Brief Definitive Report

Transforming Growth Factor β Abrogates the Effects of Hematopoietins on Eosinophils and Induces Their Apoptosis

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Summary

Hematopoietins, interleukin (IL)-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) have previously been shown to prolong eosinophil survival and abrogate apoptosis. The objective of this study was to investigate the effect of transforming growth factor β (TGF-β) on eosinophil survival and apoptosis. Eosinophils from peripheral blood of mildly eosinophilic donors were isolated to >97% purity using discontinuous Percoll density gradient. Eosinophils were cultured with hematopoietins with or without TGF-β for 4 d and their viability was assessed. We confirmed previous observations that hematopoietins prolonged eosinophil survival and inhibited apoptosis. TGF-β at concentrations $\geq 10^{-12}$ M abrogated the survival-prolonging effects of hematopoietins in a dose-dependent manner and induced apoptosis as determined by DNA fragmentation in agarose gels. The effect of TGF-β was blocked by an anti-TGF-β antibody. The anti-TGF-β antibody also prolonged eosinophil survival on its own. The culture of eosinophils with IL-3 and GM-CSF stimulated the synthesis of GM-CSF and IL-5, respectively, suggesting an autocrine mechanism of growth factor production. TGF-β inhibited the synthesis of GM-CSF and IL-5 by eosinophils. TGF-β did not have any effect on the expression of GM-CSF receptors on eosinophils. We also studied the effect of TGF-β on eosinophil function and found that TGF-β inhibited the release of eosinophil peroxidase. Thus, TGF-β seems to inhibit eosinophil survival and function. The inhibition of endogenous synthesis of hematopoietins may be one mechanism by which TGF-β blocks eosinophil survival and induces apoptosis.

The eosinophil is a short-lived, terminally differentiated cell. The death of eosinophils is characterized by DNA fragmentation, which is typical of apoptosis (1). The eosinophil is primarily involved in allergic reactions and host defense against parasites. Allergic diseases as well as parasitic infections are characterized by blood and tissue eosinophilia. The tissue eosinophilia is likely the result of increased production of eosinophils in the bone marrow and decreased apoptosis. Hematopoietins, IL-5, IL-3, and GM-CSF, have been shown to stimulate production of eosinophils in the bone marrow (2). The same cytokines inhibit eosinophilic apoptosis and prolong survival when eosinophils are cultured in their presence (3–5). Mice that are transgenic for IL-5 have profound eosinophilia (6). Antibody against IL-5 abrogates parasite-induced eosinophilia (7) suggesting a pivotal role for IL-5 in eosinophilia.

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Although there has been significant progress in our understanding of factors that control growth and survival of eosinophils, the mechanism of homeostatic regulation of these events is largely unknown. We hypothesize that the growth and survival of eosinophils are physiologically downregulated by endogenous factors. Transforming growth factor β (TGF-β) is a pleiotropic cytokine that promotes the function of resident tissue cells but largely inhibits the growth and activity of invading inflammatory cells (reviewed in reference 8). TGF-β has been shown to inhibit the differentiation of eosinophils from bone marrow cells (9). The effect of TGF-β on mature eosinophils is unknown. The objective of this study was to investigate whether TGF-β could inhibit eosinophil survival and promote apoptosis.

Materials and Methods

Polyvinylpyrrolidone-coated silica gel (Percoll) was purchased from Pharmacia LKB (Piscataway, NJ). Aniline blue, propidium

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iodide, fluorescein diacetate, lauryl sarkosinate, proteinase K, and o-phenylenediamine dihydrochloride were from Sigma Chemical Co. (St. Louis, MO). IL-3, IL-5, and GM-CSF were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). TGF-β1 was obtained from GIBCO BRL (Gaithersburg, MD) and was dissolved in 0.05 M sodium acetate buffer, pH 4.5, with 1% BSA. A monoclonal antibody (IgG1) that neutralizes TGF-β1, -β2, and -β3 was obtained from Genzyme Corp. (Cambridge, MA). 20 μg/ml of the antibody neutralizes 0.1–0.5 ng/ml of TGF-β. ^{125}I-GM-CSF (sp act, 114 μCi/μg) was purchased from New England Nuclear (Boston, MA). ELISA kits for GM-CSF were purchased from R & D Systems, Inc. (Minneapolis, MN). ELISA reagents for IL-5 were obtained from the Suntory Institute (Osaka, Japan). FCS was from Hyclone Laboratories, Inc. (Logan, UT) and was prescreened for survival and cytotoxic activity for eosinophils.

Purification of Eosinophils. Eosinophils from normal and allergic donors (peripheral blood eosinophil count, 4–8%) were isolated by sedimentation with 3% hydroxyethyl starch followed by centrifugation on a discontinuous Percoll gradient according to Gartner (10) as described previously (11). The band at the interface of 1.090 and 1.10 consisted of eosinophils of 97–99% purity as judged by staining with aniline blue (12) and Wright's stain. Eosinophils were suspended in RPMI 1640 with 10% FCS at a concentration of 10^6/ml.

Eosinophil Survival Assay. The eosinophil survival assay was carried out according to a method described previously (13). Purified eosinophils were suspended at 10^6/ml in RPMI 1640 with 10% FCS, and cultured in duplicate in multiwell plates (Falcon Labware, Oxnard, CA). Eosinophils were cultured with IL-5 (10^{-11} M), IL-3 (10^{-10} M), or GM-CSF (10^{-10} M) with or without TGF-β (10^{-10} M, unless otherwise stated). The viability of the cultured cells on day 4 was assessed by counting live and dead cells/200 total cells under a fluorescence microscope. The counting was done by a trained technician who was blinded to the protocol. Eosinophils were stained with fluorescein diacetate and propidium iodide for counting live and dead cells, respectively (13). In some experiments, eosinophils were cultured in the presence of a mouse anti-TGF-β antibody or a control mouse IgG1 antibody.

Assay of GM-CSF, IL-5, and TGF-β. GM-CSF and TGF-β were measured in eosinophil culture supernatants by using commercially available specific ELISA. IL-5 was measured by a sandwich ELISA using mouse monoclonal and rabbit polyclonal antibodies as described previously (14).

Assay of DNA Fragmentation. The DNA fragmentation assay was carried out according to a method described previously (15). Eosinophils (1–2 × 10^6/ml) were cultured for 72 h as described above, and then pelleted at 4°C. The cells were resuspended in 20 μl of 10 mM EDTA and 50 mM Tris-HCl, pH 8.0, containing 0.5% sodium lauryl sarkosinate and 0.5 mg/ml of proteinase K and incubated for 60 min at 50°C. Next, 10 μl of 10 mM EDTA, pH 8.0, containing 0.25% bromophenyl blue and 40% sucrose were mixed with each DNA extract. The individual extracts were loaded onto 2% agarose gel containing 3 μg/ml of ethidium bromide. Electrophoresis was performed in 90 mM Tris-HCl, pH 8.3, containing 90 mM boric acid and 2.5 mM EDTA until the marker dye migrated 6–8 cm. The gel was visualized and photographed in UV light with a Polaroid camera.

Assay of Peroxidase. Eosinophil peroxidase was measured by a colorimetric assay as described by Strath et al. (16). A solution of the substrate containing 0.1 mM o-phenylenediamine dihydrochloride in a 0.05 M Tris buffer, pH 8.0, containing 0.1% Triton X-100 and 1 mM hydrogen peroxide was prepared. Purified eosinophils were preincubated with TGF-β (10^{-10} M) or buffer for 10 min and then stimulated with IL-5 (10^{-10} M) for 30 min at 37°C in a water bath. Aliquots (150 μl) of the cell-free supernatants were incubated with 300 μl substrate solution for 30 min at 37°C. The reaction was stopped by addition of 200 μl of 4 M sulfuric acid. The absorbance was determined at 492 nm by using a spectrophotometer (Emax; Molecular Devices Corp., Menlo Park, CA).

Binding of ^{125}I-GM-CSF to Eosinophils. The binding of ^{125}I-GM-CSF to eosinophils was performed according to a method described previously (17). Eosinophils were suspended in RPMI 1640 + 10% FCS at a concentration of 10^6/ml. The cells were incubated overnight in the absence or presence of TGF-β (10^{-10} M) and then washed twice with HBSS + 5% FCS at 4°C. After incubation the cells were suspended in 400 μl of RPMI 1640 + 2 mg/ml of BSA. Next, eosinophils were incubated with various concentrations of ^{125}I-GM-CSF (0.5 × K_D–20 × K_D) with or without a 50-fold excess of unlabeled GM-CSF for 16 h at 4°C. After incubation, the cells were suspended and transferred onto 0.75 ml of an ice-cold mixture of 75% FCS in RPMI 1640. The cells were centrifuged for 2 min in a microcentrifuge, and the pellets were separated from the supernatants. The pellets were sliced off with a razor blade for determination of radioactivity. Specific binding was defined as the amount of binding blocked by a competition with a 50-fold excess unlabeled GM-CSF.

Data are presented as mean ± SEM. Statistical analyses were performed with Wilcoxon signed-rank test for paired samples by using the software "Epistat" (Tracy Gustafson, Round Rock, TX).

Results

Eosinophil Survival Assay. The effect of TGF-β was studied in the eosinophil survival assay. We found that TGF-β inhibited eosinophil survival in a dose-dependent manner (Fig. 1 *A*). At a concentration of ≥10^{-12} M TGF-β significantly (p <0.01) abrogated eosinophil survival. Kinetics studies showed that TGF-β exerted its maximal effect within 24 h

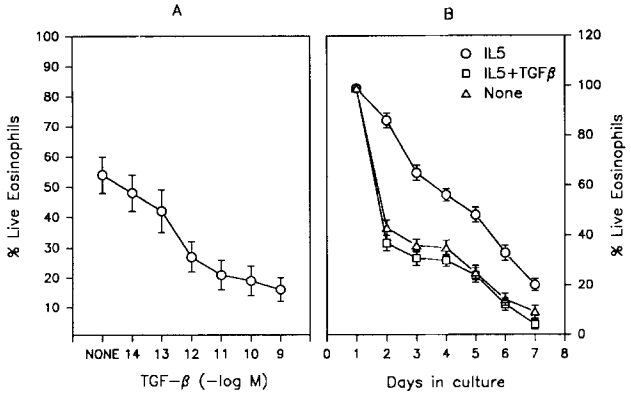

Figure 1. (*A*) The effect of TGF-β on eosinophil survival. Purified eosinophils (>97%) were cultured in the presence of various concentrations of TGF-β with added IL-3 (10^{-10} M). Live and dead cells were counted on day 4. Results of five different experiments are shown. The difference in survival is statistically significant (p <0.05) at a concentration of TGF-β ≥10^{-12} M. (*B*) The kinetics of the effect of TGF-β on eosinophil survival. Eosinophils were cultured in the medium alone (NONE), IL-5 (10^{-11} M), and IL-5 (10^{-11} M) + TGF-β (10^{-10} M). The survival was assessed every day for 7 d. Day 1 represents the start of the culture. Results of one of three similar experiments are shown.

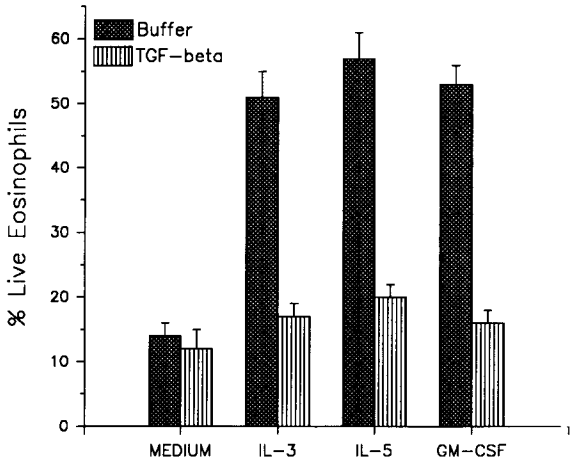

Figure 2. Inhibition of eosinophil survival by TGF-β. Eosinophils were cultured in the presence of IL-3 (10^{-10} M), IL-5 (10^{-11} M), and GM-CSF (10^{-10} M) with or without TGF-β (10^{-10} M). Live and dead cells were counted on day 4. The difference in survival in the presence of TGF-β is significant ($p < 0.01$) for IL-3 ($n = 13$), IL-5 ($n = 9$), and GM-CSF ($n = 9$).

(Fig. 1 *B*). The curve of cell survival in the presence of TGF-β is parallel to that of cells cultured in the absence of IL-5. A similar degree of inhibition of eosinophil survival occurred when IL-3, IL-5, and GM-CSF were compared as the survival-promoting agents (Fig. 2). Anti-TGF-β antibody alone prolonged the survival of eosinophils (Fig. 3). It also blocked the effect of TGF-β on IL-5–induced eosinophil survival.

Assay of DNA Fragmentation. The apoptotic cell death of eosinophils was investigated by the assay of DNA fragmentation on agarose gel electrophoresis. DNA was isolated from cultured eosinophils on day 3 and was analyzed for the presence of small molecular weight fragments ("ladder"). We found that TGF-β reversed the inhibitory effect of IL-3 and induced DNA fragmentation (Fig. 4).

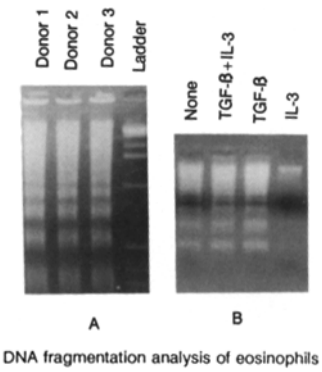

Figure 4. Analysis of apoptosis of eosinophils by agarose gel electrophoresis. (*A*) Eosinophils from three donors were cultured for 3 d in the medium alone without hematopoietins. DNA was isolated and analyzed for the presence of small molecular weight fragments. (*B*) Eosinophils were cultured in the presence of IL-3 (10^{-10} M) with or without TGF-β (10^{-10} M). DNA was isolated on day 3 and analyzed by gel electrophoresis. Results of one of three similar experiments are shown.

Effect of TGF-β on Eosinophil Function. We investigated the regulation of eosinophil cytokine synthesis. We found that IL-3 and GM-CSF stimulated the synthesis of GM-CSF and IL-5, respectively (Fig. 5), and TGF-β significantly ($p < 0.03$) inhibited production of both GM-CSF and IL-5.

To determine whether the inhibitory effect of TGF-β on eosinophil survival is due to downregulation of expression of receptors for hematopoietins, we studied the binding of ^{125}I-labeled GM-CSF to eosinophils after an overnight incubation with TGF-β. We found that preincubation with TGF-β did not alter the expression of GM-CSF receptors on eosinophils (data not shown). In another set of experiments

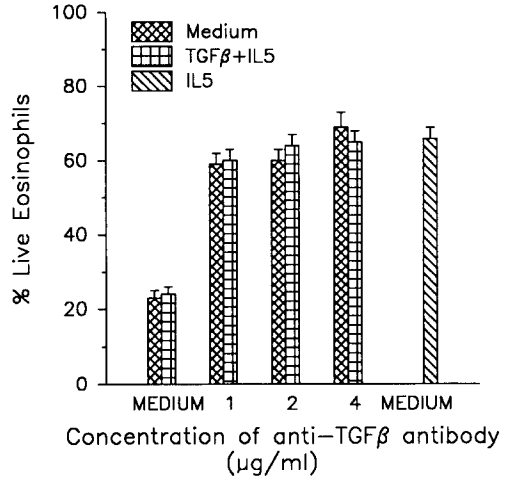

Figure 3. The effect of a neutralizing anti-TGF-β monoclonal antibody (IgG1) on eosinophils survival. Eosinophils were cultured alone or in the presence of various concentrations of the antibody with added TGF-β (10^{-10} M) and/or IL-5 (10^{-11} M). The survival was assessed on day 4 ($n = 4$). The anti-TGF-β antibody significantly prolonged ($p < 0.05$) eosinophil survival. An unrelated mouse IgG1 antibody that was used in the control experiments did not have any effect on eosinophil survival.

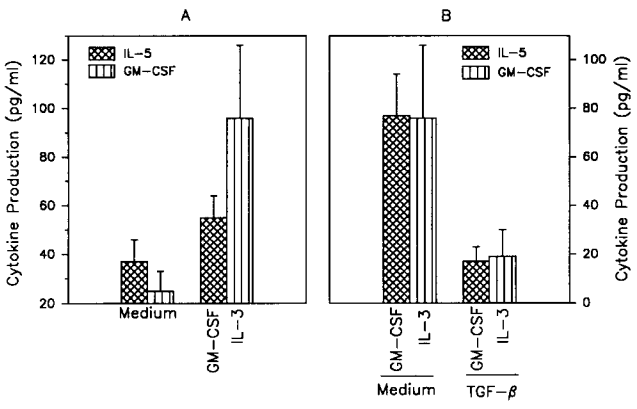

Figure 5. Production of IL-5 and GM-CSF by eosinophils (*A*) and the effects of TGF-β (*B*). (*A*) Purified eosinophils were cultured in the presence of GM-CSF, IL-3 (both 10^{-10} M), or medium for 24 h. IL-5 and GM-CSF were measured in the culture supernatant by ELISA. GM-CSF and IL-3 significantly ($p < 0.003$) stimulated the production of IL-5 ($n = 6$) and GM-CSF ($n = 6$), respectively. (*B*) Eosinophils were cultured as above in the presence (+TGF) or absence (–TGF) of TGF-β (10^{-10} M), and then IL-5 and GM-CSF were measured in the supernatants. TGF-β significantly ($p < 0.03$) inhibited the production of IL-5 ($n = 6$) and GM-CSF ($n = 9$).

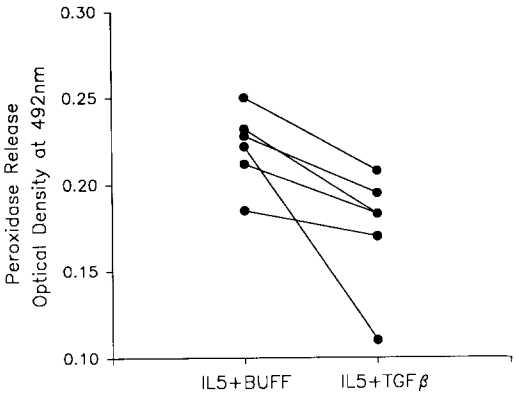

Figure 6. Inhibition of eosinophil peroxidase release by TGF-β. Eosinophils were incubated with buffer or TGF-β (10^{-10} M) for 15 min then stimulated with IL-5 (10^{-9} M) for 30 min. Peroxidase was assayed in the supernatant. Results of six duplicate experiments are shown. TGF-β significantly ($p < 0.03$) inhibited the secretion of peroxidase. Buff, buffer.

the effect of TGF-β on IL-5–stimulated peroxidase secretion was investigated. We found that TGF-β significantly ($p < 0.03$) inhibited the release of peroxidase from eosinophils (Fig. 6).

Discussion

A number of cytokines regulate growth, function, and survival of eosinophils. IL-5, IL-3, and GM-CSF are the most important known. All three cytokines enhance the function of eosinophils. Eosinophils become hypodense and primed for activation when incubated with these cytokines (18). The hematopoietins not only stimulate eosinophilic differentiation, but also prolong eosinophil survival in vitro. Prolonged survival may profoundly contribute to eosinophilia in the tissue where the hematopoietins are locally produced. Indeed, in vivo studies in patients with eosinophilia showed considerably prolonged survival ($t_{1/2}$ = 61 vs. 8–18 h in normal subjects) of eosinophils (19).

Some recent reports suggest that eosinophils themselves are the source of many cytokines. Eosinophils secrete IL-3 and GM-CSF upon stimulation with calcium ionophore, phorbol myristate acetate, and interferon γ (20, 21). In situ hybridization studies demonstrated the presence of mRNA for IL-5 in eosinophils obtained from patients with asthma (22) and those with coeliac disease (23). Thus far, there has been no report of secretion of IL-5 by eosinophils. This is the first report of production and secretion of IL-5 by eosinophils. More importantly, we identified physiologic triggers for eosinophil production of IL-5 and GM-CSF. We showed that stimulation of eosinophils with one hematopoietin resulted in the production of other hematopoietins. This observation may indicate that under some circumstances the growth and survival of eosinophils may become independent of T cells and other cytokine-producing cells. The hematopoietins produced by eosinophils may work in an autocrine and paracrine fashion.

The results of this study suggest that TGF-β is an endogenous negative regulator of eosinophil survival. TGF-β appears to completely abrogate the survival-prolonging effect of all hematopoietins, and induces apoptosis. TGF-β has been shown to cause apoptosis in hepatocytes (24). One possible mechanism of inhibiting eosinophil survival is that TGF-β blocks the synthesis of hematopoietins. TGF-β has been detected in eosinophils obtained from patients with eosinophilia (25, 26). A neutralizing anti-TGF-β antibody alone prolongs eosinophil survival suggesting an autocrine production of TGF-β. We believe that there is a fine balance between the production of TGF-β and hematopoietins by eosinophils. TGF-β may act as a homeostatic regulatory mechanism that counteracts the action of hematopoietins and programs eosinophils to die.

TGF-β inhibits the function of many cells of the immune system including T cells and monocytes. In a previous investigation of the effect of TGF-β on the expression of receptors for IFN-γ on monocytes (27), TGF-β reduced the expression of IFN-γ receptors without altering their affinity. In our study TGF-β did not affect GM-CSF receptor expression on eosinophils. Besides inhibiting cytokine synthesis and survival, TGF-β blocks the release of eosinophil peroxidase. Previously, TGF-β has been shown to downregulate the expression of CD23 on an eosinophilic cell line, Eol-1 (28). Further, this cytokine inhibits the differentiation of eosinophils from bone marrow cells (9). It seems that TGF-β has generalized inhibitory activity on all aspects of eosinophils including growth, differentiation, function, and survival. Thus, the exploration of the mechanism of action of TGF-β on eosinophils may provide new avenues for combating eosinophilia associated with eosinophilic syndromes and allergic disorders.

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