Cell cultures from bronchial subepithelial myofibroblasts enhance eosinophil survival *in vitro*

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ABSTRACT: Mechanisms of eosinophil accumulation and activation in the bronchial mucosa are crucial for the pathogenesis of asthma. The location of specialized fibroblasts, myofibroblasts, beneath the bronchial basement membrane and their proximity to infiltrating eosinophils potentially enable the myofibroblasts to modulate eosinophil survival and function in asthma. The aim of this study was to investigate the effects of bronchial myofibroblasts on eosinophil survival *in vitro*.

Eosinophils from human peripheral blood were exposed to cell cultures from bronchial myofibroblasts and to myofibroblast-conditioned media. Eosinophil viability was assessed and granulocyte/macrophage colony-stimulating factor (GM-CSF) production was examined in co-culture supernatants and as messenger ribonucleic acid (mRNA) in myofibroblasts.

Eosinophil survival was significantly increased and eosinophil apoptosis was inhibited by co-culture with myofibroblasts. Conditioned medium from tumour necrosis factor-α (TNF-α)-stimulated myofibroblasts also prolonged eosinophil survival. This effect could be blocked by GM-CSF antibody. GM-CSF mRNA and secretion from myofibroblasts were increased in co-cultures and by eosinophil-conditioned medium. Addition of antibodies to TNF-α and interleukin-1α (IL-1α) to co-cultures resulted in significant reduction both in eosinophil survival and GM-CSF levels. Blocking of fibronectin in the co-cultures did not affect the eosinophil survival enhancing activity. Prednisolone inhibited the eosinophil survival enhancing activity of the co-cultures by suppression of GM-CSF production.

Soluble eosinophil-derived cytokines are involved in the interaction of eosinophils with myofibroblasts, which results in a tumour necrosis factor-α/interleukin-1α mediated release of granulocyte/macrophage colony-stimulating factor from myofibroblasts. Bronchial myofibroblasts can, thereby, contribute to allergic inflammation by granulocyte/macrophage colony-stimulating factor-mediated inhibition of eosinophil apoptosis.
Eur Respir J., 1996, 9, 1839–1846.

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Eosinophilia in the peripheral blood and tissues is characteristic of allergic disorders, such as bronchial asthma [1–3]. The extent of eosinophil infiltration of the bronchial mucosa correlates with the clinical severity of asthma [4]. This is thought to be due to eosinophils acting as effector cell by the secretion of their unique cytotoxic cationic proteins [5], which are stored in eosinophil granules and are released upon activation. These cationic proteins have been shown to damage respiratory epithelium [6], and eosinophil major basic protein has been demonstrated in association with epithelial damage in fatal asthma [7].

Multiple mechanisms are responsible for the preferential eosinophil infiltration and accumulation in the bronchial submucosa and epithelium in asthma, including increased bone marrow production and release, and selective adhesion and migration through the vascular wall [8, 9]. The local microenvironment provided by the structural cells at the inflammatory site may have important effects on the inflammatory cell survival and activation [10], involving cell/cell interactions, extracellular matrix and cytokine networks. The population of specialized fibroblasts beneath the bronchial epithelial basement membrane, which have the contractile and synthetic cytoplasmic organelles of myofibroblasts, has been found to be increased in number in asthma in proportion to the thickening of the lamina reticularis of the basement membrane [11]. These bronchial myofibroblasts may also participate in the mucosal inflammatory response, as they are anatomically positioned so as to form close contact with and influence the function of inflammatory cells which migrate into the epithelium.

We have previously shown that cultured bronchial myofibroblasts from the bronchial epithelial lamina reticularis are capable of producing an array of cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), the production of which can be significantly enhanced by tumour necrosis factor-α (TNF-α) stimulation

[12]. In the present study, the contribution of bronchial myo-fibroblasts to mucosal inflammation in asthma has been further investigated by examining their effects on eosino-phil survival *in vitro.*

Materials and methods

Myofibroblast tissue culture

Biopsies of bronchial mucosa were obtained from asthmatic and nonasthmatic subjects undergoing fibreoptic bronchoscopy, or from lobectomy specimens for lung cancer. The specimens were washed in RPMI culture medium (Gibco, Paisley, UK) with 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin (Gibco), and were dissected under a stereomicroscope (Kowa, Tokyo, Japan) using mounted hypodermic needles [12]. The fragment consisting of only lamina reticularis and epithelium was then cultured in 5% CO_2 and 98% humidity in RPMI medium supplemented with 15% foetal calf serum (FCS) (Gibco) and antibiotics under a sterile coverslip. Once the growth of bronchial myofibroblasts was established, the primary culture was then trypsinized and plated into 25 cm^2 tissue culture flasks (Nunc, Kamstrup, Denmark) and grown to confluence.

The original tissue fragments were fixed in 3% glutaraldehyde, stained with toluidine blue and examined by microscopy for the completeness of removal of extraneous tissue: blood vessels, smooth muscle and glands from the lamina reticularis. The cultured bronchial myofibroblasts were further characterized using immunocytochemistry, as described previously [12]. They were immunostained with mouse monoclonal antibodies: CAM5.2 (Becton Dickinson, Oxford, UK) to low molecular weight (MW) cytokeratins of epithelial cells; AE3 (Dako, Oxford, UK) to general epithelial cell cytokeratins; QBEND 30 (Serotec, Oxford, UK) to CD34 on endothelial cells; α-SMA (Sigma, Poole, UK) to α–smooth muscle actin and vimentin antibody (Dako) [12]. The monolayer of myofibroblasts were also immunostained with rabbit polyclonal antibody to fibronectin (Chemicon, Harrow, UK). Cultures were, subsequently, either used for experiments, passaged or frozen in liquid nitrogen. Experiments were performed with cells up to the second passage.

Eosinophil isolation procedure

The method used was adapted from HANSEL *et al.* [13]. Eosinophils were obtained from the peripheral blood of donors with high eosinophil counts (5–10%). These donors were either asymptomatic (n=2) or had a history of rhinitis and/or conjunctivitis on exposure to grass pollen (n=2) or cats (n=1). Mean immunoglobulin E (IgE) levels were 376 IU·mL^{-1}. None of the donors had symptoms within 1 month of blood donation and none were taking medication. Fifty millilitres of heparinized blood were diluted with an equal volume of phosphate-buffered saline (PBS)/ 5% FCS. Percoll solution (Pharmacia LKB, Uppsala, Sweden) was diluted with Hanks' buffered saline solution (Gibco) to achieve a density of 1.082 g·mL^{-1}. The PBS diluted blood was then layered above the Percoll at 2:1

ratio and centrifuged at 1,613×g at 20°C for 30 min. Plasma at the top and the mononuclear cells at the interface were discarded. The granulocyte/red cell pellet was recovered. The erythrocytes were removed by cold hypotonic lysis. Anti-CD 16-coated magnetic microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) were added to the remaining granulocyte mixture (50 µL per 10^7 cells) and incubated at 4°C for 30 min. The CD16-microbead bound neutrophils were removed by retention in the column using a magnetic cell separation system (MACS) (Miltenyi Biotech). Eosinophils were collected, resuspended in RPMI and counted using the Kimura stain method.

Eosinophil survival experiments

Eosinophils-myofibroblast co-culture. Myofibroblasts were seeded in each well of 24-well plates (Costar, Cambridge, UK) at 10^5 per well, and were cultured in 1.5 mL RPMI/ 10% FCS for 24 h to allow the cells to adhere. The medium was then aspirated and eosinophils at 3×10^5 in 1.5 mL of the same medium were delivered into each well and co-cultured at 37°C for 72 h.

In separate experiments, rabbit polyclonal blocking antibodies to GM-CSF at 100 µg·mL^{-1} (Genzyme, West Malling, UK) and to interleukin (IL)-5 at 10 µg·mL^{-1} (Pharmingen, Cambridge, UK) were added to the co-cultures. In addition, rabbit polyclonal neutralizing antibodies against tumour necrosis factor-α (TNF-α) and IL-1α (Genzyme), either alone (anti-TNF-α 10 µg·mL^{-1}, anti-IL-1α 50 µg·mL^{-1}) or in combination, were added to the eosinophils for 2 h at 37°C prior to co-culture, and the antibody-eosinophil suspension was then transferred to the co-culture. Eosinophils were also incubated with equivalent concentrations of normal rabbit serum (NRS) during co-culture as a control. Eosinophil survival was examined after incubation for 72 h in all the experimental conditions. The effects of steroid on eosinophil survival in co-culture were assessed by adding varying concentrations (1, 0.5, 0.25 and 0.1 µM) of prednisolone to the co-cultures for 72 h.

To study the role of cell and matrix contact, the co-culture was performed as described above, except that eosinophils were separated from the myofibroblast monolayer by a 0.4 µm pore cell-culture insert (Costar). Bronchial myofibroblasts were also incubated with rabbit polyclonal fibronectin blocking antibody (Chemicon, Harrow, UK) prior to and during co-culture at 10, 25 and 50 µg·mL^{-1}. Eosinophils were also co-cultured with glutaraldehyde-fixed myofibroblasts using a method adapted from CHU and RICH [14], in which myofibroblasts in 24-well plates were fixed in 0.2% glutaraldehyde in PBS at room temperature for 15 min before being washed off, and eosinophils were then added to the wells and co-cultured. Furthermore, in separate experiments, eosinophils were incubated with blocking antibody to β1-integrin (clone JB1a, Chemicon) at 25 µg·mL^{-1} for 1 h prior to and during the co-culture. The eosinophil survival was measured after 72 h.

Myofibroblast-conditioned medium. Freshly isolated eosinophils were also cultured in myofibroblast-conditioned

media (CM). The CM were generated from second passage myofibroblasts cultured either in RPMI/10% FCS alone or in the medium with 5 U·mL^{-1} TNF-α (Genzyme) for 48 h. Eosinophils were cultured in CM in 96-well plates (ICN, Thame, UK) at 10^5 cells·well^{-1} in 250 µL medium for 72 h. Controls included CM to which TNF-α was added before culture with eosinophils to determine the effect of TNF-α on eosinophil survival. Eosinophils were also incubated with human recombinant GM-CSF (Genzyme) at concentrations of 1 ng·mL^{-1}, 200 pg·mL^{-1}, 40 pg·mL^{-1}, 8 pg·mL^{-1} and 1.6 pg·mL^{-1} for 96 h as positive controls for sustained viability. In a number of experiments, TNF-α stimulated myofibroblast-conditioned medium or medium containing GM-CSF were preincubated with a rabbit neutralizing polyclonal antibody against human GM-CSF (Genzyme) at 100 µg·mL^{-1} for 2 h at 37°C before eosinophils were added.

Assessment of eosinophil survival. Eosinophil survival was assessed by the trypan blue exclusion method after culturing in various conditions. Cell viability was expressed as the percentage of viable cells. All the experiments were in triplicate, counted without knowledge of the experimental conditions, and were repeated 6–8 times.

Deoxyribonucleic acid (DNA) fragmentation analysis by gel electrophoresis. Eosinophils were cultured in enriched medium alone, or with 1 ng·mL^{-1} GM-CSF, or co-cultured with myofibroblasts for 24 and 72 h. Eosinophils were then harvested, pelleted at 4°C and resuspended (0.5–1 × 10^6 cells) in 200 µL of 10 mM ethylenediamine tetra-acetic acid (EDTA) and 50 mM Tris-Cl, pH 8.0, containing 0.5% sodium dodecyl sulphate (SDS) and 0.5 mg·mL^{-1} proteinase K (Sigma), and incubated for 60 min at 50°C [15]. Ribonuclease A was then added to a final concentration of 0.2 mg·mL^{-1} and further incubated for 60 min [15]. DNA was then reconcentrated by cold ethanol precipitation and, subsequently, electrophoresed on a 2% agarose gel. The gel was visualized by ethidium bromide staining and photographed under ultraviolet (UV) light.

Measurement of GM-CSF production in co-cultures

GM-CSF mRNA in myofibroblasts. Ribonuclease protection assay (RPA) was performed as described previously [12]. Briefly, a 430 base GM-CSF antisense ribonucleic acid (RNA) probe was generated from pSP73-GM-CSF plasmid using SP6 RNA polymerase and labelled with ^{32}P-uridine triphosphate (UTP) (ICN, Thame, Kent). Using the guanidine isothiocynate/acid phenol extraction procedure, total RNA was extracted from myofibroblasts after 72 h either in co-culture with eosinophils, or cultured alone with or without TNF-α (5 U·mL^{-1}). Ten micrograms of RNA from each sample was hybridized with 10^5 counts per minute (cpm) complementary ribonucleic acid (cRNA) probe followed by ribonuclease (RNase) digestion using the RPAII™ system (Ambion Biotechnology, Witney, UK). The protected GM-CSF messenger ribonucleic acid (mRNA) fragments were detected and visualized by polyacrylamide gel electrophoresis and autoradiography. The GM-CSF mRNA expression in the cells was standardized by demonstrating β-actin mRNA

expression. The 360 base pair (bp) anti-sense mouse β-actin cRNA probe was generated using pTR1-β-actin plasmid (RPA™; Ambion) and hybridized with an equivalent amount of sample RNA under identical conditions.

GM-CSF levels in culture supernatants. GM-CSF levels were measured in culture supernatants from various conditions as described above, using an enzyme-linked immunosorbent assay (ELISA) system (Amersham, Buckinghamshire, UK). Eosinophil-conditioned medium was generated by incubating eosinophils alone in RPMI/10% FCS for 24 h. The culture supernatant was then collected and added to myofibroblast monolayers for 72 h prior to harvesting of the supernatant for ELISA measurement.

Statistical methods

Results were assessed for normality, expressed as mean ±SD, and tested for differences by Student's t test. A p-value of <0.05 was considered significant.

Results

Immunostaining of cultured bronchial myofibroblasts

Immunocytochemistry of cultured myofibroblasts showed negative staining with CAM5.2, AE3 and QBEND antibodies, indicating that there was no epithelial and endothelial cell contamination in the cultures (data not shown). Positive cytoplasmic staining for α-SM actin and vimentin was found, indicating the myofibroblastic origin of the cells. Similarly, the polyclonal blocking antibody to cellular fibronectin produced intense staining of the myofibroblast monolayers at all concentrations used in the co-culture experiments.

Co-culture enhancement of eosinophil viability

Eosinophil preparations of 97–99% purity were obtained. Initial eosinophil preparations were 99% viable, as assessed by their exclusion of trypan blue, but only 2±1% of the initial population were viable after 3 days of culture in enriched medium alone (fig. 1). In contrast, eosinophil survival was maintained at 80–90% in co-culture with myofibroblasts (p<0.001). The eosinophil survival enhancing activity of the co-cultures was reduced by half by addition of blocking antibody to GM-CSF in the co-cultures (81±4 *vs* 47±4%, p<0.001) (fig. 1a). Neutralization of IL-5 by anti-IL-5 antibody in the co-culture did not significantly affect eosinophil survival enhancing activity of the co-cultures (81±4 *vs* 75±5 %) (fig. 1a). To investigate the role of soluble mediators released from eosinophils in the co-cultures, eosinophils were preincubated with neutralizing antibodies against cytokines TNF-α and IL-1α and co-cultured with these antibodies, either alone or in combination. Both anti-TNF-α and anti-IL-1α only slightly inhibited the survival enhancing activity (92±2 *vs* 85±3 *vs* 76±5%, respectively) after 72 h culture (fig. 1b), but when used together anti-TNF-α and anti-IL-1α antibodies significantly reduced the survival enhancing activity of the co-cultures to 38±5% (p<0.001, fig. 1b).
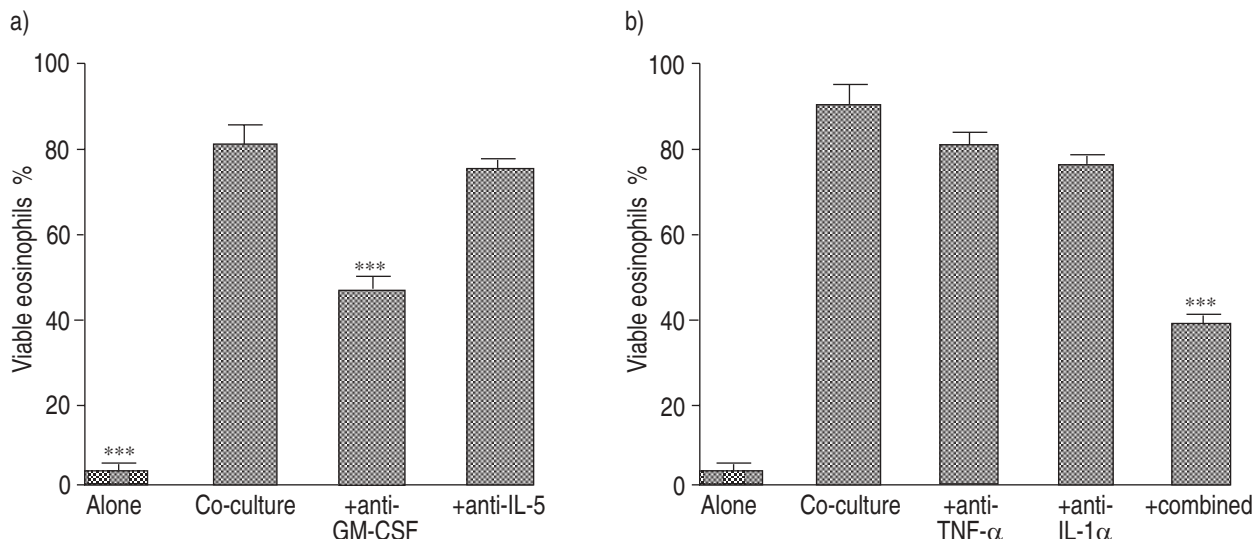
a)

b)

Fig. 1. – Eosinophil survival in co-culture with bronchial myofibroblasts. a) Alone or in co-cultures in the presence and absence of neutralizing antibodies to GM-CSF and IL-5. b) Alone or in co-cultures in the presence and absence of neutralizing antibodies against TNF-α and IL-1α either singly or in combination (combined). The data are presented as the mean±SD of eight independent experiments. ***: p<0.001 *vs* co-culture. GM-CSF: granulocyte/macrophage colony-stimulating factor; IL-5: interleukin-5; TNF-α: tumour necrosis factor-α; IL-1α: interleukin-1α.

The contribution of cell/cell contact in the prolongation of eosinophil survival was examined in co-culture experiments, in which eosinophil-myofibroblast contact was prevented by separating the two cell types using a porous cell culture insert. The viability of eosinophils from insert separated co-culture after 72 h was reduced from 84±9 to 53±12% (fig. 2), but did not reach statistical significance. The porous insert itself had no effect on eosinophil survival (90±3%) in co-culture. However, eosinophil survival was almost abolished (1.2±0.2%) after 72 h in co-culture with myofibroblasts fixed by glutaraldehyde (fig. 2), similar results to that with eosinophils cultured in medium alone (1±0.2%). The blocking of fibronectin by a polyclonal fibronectin antibody at concentrations of 10–50 µg·mL^{-1} in the co-cultures did not reduce eosinophil viability (84±9 *vs* 80±7% at 10 µg·mL^{-1}, *vs*

80±3% at 25 µg·mL^{-1}, and *vs* 78±6% at 50 µg·mL^{-1}). However, eosinophil survival in co-culture was reduced by blocking the adhesion molecule β1-integrin on eosinophils (84±9 *vs* 53±5%; p<0.01) (fig. 2). Rabbit serum controls in the co-culture did not have any effect on eosino-phil survival.

In agreement with the cell viability by trypan blue exclusion, DNA electrophoresis analysis of eosinophils cultured in medium alone after 24 and 72 h showed typical apoptotic DNA fragmentation (fig. 3). In contrast, DNA from the eosinophils co-cultured with myofibroblasts or with GM-CSF was intact.

The effects of myofibroblast-conditioned medium on eosinophil survival

To investigate effects of the mediators released from bronchial myofibroblasts on eosinophil survival, the
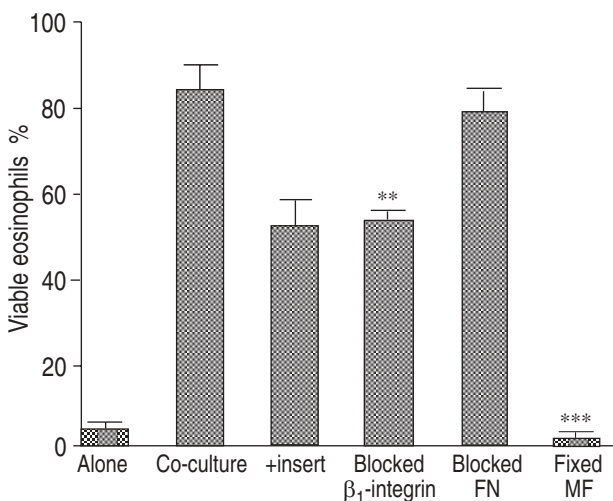

Fig. 2. – Eosinophil survival alone or in co-culture with bronchial myofibroblasts. Co-cultures were performed with cell contact or wih contact prevented by insert separation, blocking fibronectin (FN) with 50 µg·mL^{-1} of antibody, blocking β_1-integrin or glutaraldehyde fixation of myofibroblasts (MF). The data are presented as mean±SD of eight independent experiments. **, ***: p<0.01, p<0.001 *vs* co-culture.
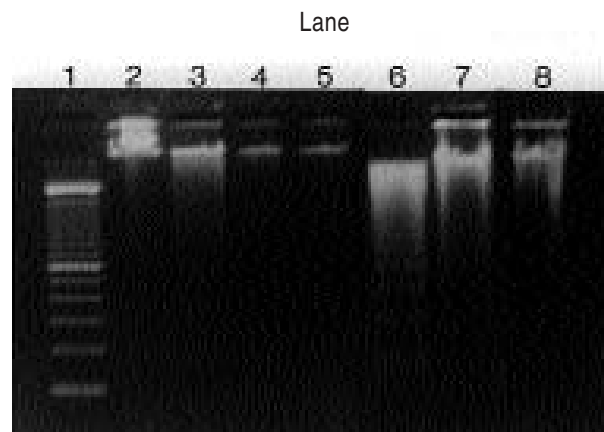
Lane

Fig. 3. – Eosinophil deoxyribonucleic acid (DNA) electrophoresis on agarose gel. Lane 1: molecular size marker; Lane 2: freshly isolated eosinophils; Lanes 3 and 6: eosinophils cultured alone for 24 and 72 h; Lanes 4 and 7: eosinophils cultured with granulocyte/macrophage colony-stimulating factor (GM-CSF) (1 ng·mL^{-1}); Lanes 5 and 8: eosinophils co-cultured with myofibroblasts for 24 and 72 h.
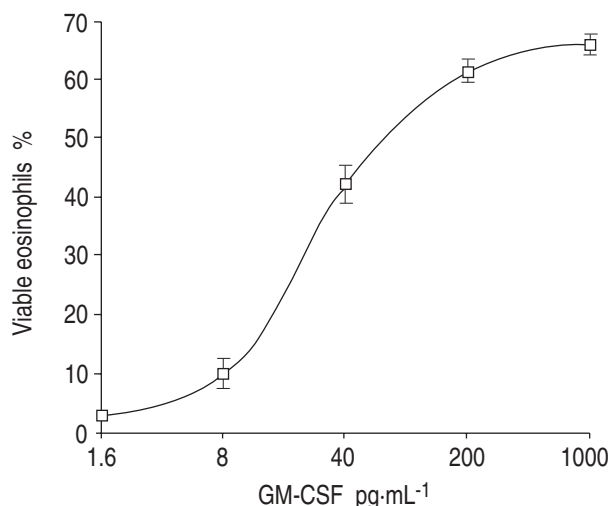
Fig. 4. – The effects of granulocyte/macrophage colony-stimulating factor (GM-CSF) on eosinophil viability after 96 h culture. The data are presented as the mean±SD of six independent experiments.

eosinophils were cultured in myofibroblast-conditioned media either from unstimulated or TNF-α stimulated myofibroblasts. Eosinophils were also cultured with GM-CSF as a positive control. Culture with GM-CSF showed a dose-dependent enhancement of eosinophil viability (fig. 4). When eosinophils were cultured in unstimulated myofibroblast-conditioned medium, their survival after 72 h was no greater than when cultured in medium alone (2.7±0.5 vs 3±0.5%) (fig. 5). However, culture of eosinophils with TNF-α stimulated myofibroblast-conditioned medium significantly increased their survival to 60±3% (p<0.001, fig. 5). The addition of TNF-α to eosinophils alone in cultures or to medium conditioned by unstimulated fibroblasts had no effect on eosinophil viability. The eosinophil viability enhancing effect of conditioned medium from TNF-α stimulated myofibroblasts was partially counteracted by a neutralizing antibody against GM-CSF (fig. 5), decreasing eosinophil survival to 24±2%.
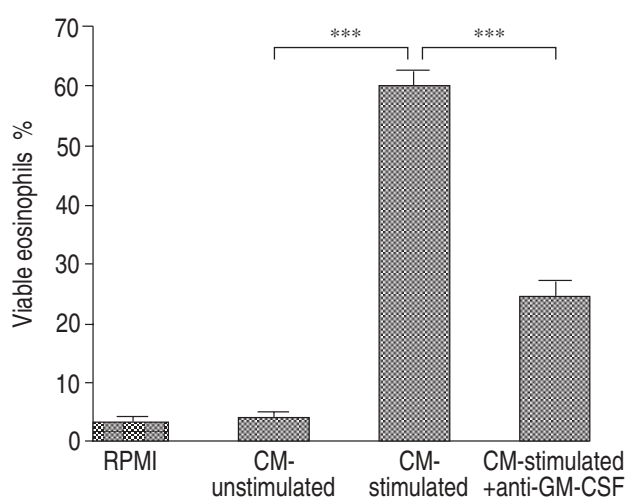

Fig. 5. – Eosinophil survival after culture for 72 h in conditioned media (CM) from unstimulated or TNF-α-stimulated myofibroblasts, with or without blocking antibody against GM-CSF. The data are presented as mean±SD of eight independent experiments. ***: p<0.001. For definitions see legend to figure 1.
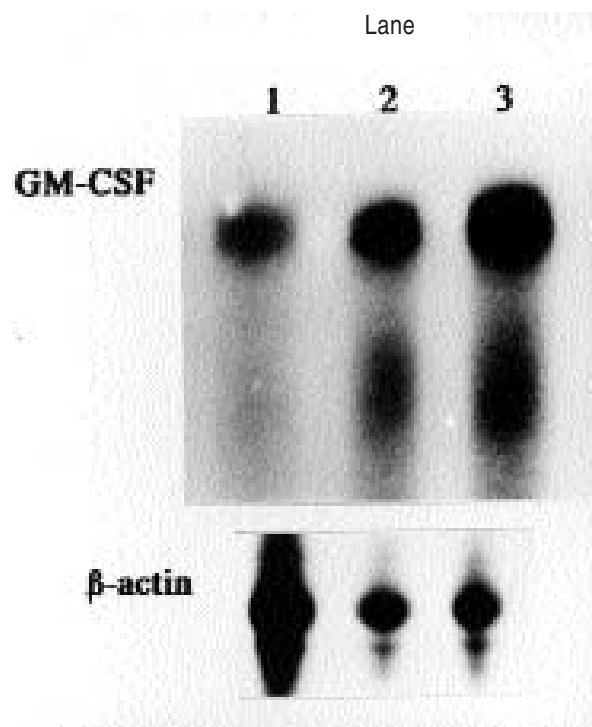

Fig. 6. – GM-CSF mRNA expressions in myofibroblasts by RPA after 72 h culture: in medium alone (lane 1); in co-culture with eosinophils (lane 2); and in the presence of TNF-α (lane 3). A 410 bp protected GM-CSF mRNA fragment was shown in all the samples. The mRNA expression was verified by detection of 260 bp protected fragment for β-actin mRNA (bottom). mRNA: messenger ribonucleic acid; RPA: ribonuclease protection assay; bp: base pair. For further definitions see legend to figure 1.

GM-CSF production in co-cultures

To determine the role of GM-CSF in eosinophil survival enhancement activity of co-cultures, GM-CSF production by myofibroblasts was examined both at the level of mRNA transcripts in the cells and of the secreted protein in the culture supernatants.

RPA demonstrated GM-CSF mRNA in the myofibroblasts from co-culture with eosinophils, or cultured alone in the presence or absence of TNF-α (5 U·mL^{-1}) for 3 days. As shown in figure 6, there was a marked increase in GM-CSF mRNA levels in the myofibroblasts from co-culture with eosinophils and also in the cells stimulated by TNF-α compared to the myofibroblasts cultured alone (fig. 6). This was independent of the levels of expression of β-actin mRNA.

Corresponding to the increased GM-CSF mRNA, GM-CSF secretion was significantly induced in eosinophil-myofibroblast co-cultures in comparison to the levels in the supernatants of myofibroblasts cultured alone (388 vs 4.17 pg·mL^{-1}; p<0.001) (table 1). The increased GM-CSF secretion in co-cultures was found to be induced by mediators released from eosinophils, since GM-CSF was not detectable in eosinophil-conditioned medium but levels increased significantly after myofibroblasts were cultured in eosinophil-conditioned medium (0 vs 500 pg·mL^{-1}); levels similar to those found in myofibroblast cultures with TNF-α (520 pg·mL^{-1}). Preincubation and co-culture of eosinophils with each of the antibodies against TNF-α and IL-1α partially reduced the GM-CSF levels compared to the levels in co-cultures without antibody. The

Table 1. – GM-CSF levels in culture supernatants

Conditions	GM-CSF pg·mL^{-1}	p-value
Effects of anti-cytokine antibodies in co-culture		
MF only	4.17±0.68	<0.001
Eos + MF co-culture	388±137	
Co-culture:		
+NRS	370±05	
+anti-TNF-α	130±23	
+anti-IL-1α	67±12	
+anti-TNF-α/IL-1α	44±10	<0.001
Effect of Eos/MF contact in co-culture		
With Eos/MF contact	318±102	
Insert separation	24±9	<0.001
Glutaraldehyde fixed MF	ND	
Effect of Eos-CM		
Eos-CM	ND	
MF cultured in Eos-CM	500±50	
MF+TNF-α	520±79	
Steroid effects in co-culture		
Co-culture only	388±137	
+prednisolone 0.25 µM	130±14	
+prednisolone 0.5 µM	60±7	<0.001

GM-CSF levels are presented as mean values±SD of six independent experiments. The p-values are for comparison with control eosinophil-myofibroblast co-culture. GM-CSF: granulocyte/macrophage colony-stimulating factor; MF: myofibroblasts; Eos: eosinophils; NRS: normal rabbit serum; TNF-α: tumour necrosis factor-α; IL-1α: interleukin-1α; CM: conditioned medium; ND: not detected.

two antibodies showed an additive effect on the reduction of GM-CSF induction in the co-cultures. Normal rabbit serum at equivalent concentrations had no effect on GM-CSF levels in co-cultures.

Cell/cell contact appeared to be required for the induction of GM-CSF secretion in co-cultures, since separation of the two cell types by a porous insert in co-cultures resulted in a marked decrease in GM-CSF secretion from 318 to 25 pg·mL^{-1} (p<0.001) (table 1), which was in keeping with the reduction in eosinophil viability in membrane-separated cultures.
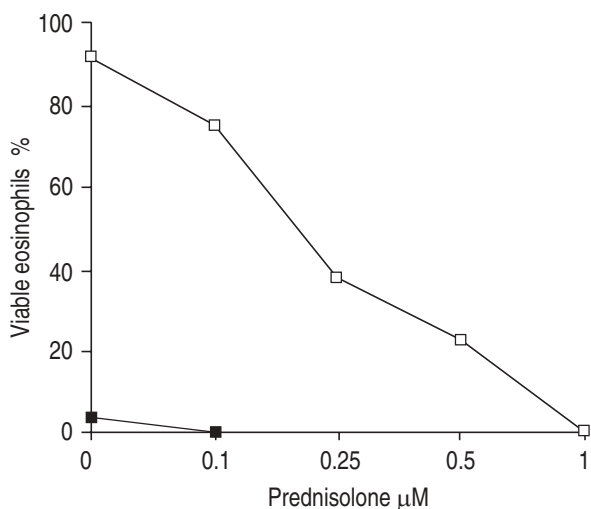

Fig. 7. – The effects of prednisolone on eosinophil survival in co-cultures with (□) or without (■) myofibroblasts after 72 h. The data are presented as mean values of six independent experiments.

Effect of steroid on eosinophil survival and induction of GM-CSF in co-cultures.

The addition of prednisolone in eosinophil-myofibroblast co-cultures produced a dose-dependent inhibition of eosinophil survival (fig. 7). At a concentration of 0.25 µM, more than 50% of the co-culture activity in sustaining eosinophil survival was inhibited, and at 1 µM almost no eosinophils survived in co-culture (fig. 7). Correspondingly, the induction of GM-CSF secretion in co-culture was also significantly inhibited by prednisolone, as shown in table 1.

Discussion

In this study, using myofibroblast-eosinophil co-culture systems, it has been demonstrated that eosinophil survival was significantly prolonged by culture with bronchial myofibroblasts compared to culture in medium alone. This was due to myofibroblast inhibition of eosinophil apoptosis. This result is in agreement with a previous study [16], which showed increased eosinophil survival in co-culture with human lung fibroblasts. It is known that eosinophils are short-lived cells with 18 h half-life in the blood [17], while survival in the extracellular compartment is determined by local conditions. It has been well-documented that the survival *in vitro* of human peripheral blood eosinophils requires continuous exposure to one of three haematopoietins: GM-CSF [15], IL-3 [18] and IL-5 [19]. Since eosinophils selectively accumulate in the bronchial mucosa in asthma [20], it would be expected that local tissue factors play a major role in the enhancement of eosinophil survival and activation, hence increasing their inflammatory effects. One of the structural tissue changes in the bronchial mucosa in asthma is an excess deposition of collagens I, III, V and fibronectin in the lamina reticularis [21], which was found to correlate with increased numbers of bronchial myofibroblasts at that site [11]. These structural cells, by close contact with inflammatory cells, may also modulate allergic inflammation by interaction with effector cells, such as eosinophils, in bronchial asthma.

The reduction in eosinophil survival when contact between eosinophils and myofibroblasts was eliminated by a porous filter suggests that cell/cell or cell/matrix interactions are involved, although not essential, in the eosinophil viability enhancing process. This is consistent with the results that the blocking of β$_1$-integrin adhesion molecules on eosinophils reduced the co-culture eosinophil survival enhancing activity by 30%, almost identical to the effect of separation of the two cell types by a porous filter. Extracellular matrix fibronectin is known to be a ligand for the β$_1$-integrin very late activation antigen-4 (VLA-4) on the surface of eosinophils [22]. The adhesion of eosinophils to fibronectin has been found to increase eosinophil survival [23, 24], and such viability enhancement could be inhibited by antibodies against fibronectin or VLA-4. However, the present study showed that fibronectin was not involved in the eosinophil survival enhancing process of the co-cultures, since blocking fibronectin did not significantly affect eosinophil survival. Thus, the contact effect appeared to act at the level of eosinophil stimulation of cytokine secretion by the

myofibroblasts in our cultures. This cell/cell interaction may expose myofibroblasts to high local concentrations of stimulatory cytokines and, similarly, eosinophils to high local concentrations of haematopoietic growth factors [25]. Moreover, direct interaction between cell surface adhesion molecules and their ligands may elicit positive intercellular signalling events, which may also be involved in the survival of eosinophils [25].

Culture of eosinophils in conditioned medium from TNF-α stimulated myofibroblasts significantly prolonged cell survival and TNF-α induced GM-CSF secretion by myofibroblasts. This study has shown that the enhanced eosinophil survival was due to GM-CSF in the culture supernatant, since the eosinophil survival increasing activity of either myofibroblasts in co-cultures, or of stimulated myofibroblast-conditioned medium, could be reduced by 38 and 60%, respectively, by the anti-GM-CSF antibody treatment. The study also demonstrated that in co-culture with glutaraldehyde-fixed myofibroblasts the eosinophil survival enhancing activity of myofibroblasts was completely abolished. Glutaraldehyde preserves cell membrane integrity but prevents active response from treated cells in culture [14], supporting a major role for cytokine secretion in the stimulation of eosinophil survival. The failure of conditioned medium from unstimulated myofibroblasts to prolong eosinophil survival is in contrast to a previous study, in which eosinophils showed increased viability when cultured with conditioned medium from unstimulated lung fibroblasts [16]. Whilst this discrepancy may be due to different experimental conditions, it is likely that bronchial myofibroblasts closely regulate their secretion of GM-CSF in order to prevent the inappropriate activation of inflammatory processes in the bronchial mucosa.

It appears that IL-5 did not play a major role in the prolongation of eosinophil survival by co-culture with myofibroblasts, since anti-IL-5 antibody treatment of the co-cultures did not alter the eosinophil survival increasing activity. The autocrine production of IL-5 by eosinophils themselves also appeared not to contribute to the survival enhancement process. These findings add further support to the hypothesis that GM-CSF is the major factor for eosinophil survival enhancing activity in co-culture.

We regard myofibroblasts as the source of GM-CSF secretion in this system since the GM-CSF mRNA level was markedly enhanced in the myofibroblasts by co-culture with eosinophils. Although it is known that eosinophils themselves synthesize GM-CSF [26], GM-CSF could not be detected in the culture supernatants from eosinophils cultured alone or in the presence of glutaraldehyde-fixed myofibroblasts, but GM-CSF levels were significantly increased after culturing myofibroblasts in eosinophil-conditioned medium. GM-CSF levels were reduced in co-cultures by the presence of antibodies against TNF-α and/or IL-1α, consistent with the extent of inhibition of eosinophil survival. The inhibition of GM-CSF induction and the survival effect of co-culture was more complete with both antibodies together. Since these two mediators have been reported to be expressed by eosinophils [27], we suggest that the production of GM-CSF from myofibroblasts was induced by TNF-α and IL-1α released from eosinophils. The contribution of cell/cell contact to the induction of GM-CSF was confirmed by the reduction in GM-CSF levels in supernatants of insert separated co-cultures.

Prednisolone was found to partially counteract the co-culture eosinophil survival enhancing activity by inhibiting the induction of GM-CSF secretion in the co-cultures, although a direct effect on eosinophils cannot be excluded. It has been reported previously [28] that prednisolone treatment was accompanied by a decrease in broncho-alveolar lavage (BAL) eosinophils and a change in the cytokine profiles of BAL cells. Thus, steroids may act by modulating local cytokine production with subsequent inhibition of bronchial eosinophilia.

In conclusion, the present study has clearly shown that bronchial myofibroblasts can prolong the survival of human peripheral blood eosinophils *in vitro*. Because of their location just beneath the bronchial epithelium, where eosinophil infiltration is most intense, bronchial myofibroblasts may be one of the important determinants of the survival and activation of eosinophils for subsequent bronchial epithelial damage and enhancement of the clinical severity of asthma.

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