

Adhesion to Fibronectin Prolongs Eosinophil Survival

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Summary

We have investigated the effect of adhesion to fibronectin (Fn) on the survival of eosinophils in culture. Peripheral blood eosinophils from normal human donors were separated by immunomagnetic selection and cultured in RPMI on Fn- (100 $\mu\text{g/ml}$) coated microtiter plates for up to 96 h. Survival was measured by trypan blue exclusion. There was a significant enhancement of eosinophil survival with Fn as compared with both bovine serum albumin-coated and uncoated wells ($p < 0.05-0.01$). Fn-induced eosinophil survival was comparable to that obtained with exogenous interleukin 3 (IL-3) or granulocyte/macrophage colony-stimulating factor (GM-CSF) and was inhibitable by antibodies against Fn, very late antigen 4 (VLA-4), IL-3, and GM-CSF. Supernatants from Fn-, but not BSA-coated wells contained picogram amounts of IL-3 and GM-CSF, and eosinophils cultured on Fn for 24 h expressed mRNA for GM-CSF as determined by in situ hybridization. Therefore, Fn prolongs eosinophil survival in culture by triggering autocrine generation of cytokines by eosinophils. Since neutrophils lack VLA-4, this could provide a partial explanation for the preferential accumulation of eosinophils at sites of allergic inflammation, as well as the predominant tissue localization of eosinophils in healthy individuals.

Eosinophils are believed to be important effector cells in the host response to infection with helminthic parasites (1) and to have proinflammatory effects in allergic inflammation, including asthma (2, 3). In healthy individuals, eosinophils reside mainly in the tissues, but the mechanism of tissue localization is incompletely understood. A number of adhesion pathways have been defined that could potentially mediate the transmigration of eosinophils through the vascular endothelium. The very late antigen 4/vascular cell adhesion molecule 1 (VLA-4/VCAM-1) pathway is of particular interest because it is not available to neutrophils (4-7). An alternative mechanism for eosinophil accumulation in tissues is prolonged survival. Like neutrophils, eosinophils are end-stage cells that in culture rapidly undergo cell death. However, eosinophil-active cytokines, such as IL-3, IL-5, and GM-CSF, prolong eosinophil survival in culture for up to 2 wk (8, 9). They also enhance eosinophil functions such as cytotoxicity for metazoan targets and mediator release (10). Activated eosinophils can also generate a number of cytokines in vitro (11, 12). mRNA for cytokines such as IL-5, GM-CSF, and TGF- α has also been detected in vivo in various disease conditions (13, 14). The physiological triggers for eosinophil cytokine generation are not clear. Extracellular matrix proteins have been shown to modulate eosinophil response to physiological soluble stimuli (15). We have recently demonstrated that eosinophils can adhere specifically to fibronectin (Fn), an abundant extracellular matrix protein, and that

VLA-4, a known receptor for Fn (16), was involved in mediating eosinophil/Fn interactions (Anwar, A. R. E., G. M. Walsh, O. Cromwell, A. B. Kay, and A. J. Wardlaw, manuscript submitted for publication). Moreover, eosinophil adhesion to Fn resulted in short-term priming of eosinophils for enhanced leukotriene C₄ (LTC₄) release. For these reasons, we have studied the ability of Fn to exert longer term effects on eosinophil function, particularly whether it could prolong eosinophil survival.

Materials and Methods

Reagents. Reagents were obtained as follows. BSA (grade V), human plasma Fn, and the control mouse myeloma protein MOPC (IgG1) were from Sigma Chemical Co. (Poole, UK). Micromagnetic beads bound to anti-CD16 mAb were obtained from Miltenyi Biotech (Bergisch-Gladbach, Germany). Magnetic-activated cell sorter (MACS) columns were supplied by Becton Dickinson UK, Ltd. (Oxford, UK).

Antibodies. Polyclonal rabbit anti-human Fn was purchased from CooperBiomedical, Inc. (Malvern, PA). This was shown to significantly inhibit adhesion of human eosinophils to Fn (Anwar et al., manuscript submitted for publication) and exhibited no cross-reactivity with other matrix proteins. Goat IgG anti-GM-CSF and polyclonal rabbit anti-IL-3 were purchased from British Biotechnology (Oxford, UK). Both were selected because of their natural ability to neutralize the bioreactivity of their natural and recombinant cytokine, i.e., IL-3 and GM-CSF, respectively. They showed

no crossreactivity with any other cytokine. Polyclonal anti-IL-5 was a generous gift from Glaxo-Biogen (Geneva, Switzerland). Mouse mAb HP2/1 (anti-CD49d, VLA-4) was a kind gift from Dr. F. Sanchez-Madrid (Hospital de La Princesa, Madrid, Spain).

Eosinophil Purification. A volume of 100 ml of blood was obtained from volunteers with a normal eosinophil count and without evidence of allergic disease. After dextran sedimentation to remove red blood cells a granulocyte pellet was obtained by density centrifugation on Percoll. The eosinophils were then separated from the neutrophils by immunomagnetic selection using the MACS system utilizing the observation that resting peripheral blood eosinophils, unlike neutrophils, do not express CD16 (17). In brief, the granulocyte pellet was incubated (40 min, 4°C) with anti-CD16 mAb bound to micromagnetic beads. By negative selection, highly purified CD16⁻ eosinophils depleted of magnetically positive neutrophils (CD16⁺) were obtained after passage of granulocytes through a steel wire column (MACS type B or C) held between a permanent magnet. This method resulted in consistently high recoveries (>99%) of pure (>99%) eosinophils.

Eosinophil Culture. Freshly isolated eosinophils (>99% viable by trypan blue exclusion), in 100 μ l of RPMI (at 2×10^5 /well), were cultured in 96-well flat-bottomed non-tissue culture-treated microtiter plates (Becton Dickinson & Co., Lincoln Park, NJ) coated with either Fn or 1% BSA, or left uncoated. To coat the wells, 100 μ l of Fn (100 μ g/ml in PBS) was added to the wells for 1 h at 37°C. The plates were then washed three times with PBS to remove unbound protein. To block nonspecific protein-binding sites, 200 μ l of 1% heat-denatured BSA in PBS was added to each well, and the plates were incubated for 2 h at room temperature. Before the cells were added, the plates were washed twice with RPMI/1% BSA. Eosinophils were cultured in RPMI with penicillin and streptomycin, but without FCS to avoid any possible interaction between eosinophils and matrix proteins contained in serum. In some experiments mAbs were added to the wells at the start of the culture. To determine eosinophil viability, cells were removed from each well by gentle pipetting, stained with trypan blue, and counted blindly. To confirm that all the cells had been removed from the well, a total cell count was performed and the well examined by direct vision using an inverted microscope. Endotoxin levels of Fn (100 μ g/ml) and BSA- (1%) treated supernatants were both <0.2 EU/ml, as determined by the *Limulus* amoebocyte lysate (LAL) test using the E-toxicate (LAL) kit (Sigma Chemical Co.).

Measurement of Cytokine Generation. Supernatants of eosinophils cultured with Fn or BSA were removed from the wells and stored at -80°C until assayed. Both IL-3 and GM-CSF were measured using commercially available ELISA kits for IL-3 and GM-CSF (Quantikine; British Biotechnology, Oxford, UK). The assay is an enzyme immunoassay using mouse mAbs against either IL-3 or GM-CSF as capture antibodies, peroxidase enzyme-linked polyclonal antibody specific for either of the two cytokines as the second antibody, and was developed by peroxide chromogenic substrate. The minimum detectable concentrations for IL-3 and GM-CSF were 10 and 7.5 pg/ml, respectively.

In Situ Hybridization. In situ hybridization was performed as previously described (11, 18). Briefly, cDNA for GM-CSF was inserted into a PGEM-1 vector, linearized, and transcribed in the presence of ³⁵S-UTP and T7 and SP6 RNA polymerases for antisense (complementary RNA) and sense (having identical sequence to mRNA) riboprobes, respectively. Cytospins were permeabilized with proteinase-K. Nonspecific binding of ³⁵S was inhibited by treating the preparations with 0.1 M triethanolamine, acetic anhydride, iodoacetamide, and *N*-ethylmaleimide. Prehybridization was performed with formamide and SSC. For hybridization, antisense

or sense probes ($1-1.5 \times 10^6$ cpm/slide) were diluted in hybridization buffer in the presence of dithiothreitol (100 nM) and heated to 60°C for 1 h to block any nonspecific binding. Hybridization with the riboprobes was >16 h at 40°C. Posthybridization washings were performed under high stringency ($4-0.1 \times$ SSC at 45-50°C) during which unhybridized single-stranded RNA was removed by RNase-A treatment. After dehydration, cytopins were processed for autoradiography and counterstained with either hematoxylin, or the specific eosinophil stain carbol chromotrope 2R.

Results and Discussion

Eosinophils cultured on Fn-coated plates had significantly prolonged survival when compared with cells cultured on plastic alone (Fig. 1) ($p < 0.01$ at 72 and 96 h). At 96 h, virtually all the eosinophils cultured on uncoated wells had died, whereas 60% of the eosinophils cultured in Fn-coated wells were still excluding trypan blue. Cells cultured on BSA were also significantly less viable at 72 and 96 h than eosinophils cultured on Fn ($63 \pm 5\%$ vs. $32.5 \pm 7\%$ and $47 \pm 4\%$ vs. $20 \pm 6\%$, respectively; $p < 0.05$). The survival curve of eosinophils cultured in uncoated wells with IL-3 (10^{-10} M) (data not shown) was almost identical to that illustrated in Fig. 1 for eosinophils cultured in RPMI alone in Fn-coated wells.

To further investigate the mechanism of prolonged Fn-induced eosinophil survival, we attempted to inhibit the effect by using a number of antibodies (Fig. 2). At 72 h, eosinophils cultured on Fn (Fig. 2, row 1) had a viability of $63 \pm 5\%$, whereas eosinophils cultured in uncoated wells had a viability of $23 \pm 6.5\%$ (row 2; $p < 0.01$) and in BSA-coated wells, $32.5 \pm 7\%$ (row 3; $p < 0.02$). Fn-induced survival was significantly inhibited by a polyclonal antibody that we have previously demonstrated inhibits eosinophil adhesion to Fn-coated wells (row 4; $p < 0.05$), whereas normal rabbit serum had no inhibitory effect (row 5). Survival was also significantly inhibited by a mAb against VLA-4 (HP2/1) (row 6; $p < 0.05$) but not by an isotype control antibody (data not shown). Lastly, survival was inhibited by antibodies against both IL-3

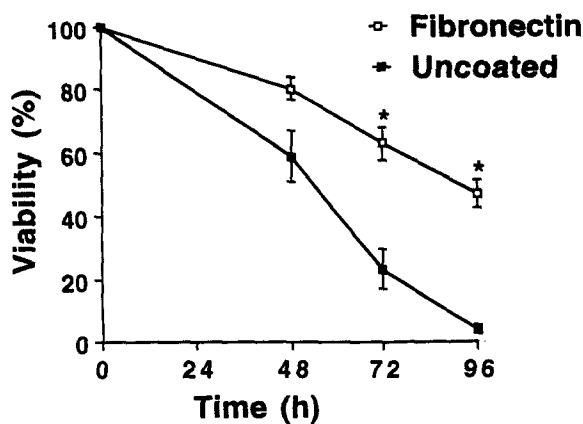


Figure 1. Survival of eosinophils cultured on Fn-coated wells or uncoated wells. Viability was measured by trypan blue exclusion at the start of culture and at 48, 72, and 96 h after culture. Fn resulted in a significant prolongation of eosinophil survival at 72 and 96 h (* $p < 0.01$) ($n = 5$).

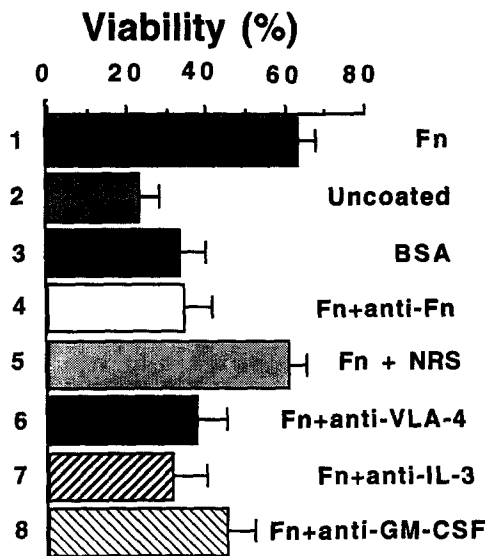


Figure 2. Inhibition of Fn-induced eosinophil survival at 72 h. Eosinophils were cultured on Fn (row 1), uncoated wells (row 2), or BSA (row 3). Eosinophils cultured on Fn had maintained survival, which was inhibited by an anti-Fn polyclonal antibody (row 4; $p < 0.05$) and anti-VLA-4 mAb (row 6; $p < 0.05$) and an antibody against IL-3 (row 7; $p < 0.05$). Normal rabbit serum (row 5) had no inhibitory effect. Inhibition by an antibody against GM-CSF (row 8) was consistently detected but was only significant at 96 h ($n = 4$).

(row 7, $p < 0.03$) and GM-CSF (row 8), although inhibition by anti-GM-CSF was only significant at 96 h ($p < 0.02$). These experiments demonstrated that the Fn-induced survival was specific, in that it was blocked by an anti-Fn polyclonal antibody, but not by normal rabbit serum, and was mediated by VLA-4. Furthermore, the blocking studies with the anticytokine antibodies suggested that the mechanism for increased survival may involve Fn-induced release of GM-CSF and IL-3 by eosinophils. To investigate this possibility we measured concentrations of IL-3 and GM-CSF in the supernatants of eosinophils cultured on Fn (Fig. 3). After 48 h in culture between 290 and 420 pg/ml of GM-CSF could be detected

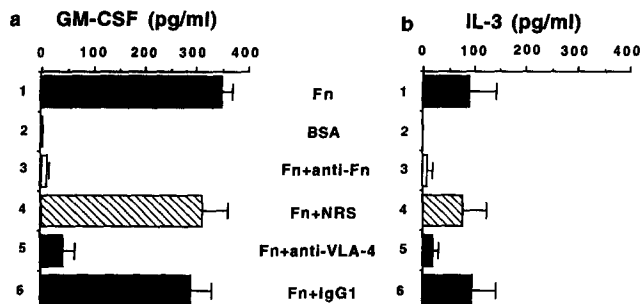


Figure 3. Release of GM-CSF (a) and IL-3 (b) by eosinophils cultured on Fn after 48 h in culture. Eosinophils released both GM-CSF and IL-3 after culture on Fn (row 1) but not after culture on BSA (row 2). Cytokine release was inhibited by anti-Fn (row 3) and anti-VLA-4 (row 5) antibodies but not by normal rabbit serum (row 4) or mouse IgG1 control (row 6) ($n = 5$).

in the supernatant of eosinophils cultured on Fn (Fig. 3 a, row 1; $n = 5$). In four of five experiments IL-3 could also be detected but in lower and more variable amounts (21–222 pg/ml) (Fig. 3 b, row 1). Negligible amounts of either GM-CSF (< 4 pg/ml) or IL-3 (< 3 pg/ml) could be detected in supernatants from BSA-coated wells (row 2). Cytokine generation by eosinophils was effectively blocked by the anti-Fn antibody (row 3) and the anti-VLA-4 mAb (row 5), but not by normal rabbit serum (row 4) or by the isotype control antibody (row 6).

To investigate if interaction with Fn induced expression of eosinophil cytokine mRNA, we undertook in situ hybridization using an 35 S-labeled riboprobe for GM-CSF using eosinophils cultured for 24 h on Fn or BSA. We chose the earlier time point because we considered that gene expression would precede secretion of GM-CSF. Expression of mRNA with antisense, but not sense, cRNA for GM-CSF was detected in eosinophils cultured on Fn but not in eosinophils cultured on BSA (Fig. 4).

Eosinophils have recently been shown to generate a number of cytokines, including IL-3, GM-CSF (11, 12), and IL-5 (14), that are active on mature eosinophils promoting, among a number of other responses, prolonged survival (8–10). However, the in vivo mechanism(s) by which eosinophils are trig-

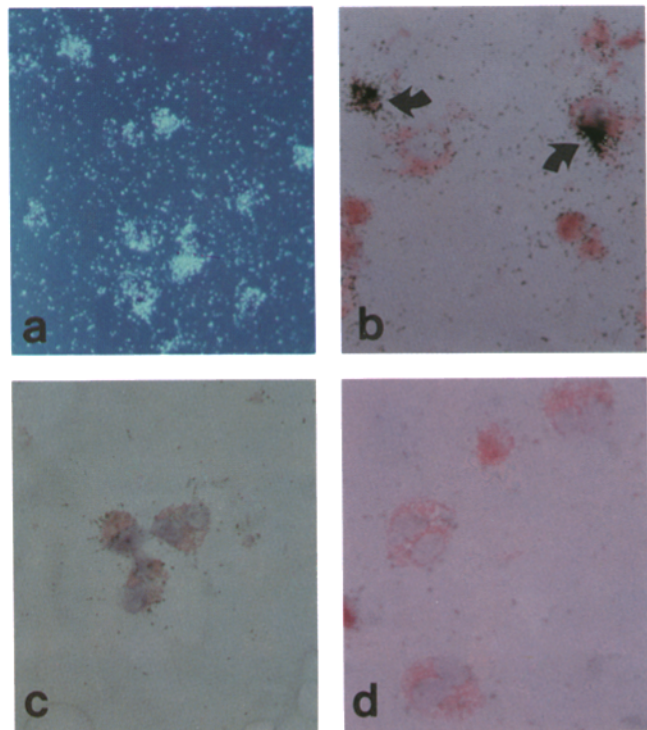


Figure 4. In situ hybridization of mRNA for GM-CSF by eosinophils cultured on Fn for 24 h. (A) Eosinophils hybridized with 35 S-labeled antisense riboprobe to detect GM-CSF mRNA, visualized under darkfield illumination; (B) positive hybridization signals (arrows) for GM-CSF mRNA colocalized to eosinophils stained specifically with carbol chromotrope 2R; (C) reduced hybridization signals in Fn-cultured eosinophils in the presence of anti-Fn antibody; (D) sense probe control.

gered to release these mediators remains unclear. In this study, we have demonstrated that eosinophil interaction with Fn induces the generation of measurable amounts of both GM-CSF and IL-3, and that these cytokines appear to induce prolonged survival of eosinophils by autocrine cytokine release. Therefore, eosinophils migrating into tissue in both healthy and diseased individuals could generate their own cytokines as a result of adhesion to Fn, thus preventing cell death and apoptosis (19). This could be one mechanism by which eosinophils accumulate in tissues where they primarily reside. We have recently demonstrated that eosinophils can adhere specifically to Fn and that this results in short-term priming of eosinophils for enhanced leukotriene C4 release (Anwar et al., manuscript submitted for publication). Adhesion to Fn was dependent on VLA-4, an integrin receptor expressed by eosinophils and mononuclear cells but not by neutrophils. Fn-induced cytokine generation by eosinophils was also inhibited by an antibody against VLA-4, suggesting that the latter is acting as a signaling as well as an adhesion receptor on eosinophils. It is now well recognized that integrins can transmit signals both inside out and outside in (20). In addition, adherence of monocytes/macrophages was shown to induce transcription of GM-CSF, whereas adherence to collagen induced IL-1 and TNF- α (21, 22). This raises the possibility that different profiles of cytokines can be generated by eosinophils depending on the nature of matrix protein/integrin receptor interaction involved. Using the immunomagnetic selection method of purifying eosinophils, the contaminating cells are usually monocytes, which, like eosinophils, do not express CD16. It is, therefore, theoretically possible that the cytokines could have been generated by contaminating monocytes. However, the purity of the eosinophils in our experiments was >99%. Moreover, the *in situ* hybridization experiments demonstrated GM-CSF mRNA expression by eosinophils cultured on Fn. Eosinophils were con-

clusively identified by the specific eosinophil stain carbol chromatrope 2R. The eosinophils used in this study were obtained from normal individuals without an eosinophilia. VLA-4 is constitutively expressed by eosinophils and, unlike Mac-1, can bind its ligands (VCAM-1 and Fn) without the need for cell activation (4). Nevertheless, it would be interesting to compare the response of eosinophils activated either *in vivo* or *in vitro* in both pattern and amount of cytokines released after culture on Fn.

We observed that eosinophils cultured in BSA had higher rates of survival compared with eosinophils cultured in uncoated wells. One explanation for this was that matrix protein contaminants in the BSA may have been interacting with eosinophils resulting in the generation of low levels of cytokines that though undetectable in our ELISA were nevertheless able to prolong survival to a limited degree. One apparent anomaly in the inhibition experiments described in Fig. 2 was the ability of the anti-IL-3 antibody to almost completely inhibit the Fn-induced survival. It would be expected that only partial inhibition would be observed especially as more GM-CSF than IL-3 was detected in the supernatant at the 48-h time point. This may be due to the kinetics of cytokine generation. We detected more IL-3 at 24 than at 48 h (140 ± 55 vs. 110 ± 52 pg/ml), whereas more GM-CSF was detected at 48 h (92 ± 18.5 vs. 362 ± 22 pg/ml). It is, therefore, possible that the antibodies were exerting their effect in the early stages of the culture when IL-3 was the more dominant cytokine generated.

In summary, we have demonstrated that eosinophil/Fn interaction results in VLA-4-mediated autocrine generation of IL-3 and GM-CSF, which leads to prolongation of eosinophil survival in culture. This could be an important mechanism for the regulation of eosinophil localization and function in health and disease.

We thank Dr. Q. Hamid for his valuable advice and Miss Janet North for her excellent technical assistance.

This work was supported by a Medical Research Council programme grant awarded to A. B. Kay.

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Received for publication 30 October 1992 and in revised form 15 December 1992.

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