Eosinophils From Patients With Blood Eosinophilia Express Transforming Growth Factor β_1

By David T.W. Wong, Aram Elovic, Karekine Matossian, Naoyoshi Nagura, Jim McBride, Ming Yung Chou, John R. Gordon, Thomas H. Rand, Stephen J. Galli, and Peter F. Weller

The infiltration of eosinophils into tissues during pathologic responses is often associated with extracellular matrix alterations such as fibrosis. Transforming growth factor-β_1 (TGF-β_1) is a well-characterized multifunctional cytokine known to exert potent effects on the extracellular matrix. In this report, we showed the production of TGF-β_1 by human eosinophils from patients with blood eosinophilia. Northern blot analysis using RNA isolated from eosinophils purified from a patient with the idiopathic hypereosinophilic syndrome (HES) detected the 2.5-kb TGF-β_1 transcript. In situ hybridization and immunohistochemistry of leukocytes from two patients with HES and two patients with blood eosinophilia localized TGF-β_1 messenger RNA (mRNA) and protein to eosinophils. No other cell type contained TGF-β_1 mRNA by in situ hybridization, whereas other leukocytes contained detectable TGF-β_1 protein by immunohistochemistry. Eosinophils from four normal donors contained little or no detectable TGF-β_1 protein by immunohistochemistry, whereas eosinophils from two of these four normal donors labeled weakly for TGF-β_1 mRNA by in situ hybridization. These results show that eosinophils in the peripheral blood of patients with blood eosinophilia can express TGF-β_1, but that eosinophils in the blood of normal donors contained little or no TGF-β_1.

TRANSFORMING growth factor-β (TGF-β) was initially detected by its ability, together with transforming growth factor-α (TGF-α), to induce the reversible transformation of rat fibroblasts in culture.[1,2] There are three distinct molecular forms of TGF-β, designated TGF-β_1, TGF-β_2, and TGF-β_3.[3-6] These three closely related TGF-β molecules are first synthesized as larger precursor polypeptides that are processed to yield 12.5-Kd monomers.[7] The biologically active, mature 25-Kd TGF-β polypeptide consists of two identical disulfide-linked monomers.[3] In their mature sequences, human TGF-β_2 and TGF-β_3 genes are approximately 80% similar to TGF-β_1, while TGF-β_3 is 72% similar to TGF-β_2.[8]

Although initially implicated to have a role in neoplastic transformation, TGF-β has subsequently been shown to have many biologic activities in a variety of normal and transformed cells.[9-11] TGF-β is now regarded as a multifunctional regulator of cell growth that can be produced by many types of normal and transformed cells.[7] Nontransformed cells that can produce of TGF-β include cultured T cells,[12] macrophages,[13] monocytes,[13,14] megakaryocytes,[15] neutrophils,[14] and fibroblasts.[16,17] Among the most striking effects of TGF-β on cellular function is its promotion of extracellular matrix formation.[7] Several recent studies have described increased expression of TGF-β in association with diseases characterized by excessive fibrosis.[7] For example, treatment of freshly isolated hepatocytes with TGF-β caused a marked elevation of α2(I)collagen messenger RNA (mRNA) expression.[18]

It is also known that significant interstitial fibrosis can occur in disorders characterized by extensive infiltrates of eosinophils, such as in the cutaneous lesions of onchocerciasis and in the pulmonary and cardiac lesions of patients with the idiopathic hypereosinophilic syndrome (HES).[19] We recently showed that eosinophils can express the cytokine TGF-α.[20,21] This finding suggested that eosinophils might also be able to express other cytokines, such as TGF-β, that could contribute to the fibrosis associated with disorders accompanied by extensive eosinophil infiltration. In this report we show that eosinophils from patients with blood eosinophilia express the multifunctional cytokine TGF-β_1, the most widely studied form of TGF-β.

MATERIALS AND METHODS

Human eosinophil isolation. Four patients with blood eosinophilia were studied. Two patients had the idiopathic HES with sustained blood eosinophilia of greater than 50%. One of these patients (HES-1) had had a mitral valve replacement due to endomyocardial disease and was receiving therapy with chlorambucil and hydroxyurea; the second patient (HES-2) had no evidence of organ damage and was receiving no therapy. The other two patients had 15% or 20% eosinophilias at the time of study, due to Job's syndrome or a drug hypersensitivity reaction, respectively. Unfractionated leukocytes, obtained from sodium citrate-anticoagulated blood after dextran sedimentation and hypotonic lysis of erythrocytes, were used for the in situ hybridization and immunohistochemistry studies. For Northern blot analyses, granulocytes obtained after dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia, Piscataway, NJ), and lysis of erythrocytes[22] were further enriched for eosinophils by sequential incubation at 4°C with the anti-CD16 monoclonal antibody (MoAb) Leulla (Becton Dickinson, Mountain View, CA) and magnetic beads conjugated with goat antimouse IgG (Advanced Magnetics, Cambridge, MA) to deplete $CD16^+$ neutrophils. Leukocytes were also isolated by dextran sedimentation from the peripheral blood (PB) of four healthy male donors, aged 27 to 44 years. These

From the Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine; the Departments of Medicine and Pathology, Beth Israel Hospital and Harvard Medical School; and the Charles A. Dana Research Institute, Beth Israel Hospital, Boston, MA.

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Address reprint requests to David T.W. Wong, DMD, DMSc, Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine, 188 Longwood Ave, Boston, MA 02115.

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preparations consisted of 1% to 5% eosinophils, 52% to 83% neutrophils, and 16% to 55% mononuclear cells.

RNA isolation and Northern blot hybridization. Total RNA was isolated from human eosinophils from patient HES-1 and from A431 human epidermoid carcinoma cells using the guanidine isothiocyanate method of Davis et al.[23] The purified human eosinophil preparation contained 74% eosinophils and 26% neutrophils. Details of Northern blot analysis using the Zetabind membrane were described previously.[24] Poly(A)$^+$RNA was further purified from the total RNA of A431 cells by oligo-dT cellulose chromatography.[23,24] Random-priming was used to label the cDNA inserts. The TGF-β_1–producing A431 cell line[11] was maintained as previously described.[21]

Molecular probes. For Northern blot analyses, the human TGF-β_1 (hTGF-β_1) cDNA was a 2.1-kb *Eco*RI fragment obtained from G.I. Bell of Chiron Corp (Emeryville, CA). The chicken β-actin cDNA was a 1.7-kb *Pst* I fragment obtained from D.W. Cleveland (UCSF, San Francisco, CA).[25] For in situ hybridization, the hTGF-β_1 cDNA was cloned into the plasmid pBSII-SK(−) (Stratagene, La Jolla, CA) such that the sense and antisense riboprobes can be produced by either T7 or T3 RNA polymerases.

In situ hybridization. Details of our in situ hybridization procedure using ^{35}S-labeled antisense and sense hTGF-α_1 riboprobes were described previously.[20,21] Immediately after isolation, blood leukocytes were embedded into 1% agar in phosphate-buffered saline (PBS) and then fixed, processed, and embedded in paraffin. To obviate potential nonspecific binding of probes to eosinophils,[26,27] we used hybridization conditions that eliminate nonspecific annealing of the riboprobes to eosinophils, as previously described.[20,21]

Immunohistochemistry. Immunohistochemical detection of TGF-β_1 protein in blood leukocytes was performed as previously described.[21] Paraffin-embedded sections (6 μm) were subjected to staining for TGF-β_1 protein using a rabbit polyclonal antibody (IgG fraction) directed against natural TGF-β_1 isolated from porcine platelets (AB-20-PB; R&D, Minneapolis, MN). TGF-β_1 is highly conserved and is identical in human and pig.[7] The rabbit polyclonal antibody at 100 ng/mL (1:100 dilution) was used to stain each section. An IgG fraction from a nonimmune normal rabbit (I-5006; Sigma Chemical, St Louis, MO) at the same concentration was used as a negative control. The Vectastain ABC-Alkaline Phosphatase (rabbit IgG, AK-5001) and alkaline phosphatase substrate I (SK-5100) (Vector Laboratories, Burlingame, CA) in the presence of 1.25 mmol/L of levamisole (SP-5000) were used for signal detection. All sections were stained with 0.2% aniline-blue for 10 minutes to identify eosinophils.[28]

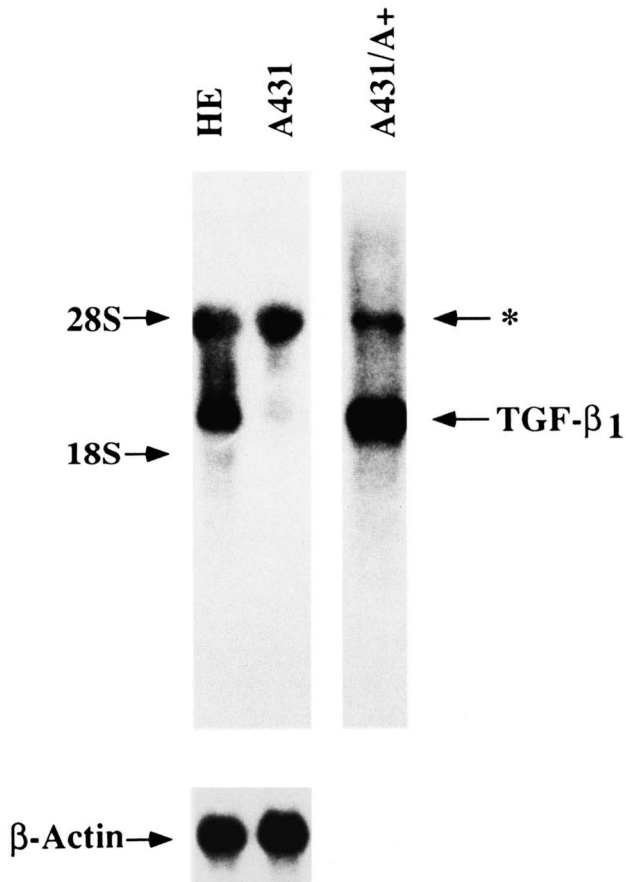

Fig 1. Detection of TGF-β_1 transcripts in preparations of human eosinophils (HE) and human A431 epidermoid carcinoma cells. RNA from HES-1 was used. Twelve micrograms of total RNA from HE (lane HE) and A431 cells (lane A431), as well as 2.5 μg of poly(A)$^+$RNA from A431 cells (lane A431/A$^+$) was used. The full-length blots were hybridized to ^{32}P-labeled hTGF-β_1 cDNA. The size of the detected TGF-β_1 hybridizable bands is ~2.5 kb. The arrows labeled 18S (~2 kb) and 28S (~5 kb) show the positions of ribosomal RNA markers that were electrophoresed at the same time. The asterisk indicates that the hybridization signals at this location represented nonspecific annealing of the ^{32}P-labeled hTGF-β_1 cDNA to the 28S ribosomal RNA. The total RNA blot was rehybridized to a ^{32}P-labeled chicken β-actin cDNA, shown in the lower panel. The size of the detected human β-actin transcript is ~2 kb.

RESULTS

Northern blot detection of TGF-β_1 mRNA in purified eosinophils. Total RNA (12 μg) was isolated from a purified human eosinophil preparation. The RNA yield from the purified human eosinophil preparation precluded us from performing poly(A)$^+$RNA selection. Total RNA from a human epidermoid carcinoma A431 cell line, which is known to contain TGF-β_1 mRNA, was used as a positive control.[11] An intense TGF-β_1 hybridization signal at ~2.5 kb was detected with eosinophil-enriched granulocytes from HES donor 1 (Fig 1). A very weak signal at the same location was detected with the A431 cells when total RNA was examined (Fig 1, lane A431). However, when poly(A)$^+$RNA (2.5 μg) of A431 cells was examined, a strong TGF-β_1 mRNA hybridizing band was observed at the 2.5-kb location (Fig 1, lane A431/A$^+$). Thus, the eosinophil preparation contained substantially more TGF-β_1 mRNA than did the A431 cells. Rehybridization of the same blot with the ^{32}P-labeled chicken β-actin cDNA (Fig 1, lower panel) showed that approximately the same amount of RNA had been loaded onto each lane and that the quality of the isolated RNA was satisfactory. Note that the signals at the 28S ribosomal location represented nonspecific annealing of the ^{32}P-hTGF-β_1 cDNA probe to the 28S ribosomal RNA. The nonspecific annealing of the radiolabeled probe to 28S ribosomal RNA was greatly reduced when poly(A)$^+$RNA of A431 cells was used (Fig 1, lane A431/A$^+$, one cycle of oligo-dT selection) when compared with the total RNA lane (Fig 1, lane A431).

Cellular localization of TGF-β_1 mRNA in human eosinophils by in situ hybridization. The human eosinophil prepa-

ration used for Northern blot analysis contained 74% eosinophils, with neutrophils representing the only identifiable contaminating cell. To localize precisely the cellular source(s) of the TGF-β_1 mRNA detected in the human eosinophil preparation, we performed in situ hybridization on blood leukocytes from two patients with HES and two patients with blood eosinophilia (Table 1). All eosinophils examined (100%) hybridized with the ^{35}S-labeled antisense hTGF-β_1 riboprobe. No neutrophils, monocytes, or lymphocytes were detectably labeled with this probe. No eosinophils or other cells detectably labeled with the ^{35}S-labeled sense hTGF-β_1 riboprobe on adjacent sections.

Figure 2A through D shows the typical TGF-β_1 mRNA labeling pattern of human eosinophils from patient HES-1 by in situ hybridization. The bright-field photomicrograph in Fig 2A shows four cells with bilobulated or multilobulated nuclei. The two cells with eosinophilic granules were prominently labeled with the riboprobe, which was especially apparent in the dark-field photomicrograph viewed with a green filter (Fig 2B). Fluorescence with a rhodamine filter was used to confirm the identification of eosinophils that had been stained with a Fisher Giemsa stain (Fig 2C). Only two of the four cells exhibited fluorescence. A double exposure simultaneously showing fluorescence and in situ hybridization labeling confirmed that the labeled cells were eosinophils (Fig 2D). By morphology, the unlabeled cells were neutrophils. Hybridization of adjacent sections to the ^{35}S-labeled sense hTGF-β_1 riboprobe did not label any cell (data not shown). These findings show the specificity of our in situ hybridization results identifying TGF-β_1 mRNA in human eosinophils.

Detection of TGF-β_1 protein in human eosinophils by immunohistochemistry. To determine if the TGF-β_1 mRNAs detected in eosinophils from the four patients with blood eosinophilia were translated into TGF-β_1 protein, immunohistochemistry was performed on paraffin-embedded sections of leukocyte preparations using a rabbit anti–TGF-β_1 polyclonal antibody. All sections were counterstained with a 0.2% aniline blue solution. Eosinophil cytoplasmic granules stained with this dye emit a blue fluorescence when viewed with a DAPI filter at 365 nm, whereas neutrophil granules do not.[28]

Table 1. In Situ Detection of TGF-β_1 mRNA and Protein in Human Eosinophils

		In Situ Hybridization				Immunohistochemistry			
		Antisense TGF-β_1		Sense TGF-β_1		TGF-β_1		Control	
Sample	Diagnosis	Eos	Others	Eos	Others	Eos	Others	Eos	Others
1	HES-1	+*	–	–	–	+†	+	–	–
2	HES-2	+*	–	–	–	+†	+	–	–
3	Eos-1	+*	–	–	–	+†	+	–	–
4	Eos-2	+*	–	–	–	+†	+	–	–
5	NL-1	–	–	–	–	–	+	–	–
6	NL-2	–	–	–	–	–	+	–	–
7	NL-3	+‡	–	–	–	–	+	–	–
8	NL-4	+§	–	–	–	–	+	–	–

Abbreviations: Eos-1, Job's syndrome; Eos-2, drug hypersensitivity reaction; NL-1 through NL-4 normal donors.

*100% positive for TGF-β_1 by in situ hybridization.

†100% positive for TGF-β_1 by immunohistochemistry.

‡Approximately 50% positive for TGF-β_1 by in situ hybridization.

§Approximately 80% positive for TGF-β_1 by in situ hybridization.

Table 1 summarizes the findings of our immunohistochemical search for TGF-β_1 protein in human eosinophils. All of the eosinophils (100%) from the four patients with blood eosinophilia exhibited positive staining for TGF-β_1. The spectrum of immunoreactivity ranged from moderate to intense. It should be noted that other leukocytes, which unlike eosinophils did not exhibit aniline-blue fluorescence, also contained immunoreactive TGF-β_1 product. Figure 3A shows typical immunostaining of leukocytes from patients with blood eosinophilia. The three cells in the field stained positive for TGF-β_1 protein (a red alkaline phosphatase substrate was used). Viewing the same field with DAPI filters at 365 nm (Fig 3B) showed that only two of the three cells exhibited aniline-blue fluorescence. This finding indicated that eosinophils from patients with blood eosinophilia contained TGF-β_1 product. Other leukocytes were also found to contain TGF-β_1 protein. Staining of adjacent sections with a nonimmune rabbit IgG preparation did not stain any cell (data not shown).

TGF-β_1 mRNA and product in eosinophils from healthy donors. PB leukocytes from four normal donors, containing 1% to 5% eosinophils, were examined for TGF-β_1 mRNA and protein by in situ hybridization and immunohistochemistry. The results of this survey are summarized in Table 1. None of the eosinophils in these specimens exhibited detectable TGF-β_1 product by immunohistochemistry. Figure 3C is a bright-field photomicrograph of a leukocyte preparation from a normal donor stained with the TGF-β_1 polyclonal antibody. It is evident that many cells in this field exhibited detectable TGF-β_1 immunostaining (arrows). When the same field was examined for eosinophils using UV illumination and DAPI filter, three aniline-blue fluorescent eosinophils were evident (Fig 3D). Two of the three aniline blue fluorescent cells in Fig 3C did not exhibit TGF-β_1 immunostaining (Fig 3C, large arrow heads), whereas one exhibited an equivocal reaction in one area of the cytoplasm (small arrow head). This finding suggests that normal human blood eosinophils contain little or no TGF-β_1 protein compared with that found in eosinophils from patients with eosinophilia. In addition, 30% to 80% of the leukocytes, negative for aniline-blue fluorescence, showed positive immunostaining for TGF-β_1 protein (arrows). Immunostaining of adjacent sections with the nonimmune rabbit IgG fraction did not result in the staining of any cell (data not shown).

When in situ hybridization was used to examine TGF-β_1 mRNA in the leukocytes from these four normal donors (Table 1), two (NL-1 and -2) showed no labeling of eosinophils or any other cells. In the other two normal donors (NL-3 and -4), ~50% and 80% of the eosinophils, respectively, labeled weakly for TGF-β_1 mRNA (data not shown). Only eosinophils were found to exhibit positive TGF-β_1 mRNA labeling. Incubation of adjacent sections with the ^{35}S-labeled sense hTGF-β_1 riboprobe did not label any cell.

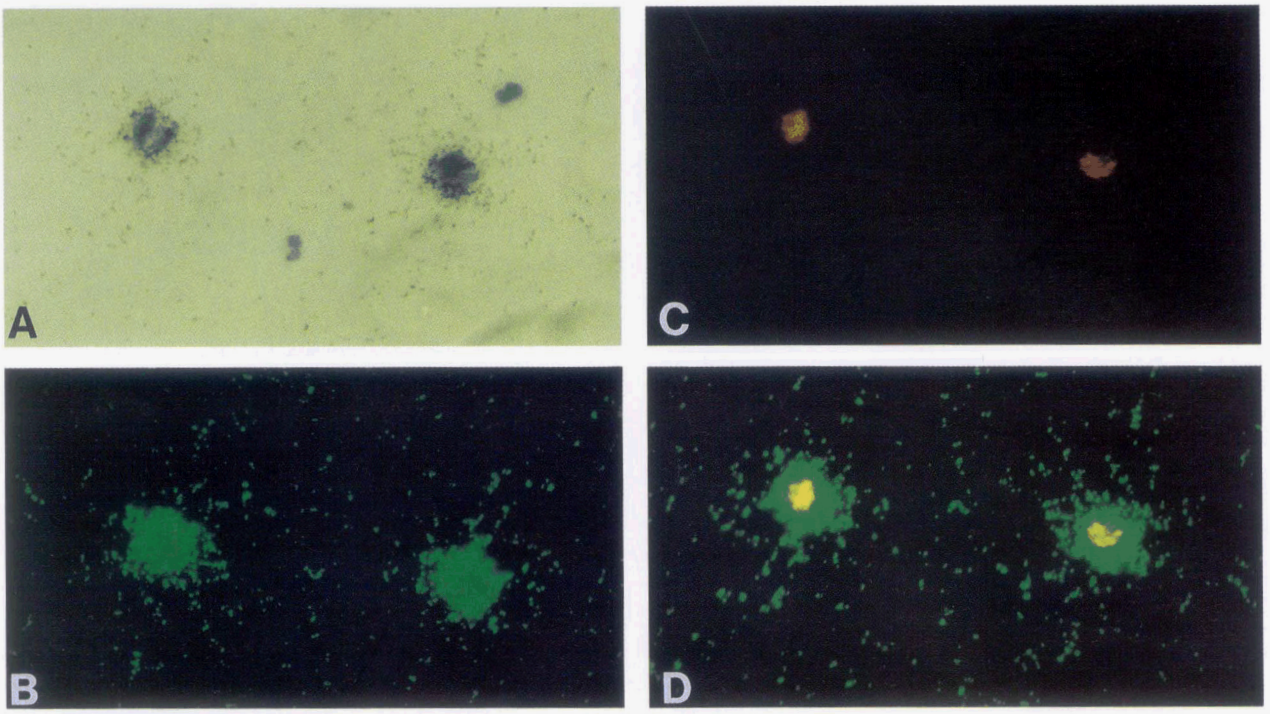

Fig 2. Localization of cellular sources of TGF-β_1 mRNA in the leukocyte preparations from patients with blood eosinophilia. The leukocyte preparation of HES-1 was used. (A to D) were hybridized to a ^{35}S-labeled antisense hTGF-β_1 riboprobe. Original magnification × 500. (A) Bright-field visualization. (B) Dark-field visualization using a green filter to highlight the autoradiographic grains. (C) Fluorescence visualization using rhodamine filter showing fluorescence in the eosinophils. (D) Double-exposure visualization, first with dark-field followed by rhodamine fluorescence. The section was stained with Giemsa (Fisher SG-28). Autoradiographic exposure time was for 48 hours at 4°C.

DISCUSSION

Little is known about the capacity of human eosinophils to elaborate cytokines. We recently established that eosinophils can produce TGF-α,[20,21] and we have now shown that human eosinophils can express the cytokine TGF-β_1. All eosinophils examined from four patients with blood eosinophilia were found to label for both TGF-β_1 mRNA and protein by in situ hybridization and immunohistochemistry, respectively. RNA isolated from a purified eosinophil preparation from a patient with HES was found to contain the 2.5-kb TGF-β_1 transcript. Thus, eosinophils from patients with blood eosinophilia can express TGF-β_1.

In contrast to the uniform detection of TGF-β_1 mRNA and protein found with eosinophils from four patients with eosinophilia, eosinophils from two of the four normal donors showed only weak labeling for TGF-β_1 mRNA by in situ hybridization and none of the blood-derived eosinophils from the normal donors had detectable TGF-β_1 protein. It should be noted that not all of these eosinophils labeled for TGF-β_1 mRNA and that levels of TGF-β_1 mRNA in those eosinophils that were positive by in situ hybridization were much lower than those seen in eosinophils from patients with blood eosinophilia. Several reasons might account for the detection of TGF-β_1 mRNA in a fraction of the eosinophils (~50% and 80%, respectively) in two of the four normal donors when these cells lacked immunohistochemically demonstrable TGF-β_1 protein. First, the TGF-β_1 mRNA in these eosinophils might not be translated into protein. Second, the translated TGF-β_1 protein might not have accumulated sufficiently in these eosinophils to permit successful immunodetection. This could be due to lower levels of synthesis, rapid degradation, and/or secretion.[7] Finally, the uniform expression of TGF-β_1 found in eosinophils from the eosinophilic patients raises the possibility that stimuli associated with the eosinophilic responses were acting to induce TGF-β_1 expression in these eosinophils in vivo.

Besides eosinophils, we consistently detected TGF-β_1 product in other leukocytes from both normal and eosinophilic donors. This result is not surprising because TGF-β_1 is produced by a number of hematopoietic cell types, including cultured T cells,[12] macrophages,[13] monocytes,[13,14] megakaryocytes,[15] and neutrophils.[14] Our leukocyte preparations contained predominantly neutrophils, eosinophils, and a small percentage of mononuclear cells. Morphologically identifiable neutrophils from both normal and eosinophilic donors were consistently immunoreactive for TGF-β_1, serving as a good positive control. However, no leukocytes other than eosinophils were found to contain TGF-β_1 mRNA by in situ hybridization.

The discovery that human eosinophils can elaborate TGF-β_1 may provide new insights into the pathogenesis of some of the striking alterations of connective tissues that have been associated with a diversity of eosinophilic diseases.[19] For example, patients with allergic asthma often exhibited a marked increase in bronchial subepithelial

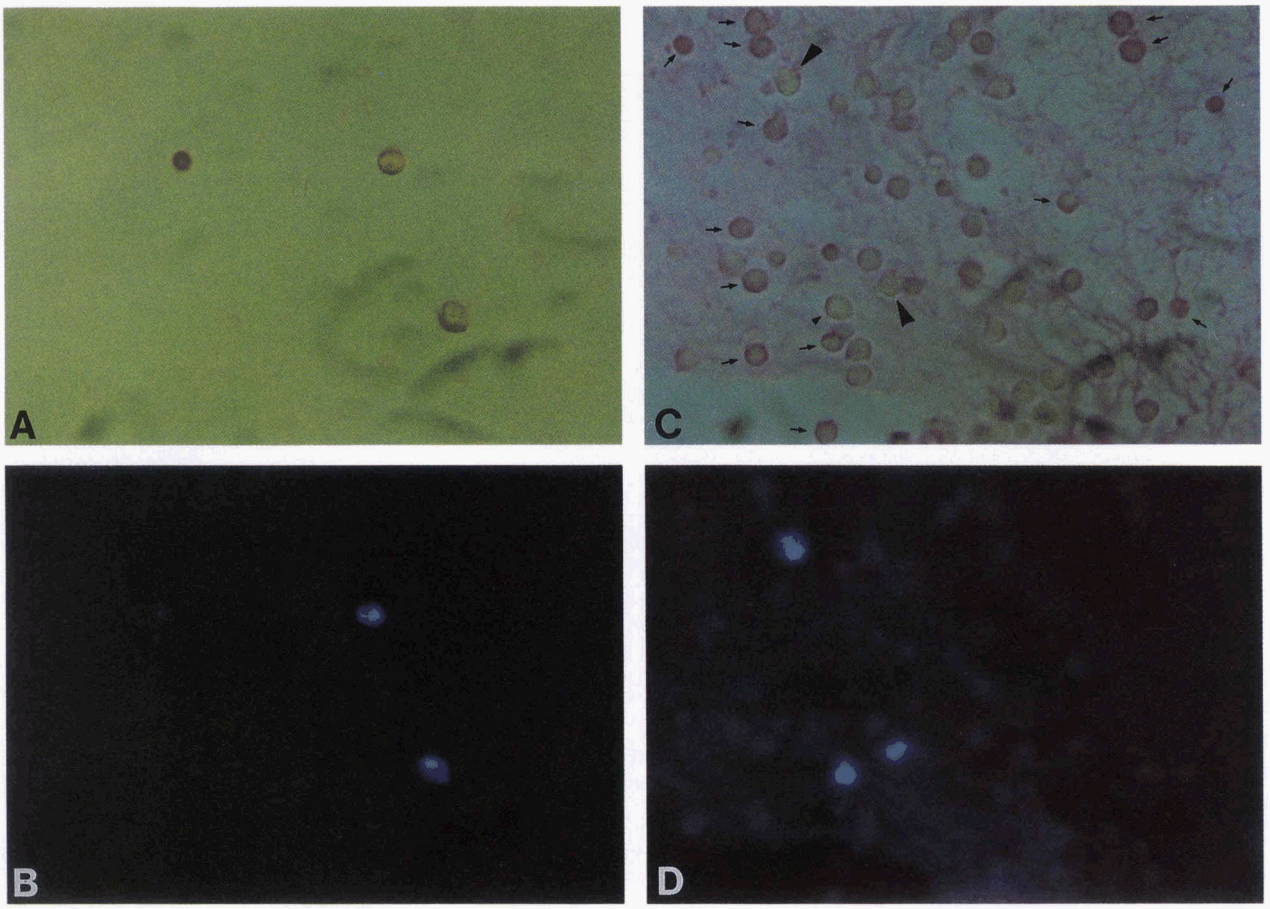

Fig 3. Immunohistochemical detection of TGF-β_1 protein in human PB leukocytes. (A and B) Patient HES-1; (C and D) normal donor NL-3. (A and C) Bright-field examination. (B and D) Visualization of aniline-blue fluorescence of eosinophils by fluorescent microscopy at 365 nm with DAPI filter. Original magnification × 500. (C) Large arrowheads, aniline-blue fluorescent eosinophils negative for TGF-β_1 protein; small arrowhead, an aniline-blue fluorescent eosinophil exhibiting an equivocal reaction in one area of the cytoplasm; arrows, other leukocytes detectable for TGF-β_1 protein.

collagen[29] and patients with onchocerciasis exhibited striking dermal fibrosis; in both instances, the pathology is associated with eosinophil infiltration. In the idiopathic HES, eosinophil infiltration is associated with significant endomyocardial fibrosis.[19] Finally, it has been shown that human eosinophils secret fibroblast stimulatory activity in culture.[30] An eosinophil-derived fibroblast growth-stimulatory factor of molecular weight 30 to 50 Kd has been noted.[31] Mature TGF-β_1, which is processed from larger precursors, and which is a potent growth stimulator of cultured fibroblasts, has a molecular weight of 25 Kd.[7] In the context of these reports, our finding that the eosinophils of patients with blood eosinophilia can express TGF-β_1 permits us to speculate that eosinophil-derived TGF-β_1 may contribute to the development of fibrosis seen in eosinophil-associated lesions.

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