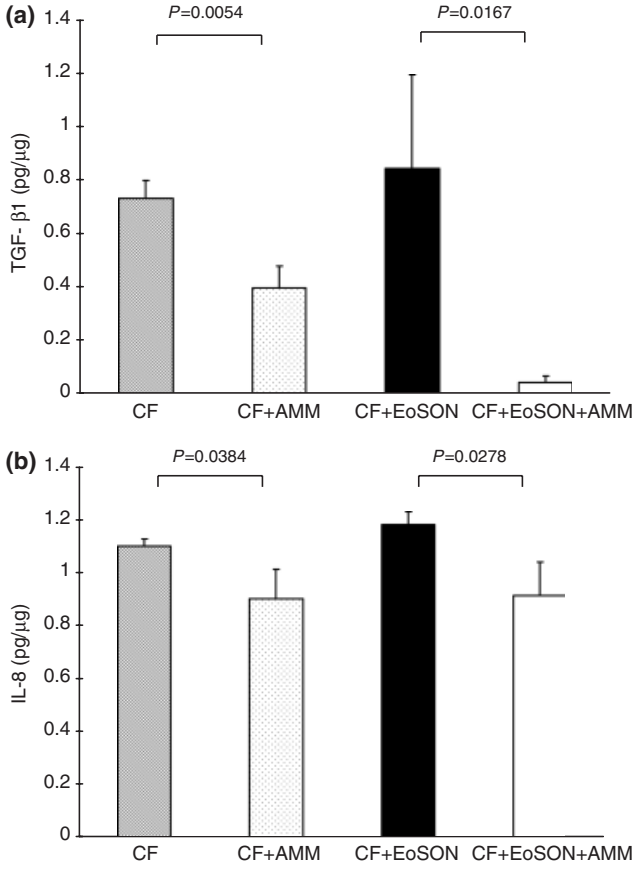

Fig. 5. Down-regulation of TGF-β_1 (a) and IL-8 (b) in CF by the AMM. The secretion of both TGF-β_1 and IL-8 from CF, with or without activation with human peripheral blood eosinophils sonicates, was down-regulated by the AMM compared with plastic cultures. Bars represent the means of three different experiments performed in three different cell lines. Experiments were carried out in triplicate. The protein concentration of TGF-β_1 is expressed as picogram cytokine per microgram of total cellular protein. CF, conjunctival fibroblasts; AMM, amniotic membrane matrix; EoSON, eosinphil sonicates; TGF, transforming growth factor.

Discussion

This study demonstrates a direct suppressive effect of the AM stromal matrix on fibrotic and inflammatory responses in human conjunctival and fetal lung fibroblasts. This was evident by suppression of proliferation of conjunctival and lung fibroblasts that were cultured on the AMM. An increased proliferation of the cells, triggered by activation with HMC-1 sonicates, was reversed when these cells were cultured on the AMM. The production of TGF-β_1, the major fibrogenic cytokine, was inhibited in fibroblasts cultured on the AMM. Again, this suppressive effect was especially pronounced when the fibroblasts were previously activated by mast cell or eosinophil sonicates. The AMM also induced down-regulation of GM-CSF and IL-8; both are cytokines that participate in the allergic inflammatory responses.

The main cellular players of the allergic inflammatory response are eosinophils and mast cells. Recent data indicate that fibroblasts are also important effector cells of the allergic inflammatory response, because of their ability to respond to different inflammatory stimuli, and release mediators that modulate and affect mast cells and eosinophils [18]. In addition, clinical observations from allergic diseases in general, such as asthma, and specifically ocular allergic conditions, such as vernal keratoconjunctivitis, show that fibrosis is a consequent and sometimes concomitant manifestation of allergy. We have therefore chosen an *in vitro* model where sonicates from either mast cells or activated peripheral blood eosinophils were added to activate either lung or CF, which were cultured on the stromal side of the AM. The anti-fibrotic effects of the AM on activated fibroblasts in the setting of an allergic stimulation were evaluated by the proliferation assay and by TGF-β_1 production, while the anti-inflammatory effect was evaluated by GM-CSF and IL-8 expression.

As a source of mast cell mediators the HMC-1 leukaemia cell line HMC-1 was used, which is an immature human leucocyte mast cell [22, 23, 27]. Mast cells can up-regulate fibrosis through the release of several mediators such as histamine and tryptase, and cytokines such as TGF-β, IL-4, and TNF-α, which are potent stimulators of collagen production and fibronectin synthesis by the fibroblasts. We have previously demonstrated that rat peritoneal mast cells can increase the proliferation of 3T3 fibroblasts in co-culture. Stimulation of these mast cells resulted in a further increase of fibroblast proliferation and an increase in collagen production [23]. In addition to mast cells, eosinophils are also capable of up-regulating fibrotic responses in fibroblasts. We have previously shown that eosinophils act as direct modulatory cells in fibroblast proliferation and collagen synthesis, partially through TGF-β [28]. For these reasons, in order to create *in vitro* conditions of allergic inflammation and fibrosis, sonicates of either HMC-1 cells or human peripheral blood eosinophils were used to activate the cultured fibroblasts.

The ability of the AMM to suppress inflammation and fibrosis has been demonstrated in both *in vitro* studies and in clinical papers. TGF-β isoforms were suppressed in corneal and limbal fibroblasts upon contact with the AMM [25]. Normal conjunctival and pterygium fibroblasts that were cultured on the AMM had decreased expression of TGF-β [19]. Although TGF-β has anti-inflammatory properties, its inhibition by the AM does not induce an overall pro-inflammatory response, suggesting that the AM has many other anti-inflammatory mechanisms. The AMM was shown in a rabbit model to trap leucocytes and to cause apoptosis [29, 30]. The expression of IL-1α and IL-1β was down-regulated in LPS-activated corneal epithelial cells when cultured on the AM stromal matrix [21]. Expression of bFGF and PDFG was down-regulated in limbal epithelial cells, while that of IL-1β and TGF-α was suppressed in conjunctival epithelial cells when cultured on AM basement membrane [31].

Many clinical reports have demonstrated the clinical efficacy of the AM in suppressing inflammation. These include suppression of acute or chronic inflammation after chemical burns [1–3], in Stevens Johnson Syndrome, in pterygium surgery [4, 32], and in symptomatic bullous keratopathy [8]. A recent study described the clinical use of the AM in managing complications of allergic inflammation. In that report, the AM was used to promote the healing of shield ulcers and plaques in vernal keratoconjunctivitis [12].

The exact mechanism by which the AM suppresses the expression of the various cytokines and mediators described above remains unclear. Two main concepts try to explain the anti-inflammatory and anti-fibrotic effects of the AMM. The

first emphasized the three-dimensional structure of the matrix environment. Studies of mammary epithelial cells have disclosed that gene expression leading to proliferation, differentiation and apoptosis is modulated by the extracellular matrix (ECM) [33]. Bi-directional communication between the matrix micro-environment and the nucleus is mediated via membrane receptors, resulting in selective gene expression. For example, overexpression of c-myc, TGF-α, fos, jun and TGF-β in a two-dimensional culture is suppressed upon contact with ECM, while that of growth inhibitor genes (p21, p27) is suppressed when cultured on plastic [34]. In this model, signalling from ECM is mediated through such receptors as β_1-integrin and EGFR, which are coordinately down-regulated upon contact with the ECM. These mechanisms may have been responsible for suppressing the inflammatory and fibrotic cytokines in our model.

The second concept involves several matrix components, which may act individually or synergistically to suppress inflammatory and fibrotic mediators. One such matrix component is decorin, which can bind and inhibit TGF-β. Decorin was found in high amounts in the AM, and it can bind TGF-β and reduce its function. Several studies had demonstrated an inhibitory effect of decorin on TGF-β activity in fibrosis [35–37]. These data may explain the lower protein levels of TGF-β found in our study in the AM cultures. Another possibility is the role of hyaluronic acid in suppression of inflammation. In human and monkey placenta, hyaluronic acid (HA) is thought to be the only glycosaminoglycan found [38], but this finding has not been verified in the AM. The synthesis of an HA-enriched matrix may play a role in scarless fetal wound healing [39]. Interestingly, a hyaluronic acid–protein–collagen complex was isolated from the fetal wound, and found to reduce TGF-β_1 and TGF-β_2 protein expression when applied to an adult skin wound [40]. Recently, high-molecular-weight HA was found to inhibit the expression of the transcription factor NF-κB, and as a result to suppress the synthesis of NF-κB-regulated cytokines such as IL-1α, IL-6 and TNF-α, all of which are potent pro-inflammatory cytokines [41].

Future studies are needed to investigate the exact mechanism by which the various matrix components are involved in the suppression of inflammation and fibrosis. The isolation of one or more matrix components from the AMM may be used clinically to tame down the inflammatory and fibrotic manifestations of severe allergic disorders. However, these individual components, when used separately, may not have the same potent effects caused by the AMM, where many factors may play in concert to induce suppression of fibrosis and inflammation, and where the three-dimensional structure of the AMM may exert effects that cannot be achieved by its single components. Our study further extends the scope of the possible uses of the AM into allergic inflammation, and turn the AMM into a potential target for exploring candidate matrix components for future therapy of severe ocular allergic diseases.

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