Eosinophils are present throughout the airway wall of asthmatics. The nature of the interaction between human airway smooth muscle cells (ASMC) and eosinophils was investigated in this study. We demonstrated, using light microscopy, that freshly isolated eosinophils from healthy donors rapidly attach to ASMC in vitro. Numbers of attached eosinophils were highest at 2 h, falling to 50% of maximum by 20 h. Eosinophil attachment at 2 h was reduced to 72% of control by anti-VCAM-1, and to 74% at 20 h by anti-ICAM-1. Pre-treatment of ASMC for 24 h with TNF-α, 10 nM, significantly increased eosinophil adhesion to 149 and 157% of control after 2 and 20 h. These results provide evidence that eosinophil interactions with ASMC involve VCAM-1 and ICAM-1 and are modulated by TNF-α.

Key words: Asthma, Eosinophils, Airway smooth muscle, ICAM-1, VCAM-1, TNF-α, IL-1β, IL-5

Introduction

Asthmatic airways are hyperresponsive, contain an increased amount of airway smooth muscle and are infiltrated by a variety of inflammatory cells, including eosinophils and T lymphocytes. It is now widely accepted that both the increased smooth muscle bulk and inflammatory cell products contribute to airway hyperresponsiveness. There is growing evidence that inflammatory cell products not only alter the contractility of airway smooth muscle cells (ASMC) but also their adhesion molecule expression, proliferation and mediator secretion. 1–4

Many groups have demonstrated that eosinophil numbers and levels of their secreted granule proteins, both in airway fluid and in peripheral blood, correlate with asthma severity. Eosinophil densities in the lumen reflect those in the inner airway wall in fatal asthma. 5 Cytokines such as interleukin (IL)-5, produced mainly by T lymphocytes, promote eosinophil recruitment, activation and survival in airway tissues. 6 Once activated, eosinophils are a source of a wide range of mediators, many of which are implicated in altering airway smooth muscle function. They release the potent smooth muscle constrictors leukotrienes C₄, D₄, and E₄. The first direct link between eosinophils and the induction of airway hyperresponsiveness was established when we demonstrated that supernatants from activated eosinophils increase the contractile responses of human bronchial rings to histamine in vitro. 7 One eosinophil product known to induce such in vitro hyperresponsiveness is PAF. 8 However, eosinophils also release a variety of growth factors and cytokines known to modulate other functions of ASMC. These include the cytokines tumour necrosis factor-α (TNF-α) and IL-1β, which are present in increased amounts in the airway fluid of asthmatics.

TNF-α and IL-1β have a variety of effects on ASMC. They modulate ASMC proliferation 9,10 and increase ASMC adhesion molecule expression. 1 More recently TNF-α and IL-1β have also been shown to induce ASMC to release cytokines and chemokines, including eotaxin and granulocyte-macrophage colony stimulating factor (GM-CSF), implicated in the recruitment and survival of eosinophils. 11,12

Since eosinophils release products that induce a variety of changes in airway smooth muscle and they are found in this smooth muscle layer, the nature of the interaction between these two cell types needs to be investigated. In this study we investigated the adhesion of human eosinophils to human ASMC in culture. The adhesion molecules involved and the effects of the cytokines TNF-α, IL-1β and IL-5 on this adhesion were studied.

Materials and methods

Materials

All cell culture requirements were obtained from Trace Biosciences (Sydney, Australia) except for
DMEM, penicillin, streptomycin, amphotericin B which were supplied by Gibco BRL (Life Technologies, Melbourne, Australia). Antibodies to ICAM-1 (clone LB-2, Cam folio and HA58, PharMingen International), VCAM-1 (El16, Cam folio and 51–10C9, PharMingen International) and irrelevant antibodies of the same isotypes were supplied by Becton Dickinson (Sydney, Australia) in PBS with $\leq 0.09\%$ NaN$_3$ and were diluted just prior to use in RPMI-1640. Recombinant human TNF-α and IL-β were supplied by R&D Systems Inc (Minneapolis, MN, USA), reconstituted in PBS with 0.1% w/v bovine serum albumin (BSA; Sigma-Aldrich, Australia) at 10 $\mu$g/ml and aliquots stored at –20°C. Recombinant human IL-5 was obtained from PharMingen International (San Diego, CA, USA), aliquots were stored at –70°C at 50 ng/μl in PBS with 0.1% BSA. All three cytokines were diluted immediately before use in RPMI-1640.

Human airway smooth muscle cell isolation and culture

Human lung was obtained from patients undergoing either surgical resection or lung transplantation. Human airway smooth muscle cells were prepared as described previously by Hawker et al.$^3$ Briefly, smooth muscle bundles (explants) were dissected out of bronchial segments, washed thoroughly and transferred to culture flasks in DMEM supplemented with 10% FBS, L-glutamine (4 mM) and antibiotics (penicillin 20 U/l, streptomycin 20 $\mu$g/ml and amphotericin B 2.5 $\mu$g/ml) and maintained at 37°C in a humidified 5% CO$_2$ in air atmosphere. Medium was changed every 5 days until the airway smooth muscle cells (ASMC) grew to confluence, when the medium on confluent ASMC was changed to RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS, Hepes 2 mM, L-glutamine 2 mM and gentamycin 20 $\mu$g/ml (RPMI) for at least 24h prior to harvest. The ASMC were then harvested using trypsin–EDTA, washed thoroughly and plated into 4-well slide-wells at 1.5–2.5 $\times$ 10$^5$ cells/well in 0.5ml RPMI and cultured for 2 days prior to the addition of eosinophils.

Eosinophil isolation

Eosinophils were isolated from 60–120 ml heparinized peripheral blood collected from healthy volunteers using modifications of the methods of Hansel and colleagues.$^{13}$ Briefly, the blood was mixed 5:1 with 6% (w/v) high molecular weight dextran in normal saline and the red cells allowed to sediment for 40 min at room temperature. The leucocyte-enriched fraction was collected and underlayered with percoll $\delta$ 1.088 g/ml and spun at 700xg for 20 min at room temperature. Leucocytes at the bottom of the gradient immediately above and in the red cell pellet, were collected and the red cells lysed with 5x the collected cell volume of lysis buffer (0.82% w/v NH$_4$Cl, 0.1% w/v K$_2$CO$_3$, 0.1 mM EDTA) for 15 min at 4°C. The remaining leucocytes were washed thoroughly, incubated with magnetic microbeads coated with antibody to CD16 for 30 min at 6°C and cells expressing CD16 (neutrophils) removed using a MACS cell separator. With this method eosinophils were isolated to $\geq 95\%$ purity and with $\geq 98\%$ viability as assessed by Kimura Light staining and trypan blue exclusion, respectively. The eosinophils were then immediately washed 2x with sterile RPMI and added to the ASMC in slide-wells at 1.5–2.5 $\times$ 10$^5$ eosinophils/well and co-cultured at 37°C in a humidified 5% CO$_2$ in air atmosphere for up to 20 h as detailed below.

Slide-well processing and assessment of eosinophil adhesion

At the appropriate harvest time the slide-wells were gently rocked and all the culture medium removed from each well. The cell layer in the wells was then gently washed 3x with RPMI, drained, fixed in 100% methanol and air-dried. The cells still adhering to the bottom of the slide-well were stained with Kimura Light and the number of eosinophils attached to the ASMC layer counted in 20 fields of view (10 fields across and 10 fields down) in each well using light microscopy and 200× magnification.

Time course

In order to determine the temporal pattern of eosinophil adhesion to ASMC, eosinophils were added to ASMC in slide-wells and duplicate slide-wells processed immediately or after 0.5, 1, 2, 4, 8, 16 and 20 h of co-culture.

Adhesion molecule identification

The role of the adhesion molecules ICAM-1 and VCAM-1 on eosinophil adhesion to ASMC was determined by incubating ASMC in duplicate slide-wells with antibodies (1.0, 3.0 or 10.0 $\mu$g/ml) to either ICAM-1, VCAM-1 or irrelevant antibodies of the same isotype, for 30 min at 37°C prior to the addition of the eosinophils. The slide-wells were then processed after 2 or 20 h of co-culture.

Effect of cytokines

**IL-1β and TNF-α.**

The effects of the cytokines IL-1β (0.1, 1.0 and 10 nM) and TNF-α (0.1, 1.0 and 10 nM), alone and in
combination, on eosinophil adhesion to ASMC were examined by adding the cytokines, or their vehicle, to duplicate slide-wells containing the ASMC, 24 h before or at the same time as the eosinophils. The eosinophils were co-cultured with the ASMC, in the presence of the cytokines, for 2 or 20 h and then the slide-wells were processed. As a result of these treatment protocols the ASMC were treated with cytokine for a total of 2 or 26 h when the eosinophils were present for only 2 h and for 20 or 44 h when the eosinophils were present for 20 h.

**IL-5**.
The effect of IL-5 on eosinophil adhesion to ASMC was examined by adding the IL-5 (10 and 100 ng/ml), or its vehicle, to duplicate slide-wells containing ASMC when the eosinophils were added. The cells were co-cultured in the presence of the IL-5 for 2 or 20 h and then the slide-wells were processed as described earlier.

The combined effect of IL-5 and TNF-α on eosinophil adhesion to ASMC was also examined. ASMC in duplicate slide-wells were pre-treated with TNF-α (10 nM) for 24 h prior to the addition of the eosinophils and IL-5 (10 and 100 ng/ml). After 2 h of coculture in the presence of both the TNF-α and IL-5, the slide wells were processed (ASMC TNF-α exposure=26 h).

**Data analysis**
The counts (2 counts of 10 fields each per well) of eosinophils attached to ASMC per 10 fields of view for the duplicate wells receiving each treatment were averaged. The number of eosinophils attached to ASMC were standardised in each experiment. For the time course experiments the data for each experiment were expressed as a percentage of the maximum number of eosinophils attached to ASMC per 10 fields of view. In the remaining experiments the data for each experiment were expressed as a percentage of the untreated control.

For each series of experiments ASMC established from bronchial tissue obtained from 3–9 different patients were used together with eosinophils isolated from 3–9 different healthy volunteer donors. The percentage of eosinophils attached was averaged and the standard error calculated for each time point/treatment. Analyses were performed on this mean data and significance (p≤0.05) determined with ANOVA and Fisher’s (PLSD) test.

**Ethical approval**
Lung samples were used with the informed consent of each patient and with the approval of the relevant Area Health Service or Hospital Ethics Committee. Blood was collected from healthy volunteer donors with their informed consent. Ethical approval for these experiments was granted by the University of Sydney Human Ethics Committee.

**Results**

**Time course**
Eosinophils adhered rapidly to ASMC, with maximum numbers being observed in most experiments by 2 h of coculture. The numbers of attached eosinophils then declined to 49.4±8.9% of maximum by 20 h (Fig. 1).

**Adhesion molecule identification**
Antibodies to the adhesion molecules ICAM-1 and VCAM-1 reduced eosinophil attachment to ASMC. Pre-treatment of ASMC with anti-ICAM-1 (3 and 10 μg/ml) for 30 min prior to and during a 20 h co-culture period with eosinophils significantly (p<0.05, n=4) reduced eosinophil attachment to the ASMC to 77.0±3.3% and 74.3±5.0% of the untreated control respectively (Fig. 2a), whereas anti-VCAM-1 had no effect (Fig. 2b). With a shorter 2 h co-culture period, there was more variability in eosinophil attachment, particularly in the presence of anti-ICAM-1 and the isotype control antibody (Fig. 2c). Anti-ICAM-1 (3 μg/ml) had no significant effect but anti-VCAM-1 (3 μg/ml) did significantly (p<0.05, n=4) reduce eosinophil attachment to ASMC (71.7±4.2% of the untreated control) compared with the isotype control (118.6±16.6% of the untreated control) (Fig. 2c).

**Effects of cytokines**

*IL-1β and TNF-α.*
IL-1β had a small effect on eosinophil attachment to ASMC, 10 nM IL-1β reduced eosinophil attachment significantly to 81.1±8.2% of the untreated control compared with 92.8±0.8% in the vehicle control.
when present for the 20 h co-culture period (20 h treatment) (Fig. 3a). All three concentrations of IL-1β were without effect, compared to the vehicle control, when added to the ASMC 24 h prior to the addition of the eosinophils and left there for the 20 h co-culture period (44 h treatment) (Fig. 3a).

In contrast to these observations, TNF-α increased eosinophil attachment to ASMC with either protocol (20 or 44 h treatment). TNF-α, 0.1 nM and 10 nM, added for the 20 h co-culture period, significantly (p < 0.05, n = 3) increased eosinophil attachment to 128.1 ± 14.2% and 139.8 ± 4.0% of the untreated control compared with 92.4 ± 6.2% in the vehicle control but these increases were not significantly different (Fig. 3b). TNF-α, 1.0 and 10 nM, when added to the ASMC 24 h prior to the 20 h co-culture period (44 h treatment), also significantly (p < 0.05, n = 3) increased eosinophil attachment in a concentration-dependent manner (0.1 vs 1.0 nM and 1.0 vs 10 nM p < 0.05, n = 3) to 119.6 ± 9.1% and 156.5 ± 5.2% of control respectively (Fig. 3b).

TNF-α increased eosinophil attachment to ASMC when the co-culture period was shortened to 2 h. TNF-α (10 nM) added 24 h before the 2 h co-culture period was started (26 h treatment), significantly (p < 0.05, n = 4) increased eosinophil attachment to 148.9 ± 13.2% of the untreated control, whereas IL-1β was without effect (112.3 ± 12.2%, n = 4). Neither 10 nM TNF-α, nor 10 nM IL-1β, had an effect when added for the co-culture period only (117 ± 10.9% and 91 ± 9.7% of untreated control respectively, n = 4). IL-1β (10 nM) did not modulate the increase in eosinophil attachment to ASMC induced by the 26 h treatment with 10 nM TNF-α. It had no effect, whether added with the TNF-α (26 h treatment) or only for the 2 h co-culture period (data not shown).

IL-5 did not modulate eosinophil attachment to ASMC under the various conditions tested. IL-5 (10 and 100 ng/ml) added at the same time as the eosinophils, had no significant effect on eosinophil attachment to ASMC following 20 h of co-culture (101.0 ± 17.6% and 91.9 ± 10.6% respectively, n = 3) (Fig. 4a). It was also without effect when the co-culture period was
shortened to 2 h (Fig. 4b). There was also no added effect of IL-5 under conditions where TNF-α increased eosinophil attachment (Fig. 4b).

**Discussion**

In this study we demonstrate for the first time that human eosinophils can rapidly attach to human airway smooth muscle cells. Maximum numbers of attached eosinophils were observed after around 2 h, with numbers falling to half that level by 20 h of coculture. Eosinophil attachment to ASMC at 2 h was significantly reduced in the presence of an antibody to the adhesion molecule VCAM-1, while at 20 h it was significantly reduced in the presence of an antibody to ICAM-1. The pro-inflammatory cytokine TNF-α significantly increased the number of attached eosinophils, whereas IL-1β and IL-5 were without effect.

Eosinophils have been shown to attach very rapidly to endothelial cells, reaching maximum numbers after 5 min of coculture. They also adhere quickly to epithelial cells with coculture periods of 30–60 min commonly being used. We hypothesized that eosinophils would also adhere rapidly to ASMC and found that maximum numbers were reached in the first 2 h and that substantial numbers were still attached after 20 h of coculture. It would appear from our observations, compared with those of Munoz and colleagues, that eosinophils attach more slowly to ASMC than they do to endothelial cells, as attached eosinophil numbers were only around 50% of maximum after 30 min.

We investigated whether the adhesion molecules ICAM-1 and VCAM-1 were involved in eosinophil adhesion to ASMC because they are both constitutively expressed on ASMC and have been shown to participate in the attachment of activated lymphocytes to ASMC. ICAM-1 and VCAM-1 both play important roles in eosinophil adhesion to and transmigration through vascular endothelium. Interestingly, although ICAM-1 is expressed on epithelial cells, only eosinophils activated with C5a, PMA or eotaxin (but not RANTES, MIP-1α, FMLP, LTB4, or PAF – activated cells) adhere in an ICAM-1 dependent manner. Our *in vitro* data are consistent with the hypothesis that VCAM-1 is important in the initial adhesion to ASMC, as antibodies to it significantly reduced the number of attached eosinophils at 2 h, but not at 20 h. They are also consistent with the hypothesis that ICAM-1 is involved in prolonged eosinophil adhesion to ASMC, as an antibody to ICAM-1 significantly reduced attached eosinophil numbers after 20 h of coculture. However, neither of the antibodies inhibited eosinophil attachment by more than 28% so other molecules must also be involved in both the initial phase and during prolonged adhesion.

Elevated levels of cytokines TNF-α and IL-1β have been found in the airways of asthmatic subjects. Macrophages, other immune cells and epithelial cells all produce TNF-α. However, mast cells not only synthesize TNF-α, but also store it in their granules and so are able to release it immediately following exposure to antigen. Interestingly, in the airways, mast cell numbers are highest in the smooth muscle layer. IL-1β is also produced by a wide range of immune and structural cells but the main source following antigen exposure is the macrophage. The cytokines TNF-α and IL-1β have been shown to affect many ASMC functions. IL-1β potentiates proliferation of guinea-pig ASMC and TNF-α stimulated human ASMC proliferation at low concentrations and was inhibitory at higher concentrations. These cytokines also stimulated ASMC secretion of a variety of other cytokines and chemokines, including some like GM-CSF, eotaxin and RANTES (regulated on activation normal T-cells expressed and secreted), with direct effects on eosinophil recruitment, activation and viability. In addition, Lazaar and colleagues demonstrated that TNF-α and IL-1β also increased the cell surface expression of ICAM-1 and VCAM-1 on human ASMC. Most importantly they found that...
activated T lymphocytes could then attach to the ASMC and induce ASMC proliferation. Thus ASMC are influenced by their inflammatory environment and may contribute further to the inflammation, regulating it locally and attracting more inflammatory cells to the muscle layer.

An additional finding of this study was that TNF-α also increases the adhesion of human eosinophils to human ASMC. TNF-α, 10 nM, increased the number of eosinophils attaching during the first 2 h of coculture only if the ASMC had been treated with it for 24 h prior to the addition of the eosinophils, whereas with an extended coculture period (20 h), it always increased the number of adhesive cells whether or not the ASMC had been treated with it for 24 h before. When TNF-α (0.1–10 nM) was present for the extended coculture period (20 h) only, it increased eosinophil adhesion period to the ASMC in a concentration-independent manner. However, when a 24 h pretreatment period with TNF-α was also included (44 h treatment), eosinophil adhesion was increased in a concentration-dependent manner. This apparent difference in effects of TNF-α may be due to time-related responses to differential recruitment of the p55 and p75 TNF-α receptors on the ASMC and/or the eosinophils by the lower concentrations of TNF-α. Prolonged treatment with TNF-α may increase adhesive eosinophil numbers by increasing ASMC adhesion molecule expression, as already demonstrated for lymphocyte adhesion to ASMC.1

IL-1β, on the other hand, was without significant effect on eosinophil adhesion to the ASMC under all conditions, except when present for just the 20 h coculture period. In this instance only, it caused a slight (19%) but significant reduction in the level of eosinophil adhesion. This lack of (or inhibitory) effect of IL-1β was unexpected as, like TNF-α, IL-1β has been shown to increase the expression of both VCAM-1 and ICAM-1 on ASMC following a 24 h treatment period.1 However, unlike TNF-α, IL-1β also induces ASMC to release PGE2.24,25 Therefore, it is possible that PGE2 released during the IL-1β treatment period, might have altered the response(s) of either cell-type and so negated any direct effects of IL-1β that might otherwise have led to increased eosinophil adhesion to the ASMC under the conditions reported here.

IL-5 was also without an effect on eosinophil adhesion to the ASMC in this study. The IL-5 used was biologically active as it increased eosinophil survival when cultured alone for 24 h by up to 27% (data not shown). As well as promoting eosinophil differentiation, growth, migration and survival, IL-5 has been shown to induce eosinophil morphological changes rapidly26 and to enhance eosinophil adhesion to immobilised ICAM-1 and VCAM-127 and to endothelial cells in an integrin-dependent (CD11/18) manner.28 Extracellular matrix proteins may also mediate eosinophil adhesion to ASMC as integrins bind to them and CD44, a molecule which binds to the matrix protein hyaluronate, plays an important role in lymphocyte adhesion to ASMC.1 However, despite increasing eosinophil surface expression of CD44 with a 24 h IL-5 treatment, Matsumoto et al. did not observe increased eosinophil adhesion to immobilised hyaluronate following IL-5 treatment.29 Under the conditions reported in this study, any IL-5 induced changes may not be of the nature required, or sufficient, to successfully increase eosinophil adhesion to ASMC, even when TNF-α does increase it.

Many studies have shown that eosinophil mediator release is induced and modulated by interactions between eosinophil adhesion molecules and their counter-ligands or extracellular matrix proteins.30–34 Interestingly, ASMC production of some extracellular matrix proteins is increased following exposure to serum from an atopic asthmatic.35 Adherent eosinophils in the airway smooth muscle layer are therefore likely to release mediators. Once activated, eosinophils may produce an extensive range of cytokines and growth factors, including IL-1β, TNF-α, PDGF, FGF, TGF-β, that have been implicated in modulating ASMC proliferation6,9,10 and/or mediator production.5,11,12 The effect on the muscle cells of eosinophil adhesion to them will vary, depending on the balance of the many inflammatory mediators present and the severity of the allergic response at the time.

TNF-α is likely to be present before and after eosinophils reach the smooth muscle layer of asthmatic airways and, of course, is likely to modulate eosinophil behaviour, as well as that of the ASMC, and so regulate their interaction. Studies in vitro have demonstrated that TNF-α-treated eosinophils have enhanced survival, up-regulated surface expression of the activation marker CD69,36 increased adhesion to immobilised ICAM-1 and VCAM-1,27 as well as degranulation, leukotriene C4 and superoxide production.26,37,38 Granule proteins cause tissue damage in vitro and have been implicated in epithelial shedding in vivo.17 We observed increased eosinophil adhesion to ASMC with no sign of ASMC detachment or overt morphological damage with eosinophil exposures to TNF-α of 2 or 20 h.

This is the first study of eosinophil adhesion to airway smooth muscle cells. We have shown that adhesion is rapid and can be prolonged. As we found it is partially mediated through ICAM-1 and VCAM-1, it is likely to result in eosinophil mediator release and altered airway smooth muscle responses. We have also demonstrated that the cytokine TNF-α increases eosinophil adhesion to the smooth muscle cells at concentrations that also induce airway smooth muscle to secrete other pro-inflammatory cytokines and chemokines. It is therefore possible that eosinophils and ASMC may regulate each other’s responses and inflammation locally in asthmatic airways, contributing to the pathogenesis and symptoms of asthma.
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