Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9660–9665, August 1999
Cell Biology

Human eosinophils regulate human lung- and skin-derived fibroblast properties *in vitro*: A role for transforming growth factor β (TGF-β)

FRANCESCA LEVI-SCHAFFER*†, EKATERINA GARBUZENKO*, ANN RUBIN‡, REUVEN REICH*, DALIA PICKHOLZ*, PHILIPPE GILLERY‡, HERVE EMONARD‡, ARNON NAGLER§, AND FRANCOIS A. XAVIER MAQUART‡

*Department of Pharmacology, School of Pharmacy, Hebrew University–Hadassah Medical School, POB 12065, Jerusalem 91120, Israel; §Department of Bone Marrow Transplantation, Hadassah University Hospital, Jerusalem 91120, Israel; and ‡Laboratory of Biochemistry, Centre National de la Recherche Scientifique 6021, Faculty of Medicine, IFR53 Biomolecules, Reims 51095, France

Communicated by Rita Levi-Montalcini, Institute of Neurobiology C.N.R., Rome, Italy, June 14, 1999 (received for review April 2, 1999)

ABSTRACT **Eosinophils have been associated with fibrosis. To investigate their direct role in fibrosis, human peripheral blood eosinophil sonicate was added to human lung or dermal fibroblasts, and proliferation ([^{3}H]thymidine) and collagen synthesis ([^{3}H]proline) were evaluated. Proliferation was enhanced significantly in the monolayers in a dose-dependent manner. The activity of the eosinophil fibrogenic factor(s) remained unaltered when heated (56°C, 30 min). Supernatants of cultured eosinophils (20 min or 18 hr) also enhanced lung fibroblast proliferation, indicating that the preformed mitogenic factor(s) can be released both promptly and with a long kinetic. Eosinophils significantly decreased collagen production in lung fibroblasts while increasing it in dermal fibroblasts. However, eosinophils containing matrix metalloproteinase 9 (zymography) in latent form and tissue inhibitors of metalloproteinases 1 and 2 (reverse zymography) did not influence either fibroblast matrix metalloproteinases or tissue inhibitors of metalloproteinases. Eosinophil sonicate added to skin and lung fibroblasts in tridimensional collagen lattices significantly enhanced lattice contraction. Transforming growth factor β (TGF-β) is a major fibrogenic cytokine produced by eosinophils. Therefore, to assess its role, eosinophil sonicate was preincubated with anti-TGF-β neutralizing antibodies. This treatment partially inhibited proliferation of lung and collagen synthesis of dermal fibroblasts and suppressed the stimulation of lattice contraction, indicating the fibrogenic role of eosinophil-associated TGF-β. In conclusion, we have shown that eosinophils act as direct modulatory cells in fibroblast proliferation, collagen synthesis, and lattice contraction, in part, through TGF-β. These data corroborate the importance of eosinophils in skin and lung fibrosis.**

The relationship between inflammatory cells and fibroblasts in areas of repair and early fibrosis has been observed for some time. Recently, attention has focused on the possibility that inflammatory cells can regulate fibroblast functions and *vice versa*, especially after the discovery that both these cell types are producers of pleiotropic cytokines (1–3). In addition, current theories implicate inflammation as the driving force that leads to fibrosis independently of the etiopathology of the specific disease.

Eosinophils are blood granulocytes that infiltrate the tissues in various pathological situations, such as allergic inflammation, parasitic infections, and neoplastic diseases (2). Eosinophils long have been associated with fibrotic conditions of different etiopathology, including endomyocardial fibrosis, scleroderma and scleroderma like-conditions, idiopathic pulmonary and retroperitoneal fibrosis, asbestos-induced lung fibrosis, wound repair, and tissue remodeling (4). More recently, eosinophils have been implicated both in the damaging of epithelial cells and in fibrosis of the subepithelium in chronic asthma (5, 6). Eosinophils possess prominent cytoplasmic granules that, among noneosinophil-specific mediators, contain characteristically preformed basic proteins such as eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), major basic protein (MBP), and eosinophil cationic protein (ECP) (2, 4). In addition, their secretory granules contain preformed cytokines such as granulocyte–macrophage colony-stimulating factor, IL-2, IL-6, IL-4, IL-5, transforming growth factor β (TGF-β), and tumor necrosis factor α (TNF-α) (1, 2).

Some of the eosinophil mediators such as TGF-β, IL-4, IL-6, TNF-α, ECP, and MBP can be fibrogenic (7–13), whereas others such as IL-2, IFN-γ, and collagenase have antifibrotic properties (14–16). Consequently, it is difficult to predict the role of eosinophils in fibrosis. Moreover, the functional heterogeneity of the fibroblast population in different anatomical locations and even from the same tissue must be considered as well (17).

Fibrosis is characterized by an excessive deposition of connective tissue, the hallmarks of which are fibroblast proliferation and/or enhanced synthesis of extracellular matrix components, mainly collagen (18). The degradation of extracellular matrix also might decrease in fibrosis (19), suggesting an alteration of matrix metalloproteinase (MMP) or tissue inhibitor of metalloproteinase (TIMP) expression or regulation. Fibroblasts from fibrotic areas also exert increased contraction forces on collagen lattices (20).

Only three studies dealt with the effects of eosinophils on fibroblast properties. They indicated that human eosinophils stimulate DNA synthesis and matrix production of dermal fibroblasts (21, 22) and that guinea pig peritoneal eosinophils enhance the replication of fetal lung fibroblasts (23).

To study the role of eosinophils in fibrotic processes that can take place in the skin and lung, we adopted the following *in vitro* approach. Human peripheral blood eosinophil sonicate was added to human lung and skin fibroblasts. Fibroblast proliferation and collagen synthesis, MMP and TIMP expression and activation, and tridimensional lattice contraction were evaluated. TGF-β has potent fibrogenic effects (5–8), and its secretion by eosinophils, a rich source of this cytokine (1, 2, 5, 6, 24–28), into the circulation or at sites of injury might play an important role in the development of fibrosis. Therefore, we specifically investigated the role of TGF-β in the eosinophil

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: MBP, major basic protein; ECP, eosinophil cationic protein; TGF-β, transforming growth factor β; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

†To whom reprint requests should be addressed. E-mail: fls@cc.huji.ac.il.

fibrogenic effects. Our findings suggest that eosinophils can play a direct modulatory role in lung and skin fibrosis and, therefore, are active contributors to the etiopathogenesis of eosinophil-associated fibrotic diseases.

MATERIALS AND METHODS

The following materials were obtained as follows: DMEM, L-glutamine, streptomycin, penicillin, FCS, and Hanks' balanced salt solution were obtained from Biological Industries, Beit Haemek, Israel; trichloroacetic acid was from Merck; ascorbic acid, β-aminopropionitrile, collagenase, *N*-ethylmaleimide, low- and high-molecular-weight markers and brij 35 were from Sigma; purified human TGF-β1 and mouse anti-human TGF-β_1-β_2-β_3 mAbs were from Genzyme Diagnostics; mouse anti-human CD3 mAb were from Ortho Pharmaceuticals; IgG-purified fraction from rabbit anti-human TGF-β_1-β_2-β_3 serum and nonimmune rabbit IgG were from Sigma; SDS and Triton X-100 were from BDH; Temed and Coomassie G 250 brilliant blue were from Bio-Rad; ^{3}H-labeled proline and thymidine were from DuPont/NEN; control MMPs were from Valbiotech, Paris; and TIMP-1 was from Calbiochem. TIMP-2 was a generous gift of H. Nagase (Kansas University, Kansas City). Other reagents and chemicals were obtained from Frutarom Chemicals (Haifa, Israel) or Prolabo (Paris) and were of analytical grade or of the best grade available. Sterile tissue culture plasticware was obtained from Falcon and Nunc.

Eosinophil Purification. Eosinophils were purified from the human peripheral blood of 11 mildly atopic volunteers (eosinophilia, <10%), not taking any oral treatment for their condition, by the MACS method as described previously (29). Written informed consent was obtained from the volunteers according to guidelines established by the Hadassah–Hebrew University Human Experimentation Helsinki Committee. Eosinophils (purity, 98–100%, Kimura staining; viability, 99%, Trypan blue staining) were resuspended in DMEM.

Preparation of Eosinophil Sonicate and Supernatants. Eosinophil sonicate was obtained by bath sonication (1 min, 0°C; Heat Systems/Ultrasonics W 380, duty cycle 5 s; output power, 50%) of a cell suspension containing 10^7 eosinophils/ml DMEM. Sonicate then was microcentrifuged for 5 min at 4°C, and debris-free sonicate supernatant ("sonicate") was collected into aliquots and stored at −80°C. For supernatant preparation, eosinophils (5×10^5/ml) were cultured in DMEM with 2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin (supplemented DMEM), containing 2% heat-inactivated FCS, in 24-well tissue culture plates, for 20 min or 18 hr (37°C, 5% CO_2). The cells then were centrifuged (5 min, 120 × *g*) and supernatants were collected and stored in aliquots at −80°C.

In heat-stability experiments, eosinophil sonicate was heated to 56°C for 30 min.

Fibroblasts. Human foreskin fibroblasts (HSG8, CRL 1635) and human fetal lung fibroblasts (FHS 738, HTB-157) were obtained from American Type Culture Collection (Manassas, VA) and cultured in supplemented DMEM/10% FCS.

Adult dermal fibroblasts were obtained from biopsies of normal skin from informed-consent volunteers who underwent minor skin surgery. The biopsies were put as explants, and fibroblasts were obtained and cultured as described (30). Because no differences were found in the foreskin and adult skin fibroblasts, the studies were carried out with either one.

Fibroblast Proliferation. Proliferation of subconfluent fibroblasts was assessed by using the [^{3}H]thymidine-incorporation assay. Fibroblasts were seeded in 96-well plates ($6–8 \times 10^3$/well) in 200 μl of supplemented DMEM/10% FCS. After overnight, wells were washed twice with supplemented DMEM/2% FCS. Eosinophil sonicate (10^3–10^6 cells/200 μl) or supernatant (0.5×10^6 cells/200 μl) was added for 24 hr. [^{3}H]Thymidine was added as a final 24-hr pulse (1 μCi/well), and cells were washed (PBS), fixed with methanol, precipitated (5% TCA), lysed (0.1% NaOH), transferred to scintillation vials, and counted in a beta-counter (LKB 1211 Rackbeta).

In a few experiments, fibroblast proliferation was evaluated by counting the fibroblasts, after trypsinization of the wells, in a hemocytometer. Data from [^{3}H]thymidine incorporation assay are expressed either as cpm/well or percentage of increase of proliferation after the addition of eosinophil products compared with medium alone.

Collagen Production. Collagen production of confluent fibroblast monolayers was assessed by [^{3}H]proline incorporation into collagenous proteins (31). Fibroblasts were seeded in 24-well tissue culture plates (10^4/well) in 1 ml of supplemented DMEM/10% FCS until confluence. Eosinophil sonicate (3×10^4–3×10^6 cells per well) in 0.5 ml of DMEM, containing 5% FCS/50 μg/ml β-aminopropionitrile/50 μg/ml ascorbic acid (modified DMEM) was added, and the cultures were incubated for 24 hr. Then [^{3}H]proline (10 μCi/well) was added for 24 hr and samples were processed as described previously (31). Data are expressed either as cpm of [^{3}H]proline incorporation in 10^4 fibroblasts or as percent increase or decrease of collagen production in cultures incubated with eosinophil sonicate in comparison with cultures incubated with medium alone.

Tridimensional Collagen Lattice Contraction. Tridimensional lattice cultures were performed in collagen matrices as described (32). Briefly, type I collagen from rat tail tendon (2 mg/ml in 18 mM acetic acid) was added to 35-mm dishes containing DMEM medium with 10% FCS and 0.1 M NaOH. Fibroblasts (10^5) were added just after collagen solution, alone or with eosinophil sonicate, before fibrillation and lattice formation. Lattice diameter was measured every day by placing the dishes on a graduated rule placed on a black surface. Cells were counted with a Malassez device after lattice digestion by clostridial collagenase (2 mg/ml in PBS containing 2.5 mM $CaCl_2$ at 37°C).

Gelatin Zymography and Reverse Zymography. SDS-substrate/PAGE was performed as described (33). Gels for zymography comprised 0.1% gelatin and 10% polyacrylamide and, for reverse zymography, comprised 0.1% gelatin, 15% polyacrylamide, and 20 ng/ml purified, activated MMP-2. Revealed inhibitory activity, which appeared as blue zones, demonstrating inhibition of lysis of gelatinase activity, was measured by automated image analysis. Linear range of enzymatic activities was assessed by using purified MMP-2 as standard. Linearity was between 10 and 200 pg of enzyme.

Addition of TGF-β1 and of Neutralizing Anti-TGF-β Antibodies to Fibroblast Cultures. TGF-β1 (2 and 20 ng/ml) was added to fibroblast cultures, and its effects on proliferation and collagen synthesis were evaluated as described. Eosinophil Sonicate was preincubated with anti-TGF-β mAbs (0.3, 3, and 30 μg/ml) or isotype control mAbs (anti-human CD3) for 30 min at 37°C before addition of the fibroblasts to evaluate their blocking effect on fibroblast proliferation and collagen synthesis. Data are expressed as percentage of inhibition. In lattice-contraction experiments, the neutralizing antibodies were rabbit anti-human (50 μg/ml) and the control antibodies were nonimmune rabbit IgG.

Statistical Analysis. The experiments were carried out in triplicate or quadruplicate. Data are expressed as mean ± SEM. Statistical analysis was performed by Student's paired *t* test with *P* values of <0.05 being considered significant.

RESULTS

Effect of Eosinophil Sonicate on Fibroblast Proliferation. To evaluate the effect of eosinophils on fibroblast proliferation, human lung subconfluent fibroblast monolayers were incubated with increasing concentrations of human peripheral blood eosinophil sonicate (10^3–10^6/well). Proliferative re-

sponse was evaluated both by [H^3]thymidine incorporation and by fibroblast counting. In both cases eosinophil sonicate caused a concentration-dependent increase in fibroblast proliferation, which started to be significant at 10^4 eosinophils per well (30%, $P < 0.05$, and 20%, $P < 0.02$, respectively, $n = 3$). Maximal increase was observed after the addition of 1×10^6 eosinophils per well. The increase in fibroblast number in this case was 54% ($P < 0.001$), and the increase of [^{3}H]thymidine incorporation was 144% ($P < 0.02$).

In subsequent experiments the effects of increasing concentrations of eosinophil sonicate (10^3–10^6/well) on human dermal fibroblast proliferation were evaluated by [^{3}H]thymidine-incorporation assay.

In this case, eosinophils induced a concentration-dependent increase in proliferation starting at 10^4 eosinophils per well (33%, $P < 0.05$, $n = 3$) and showing a maximal increase (182%, $P < 0.008$) at the highest sonicate concentration (10^6 eosinophils per well). Interestingly, eosinophil sonicate induced a comparable concentration-dependent increase of proliferation on mouse embryonic 3T3 fibroblasts (not shown). To determine whether the effect of the eosinophil sonicate is specific, increasing concentrations of human skin fibroblasts sonicate (10^3–10^6) were added to dermal fibroblast monolayers. None of these sonicate concentrations influenced fibroblast proliferation. In fact, even the highest concentration of sonicate (10^6 cells) induced [^{3}H]thymidine incorporation similar to that observed in fibroblasts incubated with culture medium alone (435 ± 38 vs. 383 ± 57 cpm/well). Next, to evaluate heat stability of the mitogenic mediator(s), heated (56°C) eosinophil sonicate was added to lung or dermal fibroblasts. Under this condition, fibroblast proliferation did not differ from that obtained after addition of untreated eosinophil sonicate. In fact, lung fibroblasts incorporated 807 ± 81 cpm [^{3}H]thymidine when incubated with untreated sonicate vs. 835 ± 35 cpm when incubated with heat-treated sonicate ($n = 3$). In dermal fibroblasts, incorporation was 1,629 ± 335 cpm with heat treated vs. 1,378 ± 95 with untreated sonicate ($n = 2$). This indicates that the mitogenic mediator(s) is heat-stable.

Effect of Eosinophil Supernatants on Fibroblast Proliferation. From the previous experiments, we concluded that eosinophil mediator(s) contained in the eosinophil sonicate enhance fibroblast proliferation. To evaluate whether eosinophils also could release this mediator(s), supernatants of eosinophils cultured in medium for either 20 min or 18 hr were added to lung fibroblasts. Eosinophil supernatant obtained after 20 min of incubation induced a significant increment in lung fibroblast proliferation (860 ± 161 cpm, $n = 7$, $P < 0.05$) compared with medium alone (403 ± 50 cpm). This mitogenic response was similar to the one obtained by the addition of sonicate from freshly isolated eosinophils (1,083 ± 85 cpm, $n = 7$, $P < 0.0001$), indicating that the preformed mediator(s) responsible for this activity is released rapidly. In addition, the eosinophil supernatants recovered after 18 hr also stimulated lung fibroblast proliferation (650 ± 125 cpm vs. 403 ± 50 cpm, $n = 7$, $P < 0.05$).

Effect of Eosinophil Sonicate on Fibroblast Collagen Synthesis. As shown in Fig. 1*A*, addition of the eosinophil sonicate at the highest concentration (3×10^6/well) decreased significantly collagen production of lung fibroblasts (50%, $P < 0.03$), whereas no significant effect was observed at lower sonicate concentrations. In contrast, dermal fibroblasts incubated with eosinophil sonicate responded with an increase in collagen production. This increase was highly significant at the highest sonicate concentration (73% increase, $P < 0.007$; Fig. 1*B*).

Effect of Eosinophil Sonicate on Fibroblast MMP and TIMP Activities. Eosinophil sonicate was found by zymography analysis to contain pro-MMP-9 and a high-molecular-weight band that may correspond to a complex with some inhibitors (Fig. 2*I*). Lung fibroblasts displayed pro-MMP-9, pro-MMP-2, and MMP-2 activities (Fig. 2*II*, lane B). Dermal

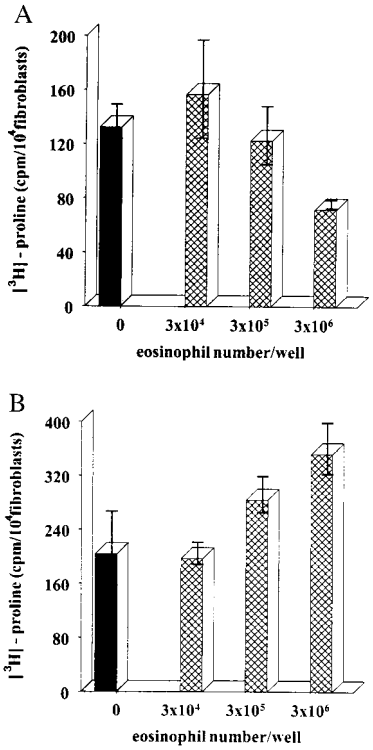

FIG. 1. Different effects of eosinophils on collagen synthesis in human lung and dermal fibroblasts. Confluent monolayers of lung (*A*) and dermal (*B*) fibroblasts were incubated with different concentrations of eosinophil sonicate for 24 hr, and collagen production was evaluated by [^{3}H]proline incorporation. Data are the mean ± SEM of three experiments performed in triplicate or quadruplicate.

fibroblasts displayed pro-MMP-2 and MMP-2 activities (Fig. 2*II*, lane D). Addition of eosinophil sonicate did not influence either lung (lane C) or dermal (lane E) gelatinase activities or pattern. Eosinophil sonicate also was shown by reverse zymography to contain TIMP-1 and TIMP-2 (Fig. 3). Its addition to fibroblast cultures did not alter TIMP expression by the cells.

Effect of Eosinophils on Collagen Lattice Contraction. Addition of increasing concentrations of eosinophil sonicate (10–10^5 per lattice) to dermal and lung fibroblasts embedded in collagen lattice enhanced significantly their contraction (1.25- to 1.4-fold, $P < 0.05$). This was particularly visible on days 1 and 2 and was evident even at a concentration as low as 10 eosinophils per lattice (not shown). Interestingly, eosinophil presence in the lattice did not increase fibroblast proliferation. In fact, the number of fibroblasts in the medium-incubated lattices and in the lattices cultured with eosinophil sonicate was comparable on the first day and last days of the experiment (90,830 ± 3,000 vs. 88,700 ± 2,450 for lung; 85,500 ± 2,500 vs. 83,750 ± 1,250 for dermal fibroblasts, respectively, $n = 4$).

The Role of TGF-β in the Fibrogenic Responses of Eosinophils. TGF-β is a fibrogenic cytokine (7, 8) and is heat-stable (34). TGF-β1 has been shown to be expressed by human eosinophils (1, 2, 5, 6, 24–28) and is the isoform particularly involved in fibrosis development (35). Therefore, dermal and lung fibroblasts were incubated with TGF-β1 and their proliferation and collagen synthesis were evaluated. At the two concentrations tested (2 ng/ml and 20 ng/ml), TGF-β1 increased the proliferation of lung fibroblasts significantly (50 and 74% increase, $P < 0.0001$ and $P < 0.02$, respectively, $n = 3$) but had no significant effect on dermal fibroblast proliferation (10 and 20% increase respectively, $P < 0.07$). Collagen production was increased significantly by TGF-β1 (2 ng/ml and 20 ng/ml) in both the fibroblasts: 27 and 129%, $P < 0.003$

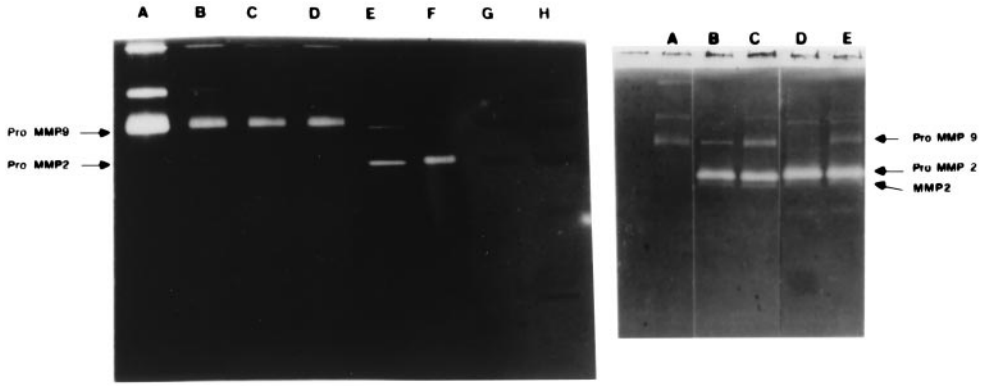

FIG. 2. Eosinophils contain MMPs but do not affect human lung or dermal fibroblast MMP production. Eosinophil sonicate was added to wells without fibroblasts (*I*) or to fibroblast cultures (*II*) at concentrations of 3×10^6 cells per well in DMEM containing 0.1% BSA for 48 hr. The culture supernatants were collected and analyzed by gelatin zymography. (*I*) Eosinophil sonicate obtained from eosinophils of four different patients was analyzed (lanes A–D). As controls on the same gel, the supernatant of HT1080 cells (lane E) and commercially available pro-MMP-2 were analyzed (lane F). Molecular weight (MW) standards of high-MW range (lane G) and low-MW range (lane H) also were run. (*II*) Eosinophil sonicate of a fifth patient (lane A), lung fibroblasts (lane B), or dermal fibroblasts (lane D) was incubated alone or eosinophil sonicate was added to either lung fibroblasts (lane C) or dermal fibroblasts (lane E).

and $P < 0.04$ for lung; 732 and 1,475%, $P < 0.007$ and $P < 0.03$ for dermal fibroblasts, respectively.

Recently, human nasal polyp eosinophils have been shown to express the three TGF-β isoforms (36, 37). Consequently, to assess the role of TGF-β in our systems, eosinophil sonicate was preincubated with neutralizing antibodies to TGF-β1,2,3 and added to lung fibroblasts. As shown in Table 1, preincubation of eosinophil sonicate with anti-TGF-β antibodies reduced their proliferative potential significantly, with variations between the different eosinophil donors. At concentrations of 0.3, 3, and 30 μg/ml, the antibodies decreased eosinophil-induced proliferation by 35% ($P < 0.005$), 30% ($P < 0.05$), and 25% ($P < 0.03$), respectively.

To evaluate the contribution of TGF-β in enhancement of collagen synthesis, dermal fibroblasts were incubated with the eosinophil sonicate from four different donors, preincubated with an optimal concentration of anti-TGF-β antibodies (3 μg/ml). Also in this case, the presence of anti-TGF-β antibodies decreased from 18 to 68% ($P < 0.05$, $n = 3$) the enhancing effect of the eosinophils on collagen production (not shown). The proliferation and collagen-stimulating effects of the eosinophil sonicate incubated with the control antibody anti-CD3 remained unaltered. The role of TGF-β in eosinophil-induced dermal and lung fibroblast lattice contraction was evaluated because it is well established that TGF-β induces tridimensional gel retraction (38). Eosinophil sonicate preincubated with neutralizing antibodies to TGF-β was added together with the dermal fibroblast to collagen lattices (Fig. 4). Anti-TGF-β antibodies totally suppressed the increase of lattice contraction induced by the eosinophil sonicate.

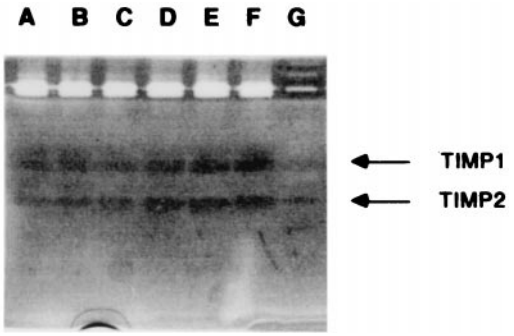

FIG. 3. Eosinophils contain TIMP-1 and TIMP-2 but do not stimulate TIMP production by dermal fibroblasts. Eosinophil sonicate (lanes: A, 0; B, 10; C, 100; D, 1,000; E, 10,000; or F, 100,000 eosinophils) was added to the lattices containing 100,000 dermal fibroblasts in DMEM containing 10% FCS. On day 7, culture medium was changed in DMEM containing 0.1% BSA and the lattices were incubated further for 48 hr. Culture medium then was collected and analyzed by reverse zymography. Eosinophil sonicate (100,000) was analyzed on the same gel (lane G). The migration positions of control TIMP-1 and TIMP-2 are indicated.

DISCUSSION

In this paper we have shown that human eosinophils have the capacity to directly affect human lung and dermal fibroblast proliferation, collagen synthesis, and lattice contraction. A large part of these effects are mediated by eosinophil-associated TGF-β.

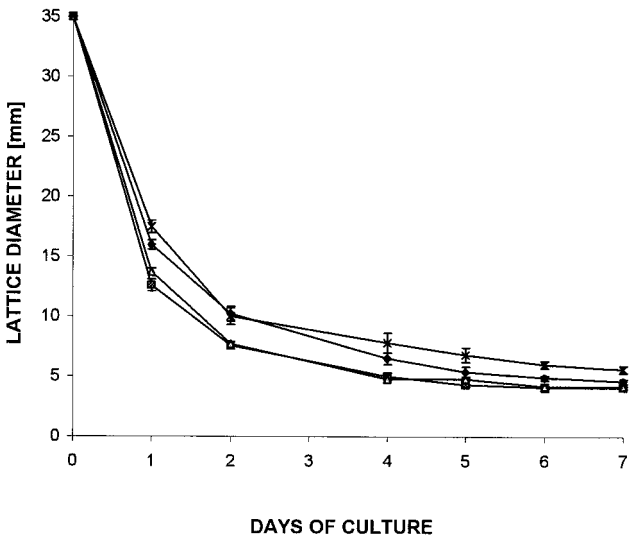

FIG. 4. Anti-TGF-β antibodies suppress the stimulation of collagen lattice contraction induced by the eosinophil. Dermal fibroblasts were seeded in collagen lattices (100,000 cells per lattice) and incubated in DMEM + 0.5% FCS alone (solid lozenges) or supplemented with the eosinophil sonicate (10 eosinophils per lattice, ■) or with the eosinophil sonicate preincubated with rabbit anti-TGF-β antibodies (crosses). Open triangles are controls consisting of the eosinophil sonicate preincubated with nonimmune rabbit IgG. Each set of data is the mean of four experiments. SDs were less than 10% of the mean value in every case and were not reported as error bars on the graph for better clarity.

Table 1. Inhibitory effect of anti-TGF-β antibodies on eosinophil-mediated lung fibroblast proliferation

Patient	Culture medium, cpm/well*	Eosinophil sonicate, cpm/well	Eosinophil sonicate + anti-TGF-β (0.3 μg/ml) cpm/well (%)†	Eosinophil sonicate + anti-TGF-β (3 μg/ml) cpm/well (%)	Eosinophil sonicate + anti-TGF-β (30 μg/ml) cpm/well (%)
1	648.6 ± 86.5	2,686.0 ± 85.4	2,049.0 ± 212.4 (18.2)	2,214.6 ± 131.4 (13.8)	1,931.0 ± 624.2 (22.5)
2	886.3 ± 30.5	3,297.0 ± 653.7	1,913.7 ± 332.5 (44.7)	1,728.0 ± 216.7 (12.0)	3,168 ± 290.9 (6.3)
3	504.3 ± 60.0	2,810.0 ± 187.3	2,258.0 ± 158.9 (20.5)	1,090.6 ± 257.5 (74.0)	1,877.0 ± 465.8 (38.2)
4	807.5 ± 51.9	3,383.0 ± 177.8	2,684.0 ± 187.8 (28.9)	3,076.6 ± 284.3 (16.7)	2,607.0 ± 196.4 (36.4)
5	610.7 ± 60.4	2,118.0 ± 502.7	1,225.7 ± 131.9 (61.7)	1,501.3 ± 156.7 (33.7)	1,915.0 ± 104.3 (18.7)

Fibroblast proliferation was assessed by [^{3}H]thymidine incorporation.
*Data are the mean ± SEM of three experiments performed in triplicate.
†Percent inhibition as: eosinophil sonicate + anti-TGF-β/eosinophil sonicate.

It has been recognized increasingly that eosinophils are involved in physiologic and pathologic connective tissue deposition and turnover. The association between eosinophils and pathologic fibrosis is supported by numerous observations. For example, eosinophil infiltration of affected tissues and the presence of ECP or MBP is prominent in diffuse or localized fibrotic conditions such as L-tryptophan-associated eosinophilia-myalgia syndrome (39), nodular sclerosing Hodgkin's disease (40), systemic sclerosis (41), pulmonary fibrosis (42), and severe asthma (5, 6).

However, the precise role of eosinophils in fibrosis has not been clarified. In fact, increased numbers of eosinophils or eosinophil-derived products in the fibrotic tissues can be circumstantial or just indicative of a role for these cells in causing or modulating other pathological changes.

In this study, we investigated the unique and direct contribution of the eosinophils to tissue remodeling. Therefore, we adopted an *in vitro* system in which human eosinophil sonicate was incubated with human lung and dermal fibroblasts, and some hallmarks of fibrosis, namely, fibroblast proliferation, collagen synthesis, and collagen lattice contraction, were evaluated. In addition, the presence of MMPs and TIMPs in eosinophils and their effects on fibroblast MMPs and TIMPs also were assessed. Eosinophil sonicate caused a dose-dependent increase in proliferation in the tested fibroblasts, similar to results reported previously (22, 23). Based on our findings, the question was raised whether the mediator(s) that caused enhancement of fibroblast proliferation could be released and, if so, what the kinetics of the release were. We found that the factors involved are both preformed, rapidly released, as demonstrated also by Pincus *et al.* (21), and released at longer time points.

Collagen production also was influenced by the eosinophil sonicate. Interestingly, whereas dermal fibroblasts produced significantly more collagen when incubated with eosinophil sonicate as reported previously with eosinophils supernatants (22), lung fibroblasts decreased significantly their collagen synthesis. The different response of dermal and lung fibroblasts to eosinophils is very important because it supports the notion of fibroblast heterogeneity and tissue-specific fibroblasts (17). It also may indicate the diverse role of eosinophils in fibrosis development in various anatomical locations and tissues.

We found that eosinophils enhanced retraction of both dermal and lung fibroblasts cultured in tridimensional collagen gels. Interestingly, the enhanced retraction capacity is a characteristic feature of fibrotic tissue fibroblasts (32). In lattices, eosinophils did not increase fibroblast proliferation as they did in monolayers. It is well established that fibroblasts respond poorly to mitotic factors when seeded in collagen lattices.

It has been shown that eosinophils infiltrating fibrotic lesions (25, 28) and circulating eosinophils (26) express elevated levels of TGF-β1 mRNA. In addition, eosinophils in chronically inflamed tissue of upper airway and in airway fibrosis in chronic asthma express TGF-β1 mRNA and immunoreactivity (5, 6, 27).

TGF-β is a potent fibrogenic cytokine that stimulates extracellular matrix formation, inhibits its degradation, and acts as a chemoattractant for fibroblasts (7, 8, 43). There are three principal isoforms of TGF-β, namely, TGFβ1, β2, and β3, with similar biologic activities (44, 45). TGF-β1 seems to be the main isoform involved in fibrosis. For example, Coker *et al.* (35) demonstrated that although TGF-β isoform gene expression is controlled in a different way during experimental pulmonary fibrosis, TGF-β1 is the predominant isoform expressed during fibrosis development whereas the expression of the two other isoforms essentially is unchanged. Therefore, to investigate the role of TGF-β in our system we incubated lung and dermal fibroblasts with TGF-β1. This cytokine enhanced both the proliferation and collagen synthesis of the fibroblasts. As mentioned earlier, human eosinophils in nasal polyps have been shown to express the three TGF-β isoforms (36, 37). Accordingly, to evaluate the role of TGF-β in the eosinophil effects and accounting for possible isoform-specific differences in both target-tissue responsiveness and mode of action (45), we preincubated the eosinophil sonicate with anti-TGF-β neutralizing antibodies against the three isoforms. Eosinophil-mediated stimulation of lung fibroblast proliferation and dermal fibroblast collagen synthesis was blocked partially by the addition of anti-TGF-β antibodies. Anti-TGF-β completely suppressed the stimulation of collagen lattice contraction induced by eosinophil sonicate. Altogether, these data indicate a major role for TGF-β in the eosinophil fibrogenic effect. However, because some of the eosinophil fibrogenic activity could not be blocked by anti-TGF-β antibodies, it is probable that other preformed mediators along with TGF-β are responsible for this effect. For example, IL-6 is known to be a fibrogenic factor (10). IL-4 stimulates collagen synthesis of human dermal fibroblasts (9), whereas IL-2 (45) inhibits fibroblast proliferation (14). Granulocyte–macrophage colony-stimulating factor, a prominent cytokine produced by eosinophils that has pleiotropic effects on these cells (47), is potentially very important in our system. In fact, recent evidence supports a major role for granulocyte–macrophage colony-stimulating factor in the development of fibrotic changes both in the skin (48, 49) and in the lung (50, 51). ECP and MBP also influence fibroblast behavior: ECP inhibits proteoglycan degradation and increases glycosaminoglycan intracellular accumulation (12), and MBP interacts in synergistic fashion with IL-1 and TGF-β to increase IL-6 production by fibroblasts (13).

The regulation of extracellular matrix protein production by eosinophils may also depend on their or fibroblast production of MMPs and of inhibition of these enzymes by TIMPs (52, 53). It has been shown that peritoneal cavity guinea pig eosinophils and probably human blood eosinophils from idiopathic hypereosinophilic syndrome patients possess a metalloproteinase that degrades types I and III collagens (16, 54). More recently, an MMP-9 activity in supernatants of human peripheral eosinophils (55) and in asthmatic airway eosinophils also has been demonstrated (56). In this study we also detected in peripheral blood eosinophils MMP-9 together with a higher

band that might correspond to a complex with an inhibitor. In addition, we have found that eosinophils possess TIMP-1 and TIMP-2 activities. However, addition of the eosinophil sonicate to the lung and dermal fibroblast monolayers did not affect their pattern of MMP and TIMP activity. This indicates that at least in our *in vitro* system, eosinophil products do not modulate extracellular matrix macromolecule degradation.

In conclusion, we have demonstrated a direct role for eosinophils in regulating fibroblast activity by showing that eosinophils increase proliferation, collagen production, and lattice retraction in dermal fibroblasts whereas they increase proliferation and lattice contraction but decrease collagen production in lung fibroblasts. Eosinophil fibrogenic effects are mediated partly by preformed TGF-β. Based on our observations, we can conclude that eosinophils play an important, direct, modulatory role in the pathophysiology of lung and skin fibrotic processes. Consequently, the immunopharmacological modulation of their mediators may have a therapeutic role in fibrotic conditions.

We thank Dr. William Hornebeck for many helpful discussions and Miss Alix Berton for technical help. We thank Ms. Maddyn Segev for her editorial and typing assistance. The work was supported by grants from the Aimwell Charitable Trust Foundation, the Centre National de la Recherche Scientifique, and the University of Reims-Champagne-Ardenne.

1. Moqbel, R., Levi-Schaffer, F. & Kay, A. B. (1994) *J. Allergy Clin. Immunol* **94,** 1183–1188.
2. Weller, P. F. (1997) *J. Allergy Clin. Immunol.* **100,** 283–287.
3. Smith, R. S., Smith, T. J., Blieden, T. M. & Phipps, R. P. (1997) *Am. J. Pathol.* **151,** 317–322.
4. Noguchi, H., Kephart, G. M., Colby, T. V. & Gleich, G. J. (1992) *Am. J. Pathol.* **140,** 521–528.
5. Hoshino, M., Nakamura, Y. & Sim, J. J. (1998) *Thorax* **53,** 21–27.
6. Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P. & Hamid, Q. (1997) *Am. J. Respir. Cell Mol. Biol.* **17,** 326–333.
7. Jutley, J. K., Wood, E. J. & Cunliffe, W. J. (1993) *Matrix* **13,** 235–241.
8. Varga, J., Rosenbloom, J. & Jimenez, S. A. (1987) *Biochem. J.* **247,** 597–604.
9. Fertin, C., Nicolas, J. F., Gillery, P., Kalis, B., Bancherau, J. & Maquart, F. X. (1991) *Cell Mol. Biol.* **37,** 823–829.
10. Duncan, M. R. & Berman, B. (1991) *J. Invest. Dermatol.* **97,** 686–692.
11. Vilcek, J., Palombella, V. J., Henriksen-Destefalo, D., Swenson, C., Feinman, R., Hirai, M. & Tsujimoto, M. (1986) *J. Exp. Med.* **163,** 632–643.
12. Hernnas, J., Sarnstrand, B., Lindroth, P., Peterson, C. G., Venge, P. & Malmstrom, A. (1992) *Eur. J. Cell Biol.* **59,** 352–363.
13. Rochester, C. L., Ackerman, S. J., Zheng, T. & Elias, J. A. (1996) *J. Immunol.* **156,** 4449–4456.
14. Rubinchik, E. & Levi-Schaffer, F. (1996) *Life Sci.* **58,** 1509–1517.
15. Duncan, M. R. & Berman, B. (1985) *J. Exp. Med.* **162,** 516–527.
16. Hibbs, M. S., Mainardi, C. L. & Kang, A. H. (1982) *Biochem. J.* **207,** 621–624.
17. Fries, K. M., Blieden, T., Looney, R. J., Sempowski, G. D., Silvera, M. R., Willis, R. A. & Phipps, R. P. (1994) *Clin. Immunol. Immunopathol.* **72,** 283–292.
18. Mauch, C. & Krieg, T. (1986) *Rheumatology* **10,** 372–394.
19. Peters, C. A., Freeman, M. R., Fernandez, C. A., Shepard, J., Wiedenrchair, D. G. & Moses, M. A. (1997) *Am. J. Physiol.* **41,** R19960–R19965.
20. Gillery, P., Maquart, F. X., Le Carre, Y., Kalis, B. & Borel, J. P. (1991) *Clin. Exp. Dermatol.* **16,** 324–330.
21. Pincus, S. H., Ramesh, K. S. & Wyler, D. J. (1987) *Blood* **70,** 572–574.
22. Birkland, T. P., Cheavens, M. D. & Pincus, S. H. (1994) *Arch. Dermatol. Res.* **286,** 312–318.
23. Shock, A., Rabe, K. F., Dent, G., Chambers, R. C., Gray, A. J., Chung, K. F., Barnes, P. J. & Laurent, G. J. (1991) *Clin. Exp. Immunol.* **86,** 185–190.
24. Kadin, M., Butmarc, J., Elovic, A. & Wong, D. (1993) *Am. J. Pathol.* **142,** 11–16.
25. Wong, D. T., Donoff, R. B., Yang, J., Song, B. Z., Matossian, K., Nagura, N., Elovic, A., McBride, J., Gallagher, G., Todd, R., *et al.* (1993) *Am. J. Pathol.* **143,** 130–142.
26. Wong, D. T., Elovic, A., Matossian, K., Nagura, N., McBride, J., Chou, M., Gordon, J., Rand, T., Galli, S. & Weller, P. (1991) *Blood* **78,** 2702–2707.
27. Ohno, I., Lea, R. G. & Flanders, K. C. (1992) *J. Clin. Invest.* **89,** 1662–1668.
28. Elovic, A. E., Ohyama, H., Sauty, A., McBride, J., Tsuji, T., Nagai, M., Weller, P. & Wang, D. T. W. (1998) *J. Immunol.* **160,** 6121–6127.
29. Levi-Schaffer, R., Temkin, V., Malamud, V., Feld, S. & Zilberman, Y. (1998) *J. Immunol.* **160,** 5554–5562.
30. Levi-Schaffer, F., Kelav-Appelbaum, R. & Rubinchik, E. (1995) *Cell Immunol.* **162,** 211–216.
31. Levi-Schaffer, F. & Rubinchik, E. (1995) *J. Invest. Dermatol.* **104,** 999–1003.
32. Gillery, P., Maquart, F. X. & Borel, J. P. (1986) *Exp. Cell Res.* **167,** 29–37.
33. Brassart, B., Randoux, A., Hornebeck, W. & Emonard, H. (1998) *Clin. Exp. Metastasis* **16,** 489–500.
34. Saita, N., Sakata, K. M., Matsumoto, M., Ionaga, K., Ando, M., Adachi, M. & Hirashima, M. (1994) *Immunol. Lett.* **41,** 279–286.
35. Coker, R. K., Laurent, G. J., Shahzeidi, S., Lympany, P. A., du Bois, R. M., Jeffery, P. K. & McAnulty, R. J. (1997) *Am. J. Pathol.* **150,** 981–991.
36. Coste, A., Lefaucheur, J. P., Wang, Q. P., Lesprit, E., Perin, F., Peynegre, R. & Esaidier, E. (1998) *Arch. Otolaryngol.–Head Neck Surg.* **1124,** 1361–1366.
37. Eisman, R. J., Allen, J. S., Lafreniere, D., Leonard, G. & Kreutzer, D. L. (1997) *Am. J. Otolaryngol.* **18,** 405–411.
38. Montesano, R. & Orci, L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4894–4897.
39. Martin, R. W., Duffy, J., Engel, A. G., Lie, J. T., Bowles, C. A., Moyer, T. P. & Gleich, G. J. (1990) *Ann. Intern. Med.* **113,** 124–134.
40. Butterfield, J. H., Kephart, G. M., Banks, P. M. & Gleich, G. J. (1986) *Blood* **68,** 1250–1256.
41. Cox, D., Earle, L., Jimenez, S. A., Leiferman, K. M., Gleich, G. J. & Varga, J. (1995) *Rheumatism* **38,** 939–945.
42. Hallgren, R., Bjermer, L., Lundogren, R. & Venge, P. (1989) *Am. Rev. Respir. Dis.* **139,** 373–381.
43. Raghu, G., Masta, S., Meyers, D. & Narayanan, A. S. (1989) *Am. Rev. Respir. Dis.* **140,** 95–100.
44. Clark, D. A. & Coker, R. (1998) *Int. J. Biochem. Cell Biol.* **30,** 293–298.
45. Manague, J. (1990) *Annu. Rev. Cell Biol.* **6,** 597–641.
46. Levi-Schaffer, F., Barkans, J., Newman, T. M., Ying, S., Wakelin, M., Hoenstein, R., Barak, V., Lacy, P., Kay, A. B. & Moqbel, R. (1996) *Immunol.* **87,** 155–161.
47. Levi-Schaffer, F., Lacy, P., Severs, N. J., Newman, T. M., North, J., Gomperts, B., Kay, A. B. & Moqbel, R. (1995) *Blood* **85,** 2579–2586.
48. Rubbia-Brandt, L., Sappino, A. P. & Gabbiani, G. (1991) *Virchows Arch. B. Cell Pathol.* **60,** 73–82.
49. Xing, Z, Gauldie, J., Tremblay, G. M., Heulett, B. R. & Addison, D. (1997) *Lab. Invest.* **77,** 615–677.
50. Andreuth, D., Gabbiani, G. & Neuville, P. (1998) *Lab. Invest.* **78,** 1493–1502.
51. Xing, Z., Ohkawara, Y., Jordana, M., Graham, F. L. & Gauldie, J. (1996) *J. Clin. Invest.* **97,** 1102–1110.
52. Lee, A. Y., Alkers, K. T., Collier, M., Li, L., Eisen, A. Z. & Seltzer, J. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4424–4429.
53. Edwards, D. R., Leco, K. J., Beaudry, P. P., Atadja, P. W., Veillette, C. & Riabowol, K. T. (1996) *Exp. Gerontol.* **31,** 207–223.
54. Davis, W. B., Fells, G. A., Sun, X. H., Gadek, J. E., Venet, A. & Crystal, R. G. (1984) *J. Clin. Invest.* **74,** 269–278.
55. Okada, S., Kita, H., George, T. J., Gleich, G. J. & Leiferman, K. M. (1997) *Am. J. Respir. Cell Mol. Biol.* **17,** 519–528.
56. Ohno, I., Ohtani, H., Nitta, Y., Suzuki, J., Hoshi, H., Honma, M., Isoyama, S., Tanno, Y., Tamura, G., Yamauchi, K., *et al.* (1997) *Am. J. Respir. Cell Mol. Biol.* **16,** 212–219.