Eosinophil Interaction With Matrix-Producing Lung Cells: An In Vitro Model System Employing AML14.3D10 Eosinophil-Like Cells To Interact With AH1F Fibroblasts And A549 Epithelial Cells

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Citation

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Abstract

The extracellular matrix protein fibronectin is found in the airway space after bronchial provocation and can extend the life of eosinophils in vitro. Herein we describe a system to study the effect of co-culture of eosinophil-like cells with cell lines derived from tissues. We compared integrin engagement on human peripheral blood eosinophils to several eosinophil-like cell lines, including EoL-3, AML 14.3D10, and HL-60 clone 15 cells. Effects of co-incubation of AML 14.3D10 cells with adherent cell AH1F fibroblasts or A549 epithelial cells were examined for adhesion, metabolic activity (reduction of cytochrome C), and fibronectin production. AML 14.3D10 cells had the ability to bind to the adherent cells, however the interaction was not reduced by blocking integrins on the eosinophil-like cells. The amount of fibronectin found in association with the extracellular matrix of AH1F fibroblasts or A549 epithelial cells as measured by ELISA was not altered when AML 14.3D10 cells were included as a co-culture. The co-cultures were separated into three fractions to measure fibronectin that was secreted into the medium or fibronectin associated with non-adherent cells, and also fibronectin associated with adherent cell extracellular matrices. Fibronectin was detected all three fractions, indicating that secreted fibronectin was not limited to incorporation within the extracellular matrix of adherent cells. Thus both fibroblasts and epithelial cells are capable of producing soluble cellular fibronectin and either may be the source of soluble cellular fibronectin found in the lungs of asthmatics after bronchoprovocation.

INTRODUCTION

Chronic asthma is characterized by airway remodeling and eosinophil efflux into the airway space. We have demonstrated a relationship between the matrix protein fibronectin and survival of eosinophils in in vitro cultures [1]. This soluble form of cellular (also known as tissue) fibronectin is found in increased amounts in the airway space BAL fluid of atopic subjects upon stimulation with antigen [2]. It is clear that the soluble form of cellular fibronectin causes human eosinophils to live longer in culture due to an autocrine production of GM-CSF [1]. It is not known the cellular origin of soluble cellular fibronectin within the airway space. Possibilities include lung tissue fibroblasts or airway epithelial cells.

Integrins are the cell surface receptors for matrix proteins and certain Ig-family adhesion molecules. Eosinophils express alpha 4-beta1 and alpha 4-beta 7 integrins that bind to fibronectin and VCAM [3]. Engagement of the beta 7 integrin with monoclonal antibodies or the soluble form of cellular fibronectin results in increased message for GM-CSF and enhanced in vitro survival [1]. However, engagement of alpha 4-beta 1 by monoclonal antibodies did not have the same effect and neither did the soluble form of VCAM indicating a specific interaction of fibronectin with beta 7 is needed. The message for fibronectin can be spliced into two major forms coding for either the plasma form of fibronectin found in the blood (produced as the primary splice variant in hepatocytes) or as the tissue (herein referred to as “cellular”) form produced by cells throughout the body such as fibroblasts. When secreted from cells in tissues, fibronectin can be integrated into the extracellular matrix and this process is highly controlled [4,5]. The fate of cellular fibronectin secreted by tissue cells that is not incorporated into matrix is not fully appreciated. At least

some of this soluble cellular fibronectin makes its way into the airway space, as demonstrated by examining BAL fluid after antigen challenge [2]. Within a matrix, fibronectin may bind soluble activators and alter cellular functions [6].

Human eosinophils produce increased amounts of superoxide anion when adherent to VCAM in the presence of activators fMet-Leu-Phe and cytochalasin B [7], GM-CSF [8] or leukotriene D_4 [9]. Blocking alpha 4-containing integrins with WAY103 (VLA-4 antagonist) inhibited superoxide production by stimulated cells, indicating an integrin-dependent production mechanism [10]. Conditioned medium obtained from human bronchial epithelial cells also causes eosinophils to release increased amounts of superoxide [11] in a manner that was dependent on the production of GM-CSF. Eotaxins have also been implicated to be involved in the increased production of superoxide by eosinophils exposed to A549 epithelial cell-conditioned medium [12]. It has been shown that eosinophils adhere to A549 epithelial cells [13] and fetal lung fibroblasts [14]. Eosinophils incubated in the presence of mouse 3T3 fibroblasts and GM-CSF exhibit enhanced survival [15] and fibroblasts incubated with conditioned medium from eosinophils exhibited enhanced replication [14]. It remains unclear how integrin engagement and interactions between eosinophils and tissue cells alter cell functions in the lung. Herein we describe a method of co-culture between eosinophils and tissue cells that may be useful in teasing apart these interactions.

Cell lines that mimic eosinophils have been useful in studying various cellular activities of eosinophils. The eosinophil cell lines AML 14.3D10, EoL-3, and HL-60 clone 15 have been used to study eosinophil adhesion and signaling mechanisms [16-18]. In this study we compare these cell lines to human eosinophils for adhesion to VCAM and monoclonal antibodies against integrins. The human foreskin fibroblast cell line AH1F produces the soluble form of cellular fibronectin known to increase survival of human eosinophils [1]. AH1F cells were therefore chosen to investigate interactions of eosinophils with fibroblasts. A549 cells are derived from human lung epithelium and have been shown to secrete eotaxin CCL26 [11]. Thus A549 cells were chosen to examine interactions with lung epithelium.

Herein we describe an experimental system to study eosinophil-like cell interaction with tissue cells. AML 14.3D10 cells demonstrated similar usage of integrins in static adhesion assays. Metabolic activity of AML 14.3D10 cells was not enhanced when co-cultured in the presence of either A549 epithelial cells or AH1F fibroblasts. The amount of fibronectin detected within matrices produced by either A549 epithelial cells or AH1F fibroblasts was not enhanced by co-culture with AML 14.3D10 cells. Examination of fibronectin found in conditioned medium or in association with non-adherent cells indicates that either fibroblasts or epithelial cells may be responsible for the soluble cellular fibronectin found within the lungs of asthmatics after bronchial provocation.

MATERIALS AND METHODS

REAGENTS:

Except otherwise noted all cell culture reagents were purchased from Sigma Chemical Company (St. Louis. MO).

HUMAN EOSINOPHIL ISOLATION:

Eosinophils were isolated from the peripheral blood of normal donors and patients with allergic rhinitis or mild bronchial asthma by an anti-CD16 magnetic bead cell separation system (MACS; Miltenyi Biotech, Auburn CA) as described previously [1]. Purity of isolated cells was verified by Diff-Quick (Baxter Health Care Corp., McGraw Park IL) staining and was at least 99% in most instances. Preparations less than 95% pure were not used in these experiments. Viability was greater than 99% as determined by trypan blue dye exclusion.

EOSINOPHIL-LIKE CELL LINE CULTURE:

AML 14.3D10 cells were a generous gift from Cassandra C. Paul (Wright State University School of Medicine, Dayton OH) and were cultured in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 5 x 10^{-5} M -mercaptoethanol, 100 U/ml penicillin, 100 g/ml streptomycin and 8% defined fetal calf serum (HyClone Laboratories, Logan, UT). EoL-3 cells were a generous gift from Richard Lynch (University of Iowa, Iowa City, IA) and were cultured in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal calf serum. Further differentiation along an eosinophil-like path was induced by the inclusion of 0.5 mM butyric acid (BA-EoL-3) within the medium for seven to ten days prior to use in assays [19,20]. Cells were observed on a daily basis and subcultured to a concentration of 1 x 10^5 viable cells per ml every two to three days in either 25 cm^2 or 75 cm^2 vent capped cell culture flasks (Corning Inc., Corning NY). In some experiments cells were pre-treated with cytokines or

activators for the times indicated in Results. Fresh medium containing activators was added to subcultured cells for the time indicated and replaced after 36 - 48 hrs during longer pre-treatment periods.

HL-60 and HL-60 clone 15 cells were purchased from ATCC (Manassas VA) and cultured in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. Cells were observed on a daily basis and subcultured to a concentration of 1 x 10^5 viable cells per ml every two to three days. In certain experiments fresh medium containing 16 nM PMA was added 20 hr before experimentation as noted in the results.

ADHERENT CELL CULTURE:

AH1F human foreskin fibroblasts were a gift from Lynn Allen-Hoffmann (University of Wisconsin, Madison WI) and were cultured in Dulbecco's Modified Eagle's medium supplemented with 3.7 g/L sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum [1,21]. A549 lung epithelial cells were purchased from ATCC and cultured in Ham's F12 medium supplemented with 2.5 g/L sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. Monolayer cultures were allowed to become confluent in either 25 cm^2 or 75 cm^2 vent capped cell culture flasks before trypsinization and subculture at 0.5 x 10^5 cells per ml in 96 well polystyrene culture plates (BD Falcon).

HYBRIDOMA CULTURE AND MONOCLONAL ANTIBODY ISOLATION:

Purified monoclonal antibodies PUJ1 to alpha 4 were purchased from Sigma and P4C10 to beta 1 from R&D Systems (Minneapolis, MN). David P. Andrew (Amgen Boulder, CO) generously provided quantities of monoclonal antibodies to beta 7 (Fib 21, Fib 22, Fib 27, and Fib 30) [22].

Hybridoma cell lines producing monoclonal antibodies to β1 integrin (TS2/16), β2 integrin (TS1/18), β7 integrin (Fib 21 and Fib 504), and to human fibronectin (HFN7.1 and P3NP/PFn) were purchased from ATCC. All hybridomas were cultured in DMEM supplemented with 2 g/L sodium bicarbonate, 4.5 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum except Fib hybridomas, which were cultured in RPMI 1640 supplemented with 4.5 g/L glucose, 15 mM HEPES, 0.1 mM non-essential amino acids, 5 x 10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. Cells were gradually acclimated to serum-free conditions before large culture in roller bottles containing either HyQ CCM1 (HyClone, Logan UT) or serum-free hybridoma medium (Sigma, St. Louis, MO). Monoclonal antibodies were isolated from hybridoma culture supernatant by passage over protein G-coupled agarose (Life Technologies, Grand Island, NY), eluted with 50 mM glycine-HCl, pH 2.5 and immediately buffer exchanged to 20 mM phosphate, 10 mM EDTA, pH 7.0.

ADHESION ASSAYS:

Pro-bind polystyrene 96 well plates (Becton Dickinson, Franklin Lakes, NJ) were coated overnight at 4 °C with either recombinant soluble VCAM-1 (R&D Systems, Minneapolis, MN) in PBS pH 6.5 or monoclonal antibodies in 15 mM $NaHCO_3$ 35 mM Na_2CO_3 pH 9.2. Non-specific sites in wells were blocked by incubation with HBSS containing calcium, magnesium and 0.1% gelatin for 30 minutes to 1 hr at 37 °C. Wells were emptied and rinsed before addition of cells for adhesion. In some cases cells were pre-treated with monoclonal antibody to integrins (or nonspecific control monoclonal antibodies) before incubation with wells. Adhesion to adherent cells was measured using cells cultured to confluence in 96 well polystyrene culture plates (Falcon). Cells were added to wells at a concentration of 2.5 x 10^5 cells per ml for human eosinophils, or 1 x 10^6 cells per ml for cell lines, and allowed to attach for 30 minutes at 37 °C in a humidified CO_2 incubator. Non-adherent cells were gently removed either by aspiration or flicking and wells were rinsed three times with assay buffer before quantification in the following procedures. Cells remaining in the wells were detected either by the activity of eosinophil peroxidase [7] (EPO assay) or by staining adherent cells with the protein-binding dye [23] bromophenyl blue (BPB assay).

EPO assay: Standard curves of known cell numbers were included with each assay measuring the activity of eosinophil peroxidase. Adherent cells were incubated in the presence of 1 mM o-phenylenediamine, 1 mM H_2O_2, 0.1% triton X-100 in Tris pH 8.0 for 30 minutes at RT as described previously [7]. Optical density of wells was measured at 490 nm using a Beckman Coulter AD 340C absorbance detector after stopping the reaction with 50 μl 4 M H_2SO_4.

BPB assay: Adherent cells in wells were fixed with 20%

trichloroacetic acid solution at 4 °C for 30 minutes and rinsed with 5 exchanges of distilled H_2O. Wells were incubated with 0.1% bromophenyl blue for 20 minutes at RT to stain cellular protein, rinsed 3 times with 1% acetic acid to remove unbound BPB and air dried. Remaining stain in the well was resuspended in Tris pH 9.6 buffer and optical density was measure at 570 nm.

REDUCTION OF CYTOCHROME C:

Analysis of the reduction of cytochrome C was performed as described previously [7]. In brief, eosinophils or eosinophil-like cell lines in HBSS containing 0.1% gelatin were incubated in the presence of 0.1 μM cytochrome C (Sigma) for 2 hr at 37 °C. Similar experiments were performed using HBSS containing calcium and magnesium, 0.1% gelatin, and 250 μM NADPH (Sigma). Optical density of each well was monitored every ten minutes for 2 hr in 2 to 4 replicate samples. Data are reported as a net change in optical density by subtracting absorbance values obtained at 0 minutes of incubation from absorbance values obtained from the same well at 120 minutes of incubation.

FIBRONECTIN ELISA:

Serum used in these assays was depleted of plasma fibronectin by two consecutive passages over a gelatin-agarose column equilibrated with Tris-buffered saline under sterile conditions. The column was stripped with 1M sodium bromide/0.05 M sodium acetate buffer and re-equilibrated before the second passage of serum over the column. Depletion of fibronectin from the serum was verified using Western blot analysis. Fibronectin-depleted serum was filtered through a 0.2 micron filter prior to addition to medium. Conditioned medium from eosinophil-like cells was prepared by culture in medium containing 10% fibronectin-depleted serum for 3 days. Medium was collected by centrifugation, filtered through 0.2 micron syringe filters and stored at 4 °C. AH1F and A549 were cultured as described above for two days prior to assay within 96 well polystyrene plates in medium containing 10% fibronectin-depleted serum. Eosinophil-like cell lines were resuspended in medium consisting of 45% adherent cell medium, 45% non-adherent cell medium and 10% fibronectin-depleted serum at 0.5 x 10^5 cells /ml. Wells containing adherent cells were incubated with either mixed medium without cells, eosinophil-like cell lines in mixed medium, or adherent cell medium mixed with conditioned medium (10% fibronectin-depleted serum).

For ELISA of fibronectin in the extracellular matrix of adherent cells, co-cultures were incubated for 24 hr, 48 hr or 72 hr prior to assay. At the beginning of the assay, cultures were viewed microscopically to ensure confluence. Wells were rinsed twice with warm HBSS containing calcium and magnesium. Anti-fibronectin antibodies HFN7.1 or P3NP/PFn were diluted to 10 μg/ml in adherent cell medium containing 5% fibronectin-depleted serum and incubated with rinsed monolayers for 1 hr at room temperature to bind to fibronectin present in the extracellular matrix of adherent cells. Wells were rinsed twice with HBSS containing calcium and magnesium before incubation with secondary alkaline-phosphatase-conjugated sheep anti-mouse antibody (Sigma Chemical Co.) diluted 1:2,000 in adherent cell medium containing 5% fibronectin-depleted serum for 1 hr at room temperature. Sigma alkaline phosphatase tablet was resuspended in Tris buffer at pH 9.0. Wells were rinsed three times with HBSS containing calcium and magnesium before incubation with substrate solution. Optical density was measured at 405 nm in each quadruplicate well. Absorbance values at 0 minutes of incubation were subtracted from absorbance values obtained at 30 minutes.

To detect fibronectin in the conditioned medium and associated with non-adherent cells, in addition to fibronectin found within the extracellular matrix of adherent cells, a fibronectin ELISA kit was purchased from Biomedical Technologies (Stoughton, MA). Co-cultures were prepared as described above except that incubation was for 48 hr only and was performed in a larger scale flask (25 cm^2 vent-capped flask instead of 96 well plates). To measure soluble fibronectin, unbound to cells, co-culture conditioned medium was harvested by centrifugation the end of 48 hours co-culture. Supernatant was stored at -20 °C until assay. To measure fibronectin bound to non-adherent cells or bound within the extracellular matrix of adherent cells, fibronectin was extracted from non-adherent cell pellets or adherent cell monolayers by treatment with urea. Non-adherent cells pelleted from harvested conditioned medium and flasks containing adherent cells from co-culture were treated with 1M urea in HBSS containing 2 mM PMSF for 1 hr at 37 °C. Samples were centrifuged at maximum speed in a microcentrifuge for 10 minutes and buffer-exchanged to HBSS by dialysis and stored at -20 °C until assay. The fibronectin ELISA was performed according to the manufacturer's specifications. All samples were run undiluted in duplicate and additional samples were diluted 1:10 and run in duplicate to ensure data points would fall

within the range of the standard curve. Results were compared to a standard curve of purified fibronectin and are reported as fibronectin concentration in ng/ml.

STATISTICAL ANALYSIS

Data were analyzed using MiniTab 15 (State College, PA). As more than two samples were compared within experiments, one way ANOVA was performed on raw optical density readings or net change of optical density values obtained after incubation. Post-test analysis was performed as Tukey's analysis using an 95% confidence interval for differences between means ($p < 0.05$).

RESULTS

I. COMPARISON OF HUMAN EOSINOPHILS WITH EOSINOPHIL-LIKE CELL LINES:

A. Adhesion to VCAM:

Eosinophils bind to recombinant soluble VCAM-1 through alpha 4-containing integrins [3]. Figure 1 shows human eosinophils are prevented from adhering to VCAM-coated wells by monoclonal antibodies to the beta 1 and 7 integrin subunits (Fig 1A). Antibodies against either beta subunit alone resulted in reduction in adhesion to recombinant soluble VCAM. When the monoclonal antibodies were combined to block both alpha 4-containing integrins, the level of adhesion was lower, however this reduction was not significantly different than the samples containing the monoclonal antibodies used separately. This experiment was repeated and showed an average of 57.9 ± 3.4% inhibition of adherence with monoclonal antibody P4C10 against beta1 integrin ($p < 0.05$ in all four experiments), 24.7 ± 15.4 % inhibition using anti-beta 7 mAb Fib 21 ($p < 0.05$ in two of four experiments), 49.8 ± 19.8% inhibition of adherence using monoclonal antibody Fib 30 against the beta 7 integrin ($p < 0.05$ in three of four experiments), and an average of 78.6 ± 10.4% inhibition of adherence when the monoclonal antibodies were combined (Fib 30 + P4C10, $p < 0.05$ in three of three experiments).

Figure 1

Figure 1. Effects of anti-integrin monoclonal antibodies on adherence to VCAM. A) Human eosinophils were pre-treated with buffer containing either no monoclonal antibody, anti-beta 1 monoclonal antibody P4C10, and beta 7 monoclonal antibody Fib 21 or a combination of both P4C10 and Fib 21 monoclonal antibodies before assessing adherence to VCAM-coated wells as described in Material and Methods using the EPO assay. Each bar represents the mean Â± standard deviation of the optical density of three wells of one representative experiment. AML 14.3D10 cells were pre-treated with buffer containing either no monoclonal antibody, anti-beta 1 monoclonal antibody TS2/16, anti-beta 7 monoclonal antibody Fib 21, or anti-alpha 4 monoclonal antibody PUJ1 as indicated before assessing adherence using the B) EPO assay or C) BPB assay as described in Materials and Methods. Bars represent the mean Â± standard deviation of the optical density of four wells of one representative experiment. (*) indicates significantly different than samples incubated without monoclonal antibody.

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Similar experiments using AML 14.3D10 cells blocking the beta 7 integrin subunit and the alpha 4 integrin subunit (to block both alpha 4-beta 1 and alpha 4-beta 7) showed that using either monoclonal antibody against the beta integrin subunit alone did not produce significant reduction in adhesion to VCAM –coated wells (Fig1B). In this experiment the monoclonal antibody TS2/16 was used against beta 1. It has been shown that this monoclonal antibody may hold the beta1 subunit in the active conformation, possibly accounting for this observation [24]. When compared to non-specific monoclonal antibody, treatment of AML 14.3D10 cells with TS2/16 resulted in a slight (4.4%) increase in adhesion to VCAM in two experiments, a slight (4.0%) decrease in adhesion in one experiment, and a significant increase in adhesion to VCAM in two experiments (65% and 32% increase in adhesion to VCAM). Blocking beta 7 with Fib 21 inhibited adhesion an average of 20.5% in three experiments. The monoclonal antibody PUJ1 against alpha 4 inhibited AML 14.3D10 adhesion an average of 84.9% when repeated in three experiments ($p < 0.05$ in all three experiments), similar to the effect seen with human eosinophils. The total amount of peroxidase activity detected in AML 14.3 D 10 cells was lower than the amount detected in eosinophils as noted in the scale of the y axis in Figure 1A,B. Considering that not all cell lines would have equivalent expression of eosinophil peroxidase, an alternate method of measuring adhesion of cells in 96-well assay plates was employed to examine the effects of blocking integrins on AML 14.3D10 cells. Total

protein was detected in adherent cells using the bromophenyl blue assay and is shown in Figure 1C. In this assay, it was again shown that anti-beta 7 mAb Fib 21 did not significantly alter AML 14.3D10 adhesion to VCAM. Anti-alpha 4 mAb PUJ1 blocked adhesion 94.3% (Figure 1C). AML 14.3D10 cells incubated with TS2/16 showed an increase in adhesion in this assay (data not shown).

Given that monoclonal antibodies to alpha 4 integrins blocked adhesion of both human eosinophils and the AML 14.3D10 cell line to VCAM, these monoclonal antibodies were used as substrates to compare integrin engagement on plates among other eosinophil-like cell lines. Table I shows adhesion of human eosinophils to anti-integrin antibodies using the eosinophil peroxidase assay reaction with o-phenylenediamine. Eosinophils became attached to wells coated with anti-beta 1 monoclonal antibody P4C10, anti-beta 7 monoclonal antibodies Fib 21, 22, 27 and 30 (data shown in Table I is attachment to Fib21, others not shown). AML 14.3D10 cells also attached to wells coated with anti-alpha 4 monoclonal antibody PUJ1, anti-beta 1 monoclonal antibody TS2/16, and anti-beta 7 monoclonal antibodies Fib 21 (Table I) and Fib 504 (not shown). Stimulation of AML 14.3D10 cells with phorbol ester for 20 hours prior to assay did not significantly alter the cell's ability to adhere to these monoclonal antibodies (Table I). Of the other cell lines tested, the only noticeable difference was seen in the ability of HL-60 cell lines to attach to monoclonal antibody coated wells (Table I). Unstimulated HL-60 cells and HL-60 clone 15 cells showed a lower level of attachment to anti-beat 7 monoclonal antibodies. After treatment with PMA, HL-60 cells increased in their ability to attach to anti-beta 7 antibodies. However, they attachment was also greatly increased to non-specific control antibodies, indicating that the attachment may not have been reliant on integrin engagement by the antibody-coated surface (Table I). The AML 14.3D10 cell line was chosen for inclusion in co-culture experiments.

Figure 2

Table I. Adhesion of eosinophil-like cell lines to antibodies coated onto a solid surface.

Wells coated with:	Eosinophils[1] (*n*=3)	AML 14.3D10[2] (*n*=4)	PMA-AML 14.3D10[2] (*n*=4)	EoL-3[2] (*n*=3)	BA-EoL-3[2] (*n*=1)	HL-60[2] (*n*=3)	PMA-HL-60[2] (*n*=3)	HL-60 clone 15[2] (*n*=4)
Control	0.1736 ± 0.114	0.1264 ±0.0310	0.1608 ±0.0190	0.1034 ±0.0142	0.075	0.0806 ±0.0166	0.4118 ±0.2943	0.1917 ±0.2056
Anti-alpha4	ND[3]	1.0272 ±0.5225	1.0998 ±0.3920	0.8986 ±0.2174	ND	ND	ND	0.9303 ±0.4695
Anti-beta1	1.1048± 0.110	1.0129 ±0.5174	1.0700 ±0.4600	0.8998 ±0.1395	0.2335	0.7263 ±0.2833	0.7757 ±0.7135	1.1411 ±0.55332
Anti-beta 2	Over[4]	1.0207 ±0.4491	1.0766 ±0.3810	0.9849 ±0.1488	1.2727	0.9397 ±0.2514	1.0577 ±0.5072	1.0150 ±0.5344
Anti-beta 7	0.5702 ± 0.227	0.4624 ±0.1585	0.4056 ±0.1083	0.7109 ±0.2493	0.2112	0.1587 ±0.0681	0.4748 ±0.4141	0.0900 ±0.0780

[1] indicates mean ± standard deviation obtained from EPO assay for peroxidase activity.
[2] indicates mean ± standard deviation obtained from BPB assay for total adherent protein.
(*n*) indicates number of experiments performed.
[3]ND indicates not determined.
[4]Over indicates that values were above the detection limit of the assay.

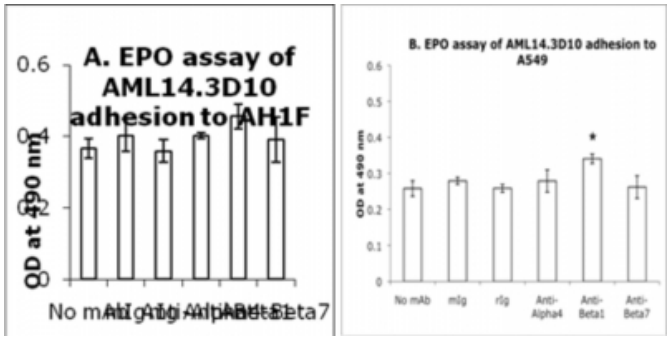
II. CO-CULTURE STUDIES:

A. Adhesion of AML14.3D10 cells to epithelial and fibroblast cells:

To investigate adhesion of the eosinophil-like cell lines to the adherent cells, the EPO assay was used to measure peroxidase activity in wells containing adherent cells. AML14.3D10 cells were allowed to adhere to confluent cultures of AH1F cells or A549 cells as described in Materials and Methods. Non-adherent cells were removed by rinsing and cells that remained in the wells were detected by the peroxidase reaction with o-phenylene diamine. In this assay AH1F cells alone showed minimal reaction with o-phenylene diamine substrate (an average of 0.003 OD units at 490 nm in wells containing confluent AH1F cells vs an average of 0.2 OD units in wells containing AML 14.3D10 cells in two experiments). In total, the amount of peroxidase detected using the OPD assay was an average of 0.2517 ± 0.0889 OD units, corresponding to an average of 26.4 ± 11.4% of the AML 14.3D10 cells added to wells in standard curve wells. A representative experiment shown in Fig 2 shows that monoclonal antibodies incubated with AML 14.3D10 cells prior to incubation with AH1F did not significantly differ in the amount of peroxidase activity detected in adherent cells (Fig 2A). This indicates that AML14.3D10 cells were able to associate with AH1F cells in an interaction that withstood rinsing even when integrins were blocked. Viability of either cell type was not altered by co-culture (data not shown).

Figure 3

Figure 2. Adhesion of AML 14.3D10 to AH1F fibroblasts or A549 epithelial cells. AML 14.3D10 cells were pre-treated with either buffer alone or buffer containing the indicated monoclonal antibody for 10 minutes at 37 Â°C before incubation with wells containing monolayers of either AH1F fibroblasts (A) or A549 epithelial cells (B). Adhesion was assessed using the EPO assay for peroxidase activity as described in Materials and Methods. Bars represent the mean Â± standard deviation of four replicate wells from one representative experiment. * indicates significant difference as compared to irrelevant antibody control.



A549 epithelial cells also showed minimal reaction in this assay, showing an average of 0.06175 ± 0.0007 OD units at 490 nm in three experiments. AML 14.3D10 cells incubated with adherent A549 cells showed an average of 0.208 ± 0.057 OD units, corresponding to 15.1 ± 6.3% of AML 14.3D10 cells in standard curve wells (Figure 2B). Incubation with anti-beta 7 integrin monoclonal antibodies did not alter the amount of peroxidase activity detected in AML 14.3D10 cells adherent to A549 cells. In one of the three replicate experiments AML 14.3D10 cells incubated with anti-alpha 4 monoclonal antibody PUJ1 demonstrated a slightly but significantly higher level of peroxidase activity than wells containing AML 14.3D10 cells incubated with irrelevant control mouse monoclonal antibody (an average of 0.2713 OD units in four replicate wells vs 0.2148 OD units in four replicate wells containing control mouse monoclonal antibody). TS2/16 monoclonal antibody to beta 1 increased the peroxidase activity of adherent cells a significant amount in each of the three experiments (an average OD of 0.2656 ± 0.0785 from cells incubated with TS2/16 vs an average of 0.2041 ± 0.0599 from cells incubated with control mouse monoclonal antibody). This could be due to either a small increase in the number of cells adherent to A549 when treated with TS2/16 or an increased peroxidase activity in the cells bound.

BA- EoL-3 cells were examined as a comparison to the AML 14.3D10 cells. BA-EoL-3 cells showed an average of 0.1565 ± 0.045 OD units, corresponding to 27.4 ± 10.2% of cells added adherent to AH1F cells (n = 3 experiments). Pre-incubation of BA-EoL-3 cells with TS2/16 monoclonal antibody to beta 1 integrin significantly increased the resulting peroxidase activity (an average of 0.2460 OD units from cells incubated with TS2/16 vs 0.1776 OD units in cells incubated with control monoclonal antibody). BA-EoL-3 cells incubated with monoclonal antibodies to alpha 4, beta 7 or beta 2 integrins did not show a significant difference in peroxidase levels produced by cells remaining in the wells with AH1F cells. BA-EoL-3 cells incubated with A549 cells showed an average of 0.1655 ±0.0538 OD units, corresponding to 26.4 ± 19% standard curve cells (n = 2 experiments). Incubation with monoclonal antibodies to block integrins did not significantly alter peroxide in either experimental repeat with A549 cells.

B. Reduction of Cytochrome C:

The reduction of Cytochrome C is one assay system used to study metabolic changes in eosinophils. The addition of superoxide dismutase in the reaction system has allowed investigators to study the amount of superoxide anion produced by stimulated eosinophils (the difference in samples with and without superoxide dismutase being an indicator of superoxide production). Human peripheral blood eosinophils incubated without stimulus resulted in a change of OD at 550 nm of 0.0061 ± 0.0077 after 2 hours incubation at 37 °C (n = 4 experiments). This level of change was significantly different than wells incubated without cells in two out of the four experiments (-0.0159 ▯ 0.0324 average of wells without cells in these experiments). When SOD is included with human eosinophils, the resulting change in OD is 0.0014 ± .0036, which was not significantly different than eosinophils incubated without SOD. In contrast, addition of the potent stimulator of the PKC pathway, PMA, changed the OD after 2 hrs by 0.4479 ± 0.0778 ($p < 0.05$ in all four experiments). When SOD was added with PMA, the resulting change in OD was only 0.1595 ± 0.0623 ($p < 0.05$ vs eosinophils incubated with PMA, n = 4 experiments). For unstimulated eosinophils, the difference between wells containing SOD and those without SOD was 0.0047 OD units, whereas the difference in PMA-stimulated wells was 0.2884 OD units, indicating a maximum superoxide dismutase-inhibitable reduction of Cytochrome C. Using the extinction coefficient of 21.1 mM^{-1} cm^{-1} for Cytochrome C, this translates to the equivalent of 35.64 nmoles of Cytochrome C being reduced per 0.5 x 10^6 eosinophils in

response to PMA, similar to previous published observations [25].

Reduction of Cytochrome C by AML 14.3D10 cells incubated with PMA was also enhanced. In one experiment the average of four wells showed a change in OD of -0.0125 without PMA, whereas the presence of 1 ng/ml PMA showed a change of OD of 0.1130 and 10 ng/ml showed 0.3578 (n = 4 wells each sample). Reduction of Cytochrome C was slightly enhanced by the addition of cytokine activators IL-5 or GM-CSF to AML 14.3D10 cells, however the change in OD was not significantly enhanced over AML 14.3D10 incubated without cytokine activators. In an average of three separate experiments, the resulting change in OD after 2 hr incubation was 0.0126 ± 0.016 for unstimulated AML 14.3D10, 0.0233 ± 0.0215 for GM-CSF-treated AML 14.3D10 and 0.01794 ± 0.0142 for IL-5-treated AML 14.3D10 cells (each not significantly different vs control).

Engagement of integrins with monoclonal antibodies does not significantly increase the amount of Cytochrome C reduced by human eosinophils when compared with human eosinophils incubated without monoclonal antibodies. When human eosinophils were incubated in the presence of monoclonal antibodies to engage integrins on their cell surfaces, the resulting change in OD was 0.0789 SOD-inhibitable units when beta 1 integrin was engaged and 0.1206 SOD-inhibitable units when beta 7 integrin was engaged, which was not significantly different that eosinophils incubated without monoclonal antibodies (a change of 0.1142 SOD-inhibitable units, n = 4 experiments). Similar findings were observed when the monoclonal antibodies were coated onto the bottom of 96-well plates instead of incubating with the cells in solution (SOD-inhibitable change of 0.0928 for coated anti-beta 1 and 0.0545 for coated anti-beta 7, n = 4 experiments). Similar results were obtained using AML 14.3D10 cells. The level of change in OD after 2 hr was very low and in some cases was negative, meaning the OD had decreased during the assay. The antibody against beta 1 (TS2/16) did show a significant increase in the amount of Cytochrome C reduced in one of four experimental trails (exhibiting a change in OD of 0.0009 □ 0.0641 with anti-beta 1 compared to -0.0211 □ 0.0606 units when incubated without mAb). No difference was observed when AML 14.3D10 were incubated with non-specific mouse immunoglobulin (mIg), non-specific rat immunoglobulin (rIg), anti-beta 2 or anti-alpha 4 monoclonal antibodies (data not shown). Whereas there does seem to be a small spontaneous generation of superoxide anion by human eosinophils, there does not seem to be a corresponding spontaneous generation of superoxide in AML 14.3D10 cells. Engagement of integrins by monoclonal antibodies did not increase superoxide production in either cell type. Human eosinophils incubated with VCAM protein to engage alpha 4 integrins showed an increase in the amount of Cytochrome C reduced by 0.0564 OD units in one experiment, and AML 14.3D10 cells showed a slight increase of 0.0337 OD units in one experiment, however with both of these cells the amount changed was not significantly different than cells incubated without additional ligand. This observation correlates with prior observations that eosinophils incubated in VCAM-coated wells produce more superoxide ion when formyl-methionine-leucine-phenylalanine (f-MLP) and cytochalasin B are present [7], which they were not in our current assay observing baseline levels in these cells.

The reduction of Cytochrome C can be accomplished by other metabolic reactions producing anions in the cell. In an attempt to measure general metabolic activity of the eosinophil cell lines in the presence of adherent cells, the Cytochrome C reduction assay was modified to include NADPH as a substrate. The change in OD of Cytochrome C by AML 14.3D10 cells after 2 hr was increased from a 0.0015 OD units in the absence of NADPH to 0.075 OD units in the presence of NADPH in one experiment. When AML 14.3D10 or BA-EoL-3 cells were incubated in this assay system for 2 hr in the presence of SOD, the change in absorbance was not different than incubation without SOD, indicating that the reduction of Cytochrome C was not due to production of the superoxide anion (AML 14.3D10: 0.0782 OD units without SOD vs 0.0789 OD units with SOD, average of two experiments; BA-EoL3: 0.0229 OD units without SOD vs 0.0309 OD Units with SOD, average of three experiments).

Co-culture of AML 14.3D10 cells with AH1F fibroblasts or A549 epithelial cells in the presence of NADPH did not alter the amounts of change in OD after 2 hrs of incubation as AML 14.3D10 incubated without adherent cells. AML 14.3D10 cells incubated alone yielded a net change in absorbance of 0.1087 □ 0.1632. When AML 14.3 D 10 cells were incubated with AH1F cells, the net change was 0.0486 □ 0.0296 (a significant change in only one of four experiments). When AML 14.3D10 cells were incubated

with A549 cells, the net change was 0.0874 □ 0.0220 (not significant in four out of four replicate experiments). Similar results were obtained when BA-EoL-3 cells were used in the co-cultures instead of AML 14.3D10 (data not shown). When cells were co-cultured, the amount of change in OD after 2 hr was not significantly lowered by the presence of SOD or monoclonal antibodies against integrins (data not shown). Therefore no enhancement in metabolic activity was detected in co-cultures using this assay system.

C. Production of Fibronectin by adherent cells in contact with eosinophil-like cell lines:

To see if the amount of fibronectin within the extracellular matrix was altered by incubation with eosinophil-like cell lines, an ELISA to measure the presence of fibronectin on living adherent cells was performed. Adherent cells were cultured in medium containing fibronectin-depleted serum for 2 days prior to addition of AML 14.3D10 or BA-EoL-3. Cultures were rinsed after the indicated time of incubation and monoclonal antibody HFN7.1 or P3NP/PFn was used to detect the amount of fibronectin associated with the extracellular matrix of adherent cells. AH1F fibroblasts secreted fibronectin that was incorporated into the extracellular matrix (Fig. 3A). Fibronectin was also detected in the extracellular matrix formed by A549 epithelial cells, however at a lower level than that found in fibroblast extracellular matrix (a maximum change in absorbance of 0.208 ± 0.004 from A549 cells cultured for 3 days compared with 0.688 ± 0.088 from AH1F cells incubated for the same period of time). No significant increase was observed in the amount of fibronectin associated with monolayers of AH1F cells or A549 cells when cultured in the presence of either eosinophil cell lines as detected by monoclonal antibody HFN7.1 (Figure 3A,B). Lower levels of detection were obtained when monoclonal antibody P3NP/PFn was used as the primary detection antibody (for example; AH1F cells incubated with HFN7.1 exhibited a change in absorbance of 0.688 ± 0.088 compared with a change in absorbance of 0.365 ± 0.028 with P3NP/PFn), however the same trends of levels were observed. Conditioned medium from AML 14.3D10 or BA-EoL-3 also did not alter the amount of fibronectin associated with the extracellular matrix of adherent cells (data not shown). This ELISA measures only fibronectin associated with cells adherent in the culture dish, as culture medium and non-adherent cells were rinsed away prior to detection.

Figure 3: ELISA results measuring fibronectin in co-cultures. The amount of fibronectin associated with the extracellular matrix of monolayers of either AH1F (A) or A549 (B) cells after co-culture with the indicated eosinophil-like cell line for the times indicated was measured by ELISA as described in Materials and Methods using monoclonal antibody HFN7.1. Bars represent the mean ± standard deviation of four replicate wells from one plate incubated as co-culture for the indicated number of days prior to assay. Samples from 48 hour co-culture were assessed for fibronectin quantities using the Biomedical Technologies ELISA as described in Materials and Methods (C, D). Dark bars represent the quantity of fibronectin measured from urea-extracted monolayers as indicated. Shaded bars represent the quantity of fibronectin measured in samples of urea-extracted cells pelleted from conditioned medium of co-cultures. Clear bars represent the quantity of fibronectin measured in conditioned medium from co-cultures as indicated. Each bar represents the average of two replicate wells for each sample.

To measure if a change in soluble or non-adherent cell-associated fibronectin occurred in co-cultures, an alternate ELISA assay protocol was used. Co-cultures were performed as described above except that samples were all taken at 48 hours of incubation. Fibronectin was extracted from the cell surfaces of adherent cells (dark bars in Figure 3C,D) and from the surfaces of non-adherent cells rinsed from the wells (shaded bars in Figure 3C,D). Clear bars represent medium collected after co-incubation after non-adherent cells were removed by centrifugation. The amount of fibronectin detected was quantitated by comparison with the results obtained using a standard curve of known fibronectin concentrations. The amount of fibronectin associated with monolayers of AH1F cells was greater than the amount associated with A549 cell monolayers, confirming the whole cell ELISA observations (Figure 3CD). The amount of fibronectin associated with monolayers incubated with conditioned medium from AML 14.3D10 or BA-EoL-3 cells was not different than the amount seen with monolayers cultured with cells. The conditioned medium from AH1F cells incubated alone contained 1,022 ± 85 ng/ml fibronectin, as compared with 1,034 ± 90 ng/ml of fibronectin within the AH1F conditioned medium containing AML14.3D10 conditioned medium (n = two wells each, no significant difference). There was 1,245 ± 24 ng/ml of fibronectin detected in the conditioned medium of A549 cells. When AML14.3D10 conditioned medium was

included in the culture, 1,080 ± 31 ng/ml fibronectin was detected (n = two wells each, no significant difference). It is of note that whereas the amount of fibronectin detected in A549 matrices was much smaller than the amount found in AH1F matrices, the amounts detected from the conditioned medium from either fibroblast or epithelial cells was of a similar level (over 1,000 ng/ml).

Pelleted cells from conditioned medium of co-cultures of AML 14.3D10 or BA-EoL-3 cells incubated with A549 epithelial (non-adherent AML 14.3D10 or BA-EoL-3 cells in the co-culture, shaded), showed a significantly greater amount of fibronectin than was observed in co-cultures of AML 14.3D10 or BA-EoL-3 cells incubated with AH1F fibroblasts (Fig 3CD). In co-cultures of A549 + AML 14.3D10, the amount of fibronectin extracted from the surface of non-adherent cells (presumably AML 14.3D10 cells that did not adhere to AH1F cells) was 351 ± 25 ng/ml. In co-cultures of AH1F + AML 14.3D10, the amount of fibronectin extracted from the surface of non-adherent cells was 145 ± 75 (n = 2 wells each, $p < 0.05$). The amount of fibronectin quantitated in medium from any of the co-cultures was not significantly different than monolayers incubated without co-culture. Eosinophil-like cells may have contributed a small amount of fibronectin detected within the conditioned medium, however it was not an amount sufficient to be considered a significant change in these levels. Fibronectin was not extracted from eosinophil-like cells cultured in the absence of A549 or AH1F cells, so we cannot state with certainty the origin of fibronectin that was extracted from the surface of these non-adherent cells.

DISCUSSION

Asthma is a disease characterized by both airway remodeling and chronic inflammation. The relationship between these phenomena and how they relate to pathogenesis is just beginning to be elucidated [reviewed in 6,26]. Airway remodeling includes the deposition of fibronectin within the extracellular matrix of airway tissues [6] and an increase in the soluble form of cellular fibronectin within the airway space [2]. The source of this increased soluble fibronectin is not yet known, however several cell types present within airway tissue or airway space are potential contributors. Eosinophils are known to produce several cytokines that could potentially alter airway matrix production [6]. TGF-β has recently been investigated for a potential role in remodeling, as eosinophils have been shown to be a potent source of this cytokine [27]. Herein we show by two different ELISA methods that the amount of matrix fibronectin is not changed by either fibroblasts or epithelial cells incubated with eosinophil-like cells. This observation agrees with the findings of Fattouh and colleagues [28,29] of airway remodeling (collagen deposition) occurring independently of TGFβ or eosinophils. We are the first to examine the role of eosinophils in soluble fibronectin production. Herein we have shown using an in vitro model system that both fibroblasts and epithelial cells are capable of producing soluble fibronectin.

The fibronectin that was produced within co-cultures was not limited to incorporation into extracellular matrix (Figure 3C,D). By examining medium from co-cultures and isolating non-adherent cells from co-cultures, we have shown that fibronectin is secreted into the medium by both fibroblasts and epithelial cells and that fibronectin becomes associated with non-adherent cells. We assume fibronectin in the co-cultures was produced by the adherent cell population and not by the eosinophil-like cells. The fact that more fibronectin was isolated from eosinophil-like cells incubated with A549 epithelial cells could be due to more fibronectin being produced by A549 in co-cultures, which in turn binds more sites on the non-adherent cells. One must also consider that fewer eosinophil-like cells were able to become adherent to A549 cells (Fig 2), therefore more cells may have been isolated from the conditioned medium of these co-cultures. Either way, it is clear that this soluble form of cellularly-derived fibronectin was able to become associated with the eosinophil-like cells. This supports our previous observations that the soluble form of cellular fibronectin enhances eosinophil survival in vitro [1].

Eosinophil-like cell lines have similar levels of interaction with VCAM as unstimulated eosinophils. Adhesion of eosinophil-like cells was strong enough to withstand the gentle washing procedures of the adherence assays. Sufficient monoclonal antibodies were presumed to be used as doubling the concentration of monoclonal antibodies used in the assays did not alter the results (data not shown). These antibodies were able to block adhesion of cells to VCAM coated onto culture dishes (Figure 1). Antibody P4C10 consistently blocked eosinophil adhesion to VCAM, whereas TS2/16 yielded inconsistent results with AML 14.3D10 cells (Figure 1). However, blocking interaction through both beta 1 and beta 7 inhibited eosinophil and AML 14.3D10 adhesion to VCAM by a similar extent (78.6% and 84.9%, respectively), indicating similar usage in adhesion.

Eosinophils isolated from Dunkin-Hartley guinea pigs have been shown to bind fetal lung fibroblasts in an interaction that is enhanced by stimulation of the eosinophils with PMA or of the fibroblasts by IL-1 or TNF [14]. AML 14.3D10 cells bound to AH1F fibroblasts (Figure 2A). Blocking integrins with monoclonal antibodies did not inhibit the interaction of eosinophil-like cell line AML 14.3D10 with AH1F fibroblasts. The interaction of unstimulated guinea pig eosinophils to fibroblasts was not inhibited by RGD-containing peptides [14], in agreement with our findings that beta 1 integrin engagement is not required for this adhesion. Monoclonal antibodies blocking beta 2 integrins also had no inhibitory effect on adhesion when used either alone or in combination with antibodies blocking alpha 4-containing integrins (data not shown). Enhanced adhesion after cell stimulation was presumably integrin-mediated [14] via adhesion to ICAM.

We have previously shown that human eosinophils adhere to bronchial epithelial cell line A549 and primary cultures [13]. Adhesion was enhanced by prior stimulation of the eosinophils with PMA or epithelial cells with TNF [13]. Here we have shown eosinophil-like cell line AML 14.3D10 adheres to A549 cells (Figure 2B). Whereas AML 14.3D10 cells showed a lower level of adhesion to A549 cells than AH1F cells, BA-EoL-3 cells exhibited a similar level of adhesion to either adherent cell type (data not shown). The levels of adhesion of the eosinophil-like cell lines was somewhat higher than our previous observations with unstimulated human eosinophils with epithelial cells (15% of AML 14.3D10 cells or 20% of BA-EoL-3 cells compared with 2.4% of eosinophils becoming adherent to A549 cells without PMA stimulation). However, these levels were similar to those seen after stimulation (15 to 20% of PMA-stimulated eosinophils becoming adherent). Blocking beta 2-containing integrins had a significant, albeit partial, inhibitory effect on adhesion of PMA-stimulated eosinophils to A549 cells [13]. Blocking alpha-4-containing integrins did not show any inhibitory effect on adhesion of either AML 14.3D10 cells nor BA-EoL-3 cells to A549 cells (Figure 2B and data not shown). Indeed, engagement of beta 1 integrins by TS2/16 may have increased adhesion (Figure 2). Blocking beta-2 integrins also had no effect on these interactions (data not shown).

Examining superoxide production has been used to measure the effects of activators on eosinophil function [7-10]. Adhesion to VCAM has been shown to increase superoxide production when eosinophils are activated in the presence of f-MLP and cytochalasin B [7]. In this study we wished to look at the effect of adhesion or integrin engagement in the absence of other artificial stimuli. Integrins did not seem to be involved in metabolic changes in these cells, either as superoxide production or more generic metabolic activity. We chose to represent that data without manipulation in this study because reactions were not completely inhibited by superoxide dismutase. Laudanna et al showed a small increase in superoxide production (up to 3 nmoles O_2^-/2 x 10^5cells) from eosinophils incubated in wells coated with anti-alpha 4 or anti-beta 2 integrin monoclonal antibodies [30], which translates to within the range of change of OD reported herein. However, no statistical analysis was described by Laudanna, and thus it is hard to compare results in terms of significance. We have reported herein that the level of change seen our experimental conditions was not significantly different than controls. It may be that we have discounted possible effects using our method of analysis as the degree of the effects were similar to previously published reports.

Conditioned medium obtained from normal human bronchial epithelial cells has been shown to increase superoxide generation in human eosinophils [11]. In our study we were unable to detect an alteration in metabolic activity in eosinophil-like cells incubated directly with either A549 epithelial cells or with AH1F fibroblast cells. Differences in assay conditions could account for this discrepancy. Our experiments were of a shorter duration (2 hr co-culture assay instead of three hour assay of cells exposed to conditioned medium that was collected after 24 hrs of incubation). Given that our experiments show a lower basal level of superoxide production by cell lines it may be that stronger or longer periods of stimulation are required to see effects on superoxide production with activators other than PMA.

In our hands, AML 14.3D10 cells demonstrated similar usage of integrins in static adhesion assays. Metabolic activity of AML 14.3D10 cells was not enhanced when co-cultured in the presence of either A549 epithelial cells or AH1F fibroblasts. The amount of fibronectin detected within matrices produced by either A549 epithelial cells or AH1F fibroblasts was also not enhanced by co-culture with AML 14.3D10 cells. Examination of fibronectin found in conditioned medium or in association with non-adherent cells indicates that either fibroblasts or epithelial cells may be responsible for the soluble cellular fibronectin found

within the lungs of asthmatics after bronchial provocation. Further examination of the interaction of eosinophils with epithelial cells may contribute to our understanding of the various factors that lead to the chronic stage of asthma, including tissue damage and fibrosis. Whereas targeting integrins with pharmacologic therapies may disrupt survival of eosinophils within the lung, it may not be enough to prevent tissue damage.

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