American Journal of Pathology, Vol. 147, No. 2, August 1995

Cellular Localization of Transforming Growth Factor-β Expression In Bleomycin-Induced Pulmonary Fibrosis

Kai Zhang,* Kathleen C. Flanders,† and Sem H. Phan*

From the Department of Pathology, University of Michigan, Ann Arbor, Michigan, and Laboratory of Chemoprevention,† National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

Bleomycin-induced pulmonary fibrosis is associated with increased lung transforming growth factor-β (TGF-β) gene expression, but cellular localization of the source of this expression has not been unequivocally established. In this study, lung fibrosis was induced in rats by endotracheal bleomycin injection on day 0 and, on selected days afterwards, lungs were harvested for* in situ *hybridization, immunohistochemical and histochemical analyses for TGF-β_1 mRNA and protein expression, and cell identification. The results show that control lungs express essentially no detectable TGF-β_1 mRNA or protein in the parenchyma. Before day 3 after bleomycin treatment, scattered bronchiolar epithelial cells, mononuclear cells, and eosinophils expressed elevated levels of TGF-β_1. Between days 3 and 14, there was a major increase in the number of eosinophils, myofibroblasts, and fibroblasts strongly expressing TGF-β_1 mRNA and protein. TGF-β_1-producing cells were predominantly localized within areas of injury and active fibrosis. After day 14, the intensity and number of TGF-β_1-expressing cells significantly declined and were predominantly found in fibroblasts in fibrotic areas. The expression of TGF-β_1 protein was generally coincident with that for mRNA with the exception of bronchiolar epithelial cells in which strong protein expression was unaccompanied by a commensurate increase in mRNA. The study demonstrates that myofibroblasts, fibroblasts, and eosinophils represent the major sources of increased lung TGF-β_1 expression in this model of pulmonary fibrosis. (Am J Pathol 1995, 147:352–361)

Transforming growth factor-β (TGF-β) is a multifunctional mediator capable of regulating cell proliferation and differentiation as well as synthesis of many components of the extracellular matrix.[1] Increased production of TGF-β has been associated with normal reparative as well as pathological fibrotic processes in many organs including lung.[2–13] Some of the experiments suggest that modulation of tissue repair and scar formation can be approximated to some degree by either exogenous administration of TGF-β_1 or neutralizing antibody to TGF-β_1 under certain experimental conditions.[14,15] Up-regulation of lung TGF-β_1 mRNA expression in rodent bleomycin-induced pulmonary fibrosis correlates with the increase in lung extracellular matrix production.[7–9] *In vivo* studies of human idiopathic pulmonary fibrosis and bleomycin-induced lung fibrosis suggest that epithelial cells and macrophages may represent possible cellular sources of TGF-β_1 during the period of active fibrosis, although the distribution or pattern of TGF-β_1 expression is not identical in these studies.[10–13] These studies also do not unequivocally identify the cells expressing this cytokine, especially in terms of specific phenotypic characteristics. Other recent studies suggest that the newly reactive myofibroblasts and/or fibroblasts and eosinophils may play an important role in terms of *in vivo* cytokine production during reparative and fibrotic processes.[16–19] In this study, these issues are examined by using combined *in situ* hybridization and immunohistochemical and histochemical methods to more specifically identify the cells responsible for TGF-β_1 up-regulation in fibrotic lungs during evolution of this disease. The influence of the methods of tissue fixation and differences in staining pattern, intensity, and specificity between various TGF-β antibodies are also examined.

Supported in part by NIH grants HL28737, HL31963, HL46487, DK46469, and HL52285.

Accepted for publication April 18, 1995.

Address reprint requests to Dr. Sem H. Phan, Department of Pathology M0602, University of Michigan Medical School, Ann Arbor, MI 48109–0602.

Materials and Methods

Animals and Tissue Sampling

Specific pathogen-free, male Fisher 344 rats weighing 200 to 250 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). These animals arrived in filtered cages and were maintained in clean animal quarters separate from other laboratory animals. Animals were randomly assigned to control or experimental groups. Pulmonary fibrosis was induced on day 0 by the endotracheal injection of 0.75 U of bleomycin per 100 g of body weight in 300 μl of sterile saline as previously described.[19,20] Control animals received sterile saline only. Animals (13 per group) were sacrificed on days 1, 3, 7, 14, 21, and 28 after bleomycin treatment. The lungs were rapidly dissected free of extraneous tissues and those from 5 animals in each group were immediately filled with either neutral buffered formalin, pH 7.2, or methacarn (methanol/chloroform/glacial acetic acid; 60:30:10, v/v) perfused through the trachea. After at least 16 hours of fixation, the lungs from the anterior, middle, and lower right segments as well as posterior and anterior left segments were cut with a razor blade and embedded in paraffin.[19,20] Three- to five-micron sections of formalin-fixed tissues were prepared for *in situ* hybridization, immunochemistry, or combined *in situ* hybridization and immunochemistry. Sections of methacarn-fixed tissues were used for immunochemistry only. The general histological appearance of tissue was assessed after routine hematoxylin and eosin staining. The methacarn fixation allows for greater preservation of antigenic properties, and thus enhanced sensitivity for immunochemical studies, but rendered the tissues unsuitable for *in situ* hybridization analysis.[19,20]

Histochemical and Immunochemical Analyses

Three different anti-TGF-β antibodies were evaluated for this study. Polyclonal rabbit anti-TGF-β antibody (AB-10-NA), which cross-reacts with TGF-β_1 and -β_2, was purchased from R&D Systems (Minneapolis, MN). Polyclonal rabbit antibody to the NH_2-terminal 1–30 amino acids of mature TGF-β_1 (anti-LC) was prepared as previously described.[10,11] A mouse monoclonal anti-TGF-β antibody (1D11.16), which also cross-reacts with TGF-β_1 and β_2, was a generous gift of Celtrix Pharmaceuticals (Santa Clara, CA).[21] Rabbit affinity-purified nonimmune immunoglobulin (Ig)G, chromotrope 2R, and toluidine blue were purchased from Sigma Chemical Co.(St. Louis, MO). For cell phenotype identification the following antibodies were used: anti-human eosinophil major basic protein monoclonal antibody (BMK-13, IgG_1) from Monosan (Uden, The Netherlands), mouse anti-rat macrophage/monocyte antibody (ED1) from Serotec (Indianapolis, IN), rabbit anti-T-lymphocyte antibody (CD3) and rabbit anti-human von Willebrand Factor (vWF) from Dako (Carpinteria, CA), mouse monoclonal anti-α-smooth muscle actin (α-SM actin) from Boehringer Mannheim Biochemicals (Indianapolis, IN). Secondary antibodies and related reagents were biotinylated and affinity-purified horse anti-mouse IgG (rat adsorbed), biotinylated affinity-purified goat anti-rabbit IgG, avidin-biotin amplification and ABC Elite kits from Vector Laboratories (Burlingame, CA), and mouse myeloma IgG_1 and IgG_{2a} from Zymed (South San Francisco, CA).

The newly reactive myofibroblasts or fibroblast-like cells were identified as spindle-shaped cells possessing oval or elongated nuclei and positive staining with anti-α-SM actin antibody.[20,22] Eosinophils were identified by their polymorphonuclear morphology and specific characteristic staining by chromotrope 2R[19,23] or by cytoplasmic staining with anti-human eosinophil major basic protein antibody.[19,24] Neutrophils exhibited similar polymorphonuclear morphology but did not stain with chromotrope 2R. Monocytes and macrophages were identified by their mononuclear morphology and cytoplasmic staining with the ED1 antibody.[19] T lymphocytes, mast cells, and endothelial cells were identified by cytoplasmic staining with CD3 antibody, toluidine blue, and anti-human vWF polyclonal antibody, respectively.[19,24,25]

For identification of eosinophils or mast cells, the paraffin sections were dewaxed through xylene, rehydrated through an ethanol series, and either stained with chromotrope 2R or toluidine blue, respectively.[19] The slides were then rinsed in distilled water and coverslipped. Eosinophils exhibited bright red cytoplasmic staining whereas mast cells varied in color from purple to red-violet with their respective histochemical stains.

Immunochemistry was performed by the immunoperoxidase technique as previously described.[20,26] Briefly, endogenous peroxidase activity in sections was inactivated with 3% H_20_2 for 10 minutes. The primary antibodies were used at the following concentrations or dilutions: polyclonal rabbit anti-LC (10 to 15 μg/ml), polyclonal rabbit anti-TGF-β (8 to 10 μg/ml), mouse monoclonal anti-human TGF-β (150 μg/ml), BMK-13 (1:5 dilution), ED-1 (1:800 dilution), anti-α-SM actin (1:500 dilution), anti-CD3 (1:100 dilution), and anti-vWF (1:200). Biotinylated and affinity-purified horse or goat anti-rabbit IgG was used as the secondary antibody. Avidin-biotin

amplification (ABC Elite) was followed by incubation with the substrate, 3′,3′-diaminobenzidine. Nuclear counterstain with hematoxylin was followed by graded sequential dehydration in ethanol.

Negative and positive control slides included (1) omission of primary antibody, (2) substitution of primary antibody for corresponding nonimmune IgG isotype, and (3) use of rat spleen and intestine as positive controls for ED-1 and CD3 antibodies and for α-SM-actin antibody, respectively. Cytospun human blood samples were used as positive controls for eosinophil immunostaining by the BMK-13 antibody. Rat carotid blood vessels were used as positive controls for the anti-vWF antibody.

In Situ *Hybridization*

In situ hybridization was performed as previously described.[19,20] The 30-mer oligonucleotide antisense probe for TGF-β_1[9] was 5′-GAAGTTGGCATGGTAG-CCCTTGGGCTCGTG-3′.

The corresponding sense probe was used for control purposes. These probes were synthesized by an automated DNA synthesizer and then purified by high pressure liquid chromatography before use. The oligonucleotide probes were labeled with ^{35}S by the 3′-end-labeling method and then purified by electrophoresis on a 12% polyacrylamide gel.[19,20] Briefly, the paraffin sections were dewaxed, pretreated with chromotrope 2R,[18,19] and sequentially treated with 0.2 N HCl and 0.25% (v/v) acetic anhydride in triethanolamine. After sequential washes in 2X standard saline citrate (SSC), the slides were overlaid with prehybridization buffer. This was followed by the addition of the indicated radiolabeled oligonucleotide probe diluted in hybridization buffer and the slides were then coverslipped. After 18 hours, the coverslips were gently removed and the slides sequentially washed in various dilutions of SSC. After dehydration in alcohol, the slides were air dried and dipped in Kodak NBT-2 emulsion for autoradiographic detection. The slides were allowed to air dry and then stored in a desiccated chamber at 4°C for 2 to 3 weeks. They were then developed in Kodak D-19 developer, washed, and fixed. After staining with hematoxylin and eosin, the slides were coverslipped.

Combined in Situ *Hybridization and Histo- or Immunochemical Staining*

Selected slides from different time points were subjected to combined *in situ* hybridization for TGF-β_1 mRNA and either histochemical staining with chromotrope 2R or immunochemical staining with anti-α-SM actin antibody. After *in situ* hybridization was performed with TGF-β_1 antisense probe as described above and finally washed in 0.5X SSC, slides were stained with chromotrope 2R or toluidine blue or subjected to immunohistochemical staining with the indicated antibodies with avidin-biotin peroxidase as described above. The slides were then dehydrated and dried. Emulsion and autoradiography were then undertaken as described above for *in situ* hybridization.

Controls for *in situ* hybridization consisted of (1) previous digestion of lung tissue with 100 μg/ml RNAse A at 37°C for 45 minutes before addition of antisense probe, (2) substituting sense probe for antisense probe, (3) combined *in situ* hybridization of the TGF-β_1 sense probe or antisense probe before RNAse digestion and chromotrope 2R histological staining, and (4) cytospin slides made from normal rat lung fibroblasts treated with bleomycin (100 ng/ml) for 16 hours *in vitro* as positive control for *in situ* hybridization analysis of TGF-β_1 mRNA.[25]

Morphometric Analysis

Five segments from each rat and a total of four to five rats per group were analyzed. At least 50 randomly chosen noncontiguous and nonoverlapping (at high

Table 1. *Summary of Effects of Antibody Type and Fixation Method on Immunohistochemical Staining for TGF-β*

		Cells stained							
		Eos		Epi		Mon		Myo/F	
Antibody	Antigen specificity	For	Met	For	Met	For	Met	For	Met
AB-10NA (R&D)	TGF-$\beta_{1\&2}$	–	+++	+++	+++	++	+++	–	+++
1D11.16 (Celtrix)	TGF-$\beta_{1\&2}$	–	++	+++	+++	++	++	–	++
Anti-LC	TGF-β_1	–	–	++	++	+	++	–	–/+

Relative cell staining intensity is shown as none (–), weak (+), moderate (++), or strong (+++). All antibodies were polyclonal except 1D11.16, which was monoclonal.

Eos, eosinophils; Epi, epithelial cells; Mon, mononuclear cells (includes macrophages, monocytes, and lymphocytes); Myo/F, myofibroblasts or fibroblasts; For, formalin-fixed tissues; Met, methacarn-fixed tissues.

* This antibody is made against a synthetic peptide of TGF-β_1 (NH_2-terminal 1-30 amino acids of the mature protein).

power) fields (×40 objective) were counted in each lung segment. For *in situ* hybridization data, cells containing more than five grains were considered as TGF-β_1-positive cells. Background staining revealed less than five grains per cell based on evaluation of control slides with the sense probe and/or pretreatment of the tissue section with RNAse.

Positive cell counts were expressed as the average number of cells per high power field ± SEM. Means from lungs of bleomycin-treated animals were compared with the corresponding values obtained from control animals at the respective time points by the paired Student's *t*-test.

Results

Morphological Analysis of Control Lungs

Control lungs from various time points exhibited no significant TGF-β expression when assessed by both immunohistochemistry (using all three antibodies in methacarn-fixed tissues) and *in situ* hybridization. There was no evidence of infection or pulmonary inflammation. The number and distribution of ED-1-positive macrophages, CD3-positive T cells, and mast cells were consistent with a previous report.[19] Hybridization with the sense TGF-β_1 probe and combined *in situ* hybridization with the TGF-β_1 sense probe and chromotrope 2R histological staining revealed less than five grains per cell. Hybridization of previously RNAse-digested sections with the antisense probe also showed similarly low background. Cytospin slides made from normal rat lung fibroblasts stimulated with bleomycin were used as positive controls for *in situ* hybridization analysis with the TGF-β_1 antisense probe. A significant increase in the TGF-β_1 hybridization signal was shown in fibroblast-like cells after treatment with bleomycin compared with control slides (data not shown).

Morphological Analysis of Injured and Fibrotic Lungs

Day 1 Lung After Bleomycin Treatment

There was minimal morphological alteration at this time point. Immunohistochemical staining by all three anti-TGF-β antibodies revealed weak to moderate signals in the epithelial cells of certain bronchioles in both formaldehyde and methacarn-fixed lung tissues (data not shown). Only a few scattered inflammatory cells were present, including neutrophils, ED-1-positive macrophages, CD3-positive T lymphocytes, and occasional chromotrope 2R- and BMK-13-positive eosinophils, around major airways and adjacent blood vessels and small venules as well as within the alveolar septa and alveolar space. These cells at this time point did not stain with all anti-TGF-β antibodies tested and with both types of tissue fixation. No significant and specific *in situ* hybridization signal for TGF-β_1 mRNA was detected in the lung at this time point (Figure 1).

Day 3 Lung After Bleomycin Treatment

At this time point, there was increasing infiltration by neutrophils, macrophages, and T lymphocytes, primarily in peribronchiolar and perivascular areas, within the alveolar septa, and in the alveolar space. More chromotrope 2R- and BMK-13-positive eosinophils could also be identified in the infiltrating cells. No significant change in the number and distribution of mast cells was observed and the lung architecture was for the most part intact. Immunohistochemical and *in situ* hybridization analyses revealed similar cellular distribution patterns of moderate to high increase in TGF-β expression within the areas of injury and inflammation. Most of the epithelial cells of affected bronchioles showed strong positive staining with all three anti-TGF-β antibodies and under both fixing conditions, although they are essentially negative for TGF-β_1 mRNA expression (Figure 2a). The remainder of the cells expressing heightened levels of TGF-β protein and mRNA consisted primarily of eosinophils and a few scattered mononuclear cells (Figure 2b). The identity of the former was confirmed by histochemical staining of the same sections with chromotrope 2R and immunohistochemical staining of serial sections with anti-major basic protein antibody.

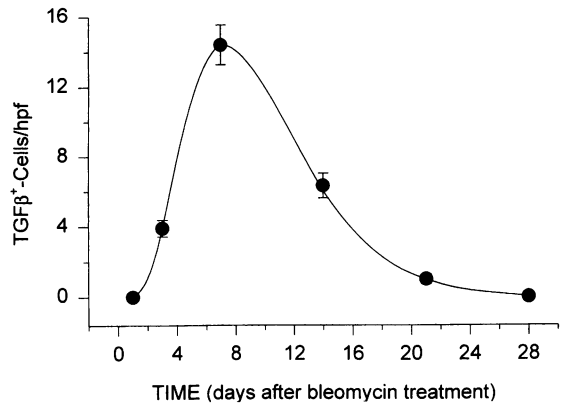


Figure 1. *Kinetics of lung cells expressing TGF-β_1 mRNA. Lung sections from the indicated time points after bleomycin treatment were subjected to* in situ *hybridization for detection of TGF-β_1 mRNA, and the number of positive cells were counted and expressed as cells per high power field (×400 magnification) as described in Materials and Methods. Data were expressed as means ± SE, with n = 5 animals at each time point.*

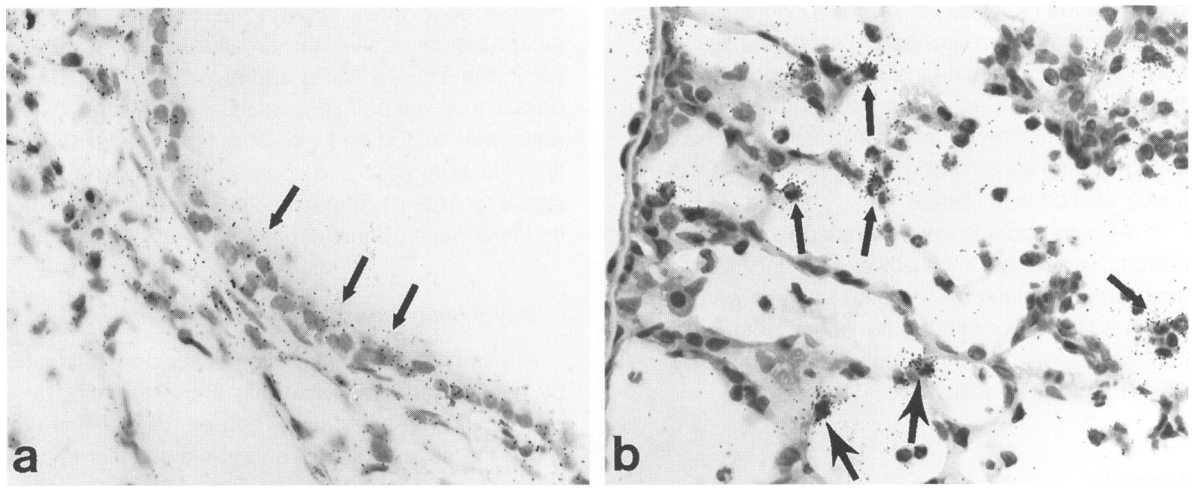


Figure 2. *TGF-β_1 mRNA expression at day 3 after bleomycin treatment. Lungs from day 3 were subjected to* in situ *hybridization for detection of TGF-β_1 expression. Bronchiolar epithelial cells* (a) *were essentially negative, whereas scattered eosinophils (small arrows) and mononuclear cells (large arrows) exhibited strong signals for this cytokine* (b). *All micrographs were photographed at ×400.*

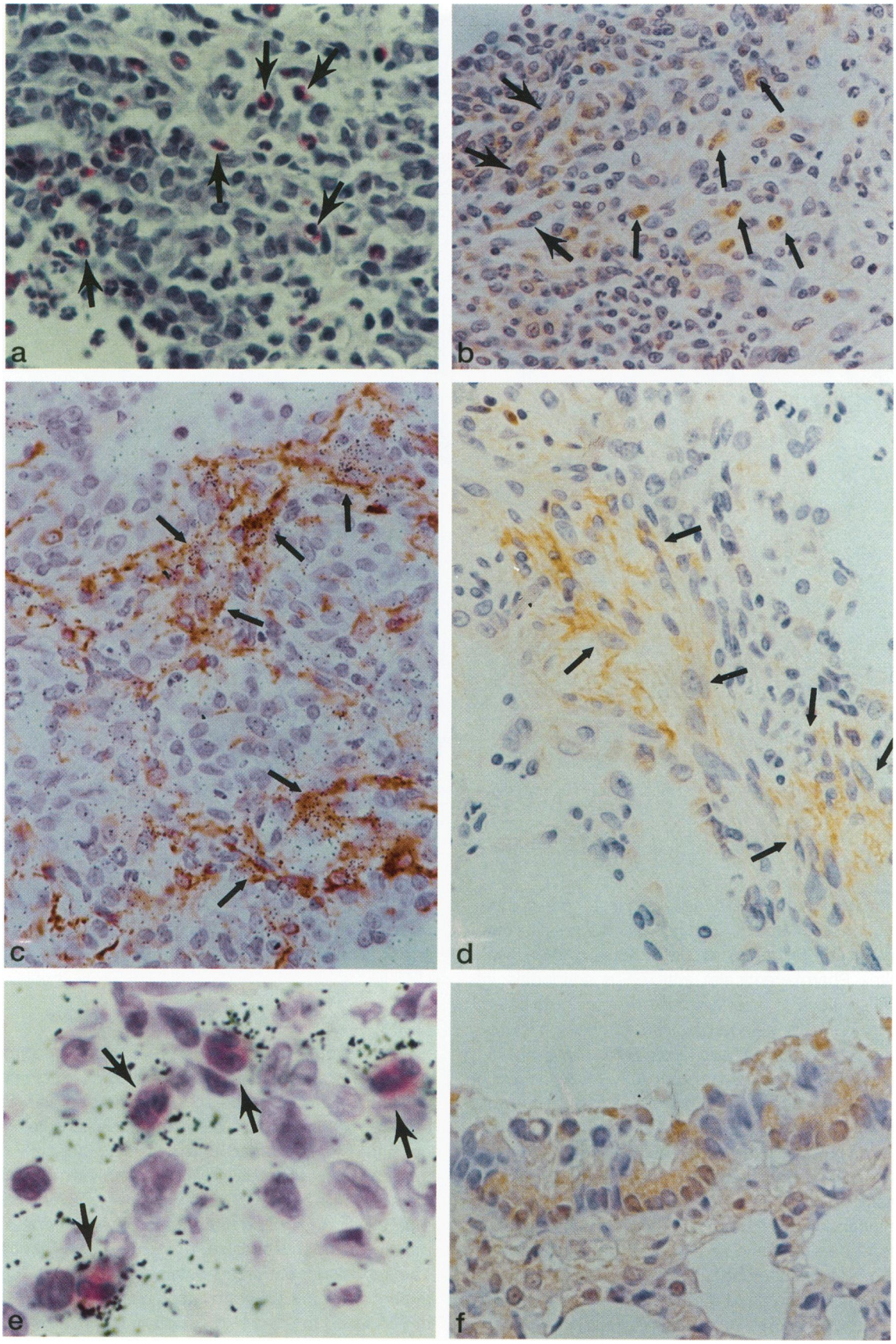
Many of the mononuclear cells demonstrated positive staining with anti-CD3 antibody in serial sections. Positive immunohistochemical staining of eosinophils could not be demonstrated with the anti-LC antibody under both methods of tissue fixation, nor was it observed with the other two antibodies if the tissues were fixed in formalin. Thus, demonstration of expression in eosinophils could be detected only in methacarn-fixed tissues with the rabbit polyclonal (R&D Systems) and the mouse monoclonal (Celtrix Pharmaceuticals) anti-TGF-β antibodies, and this was true for all tissues from other time points as well.

Days 7 and 14 After Bleomycin Treatment

Severe distortion of lung parenchyma now became prominent. Maximal active fibrosis was indicated by the large number of newly reactive myofibroblasts within fibrotic lesions and by a peak of increase in collagen production at these time points.[20] Associated with this was a further increase in the number of chromotrope 2R- and BMK-13-positive eosinophils, ED-1-positive macrophages, and CD3-positive lymphocytes. The number of neutrophils was significantly decreased at these time points, whereas the number and distribution of mast cells remained unchanged.[19]

There was a dramatic and peak increase in TGF-β_1 mRNA-expressing cells at these time points (Figure 1), which were mainly distributed within the fibrotic and inflamed areas. On day 7 most of these positive cells were identified as eosinophils on the basis of polymorphonuclear morphology and positive staining with chromotrope 2R and BMK-13 antibody (Figures 3, b and e, and 4a). This coincided with the peak increase in eosinophil count in lung sections[19] (Figure 1). By day 14, the number of eosinophils was substantially decreased and most of the cells strongly expressing TGF-β_1 mRNA and protein resembled myofibroblasts on the basis of strong staining with α-SM actin and elongated cellular morphology (Figures 3, d and c, and 4, b and c). These myofibroblasts appeared hypertrophic and resembled the cells expressing high levels of collagen mRNA as reported in a previous study.[20] Scattered mononuclear cells also expressed significant levels of TGF-β_1 mRNA near these fibrotic areas (Figure 4d), and these appear to be mainly macrophages and lymphocytes on the basis of ED-1 and anti-CD3 antibody staining of serial sections (data not shown), respectively. In contrast to the strong anti-TGF-β antibody staining (Figure 3d), TGF-β_1 mRNA signal was conspicuously absent from

Figure 3. *Cellular distribution of TGF-β expression. TGF-β expression was determined either by immunohistochemistry* (b, d, *and* f) *or by* in situ *hybridization* (c *and* e) *as described in Materials and Methods. Large numbers of eosinophils were present on day 7 as confirmed by chromotrope 2R staining* (a) *and they exhibited peak TGF-β protein* (b) *and mRNA* (e) *expression. The identity of these cells was directly confirmed by combined* in situ *hybridization for TGF-β_1 mRNA and chromotrope 2R staining of the same tissue section* (e). *Chromotrope 2R stained eosinophils (arrows) red* (e). *Fibroblast-like cells showed strong TGF-β protein* (d) *and mRNA* (c) *expression in a day 14 lung, and their identities were confirmed as myofibroblasts (arrows) by combined* in situ *hybridization for TGF-β_1 mRNA and positive staining with anti-α-SM actin antibody* (c). *Bronchiolar epithelial cells showed strong positive TGF-β protein expression in this section from a day 7 lung* (f). *Similar positive staining was seen in sections from days 3 and 14. All panels were photographed at ×400 except for* e, *which was at ×1000.*


a
b
c
d
e
f

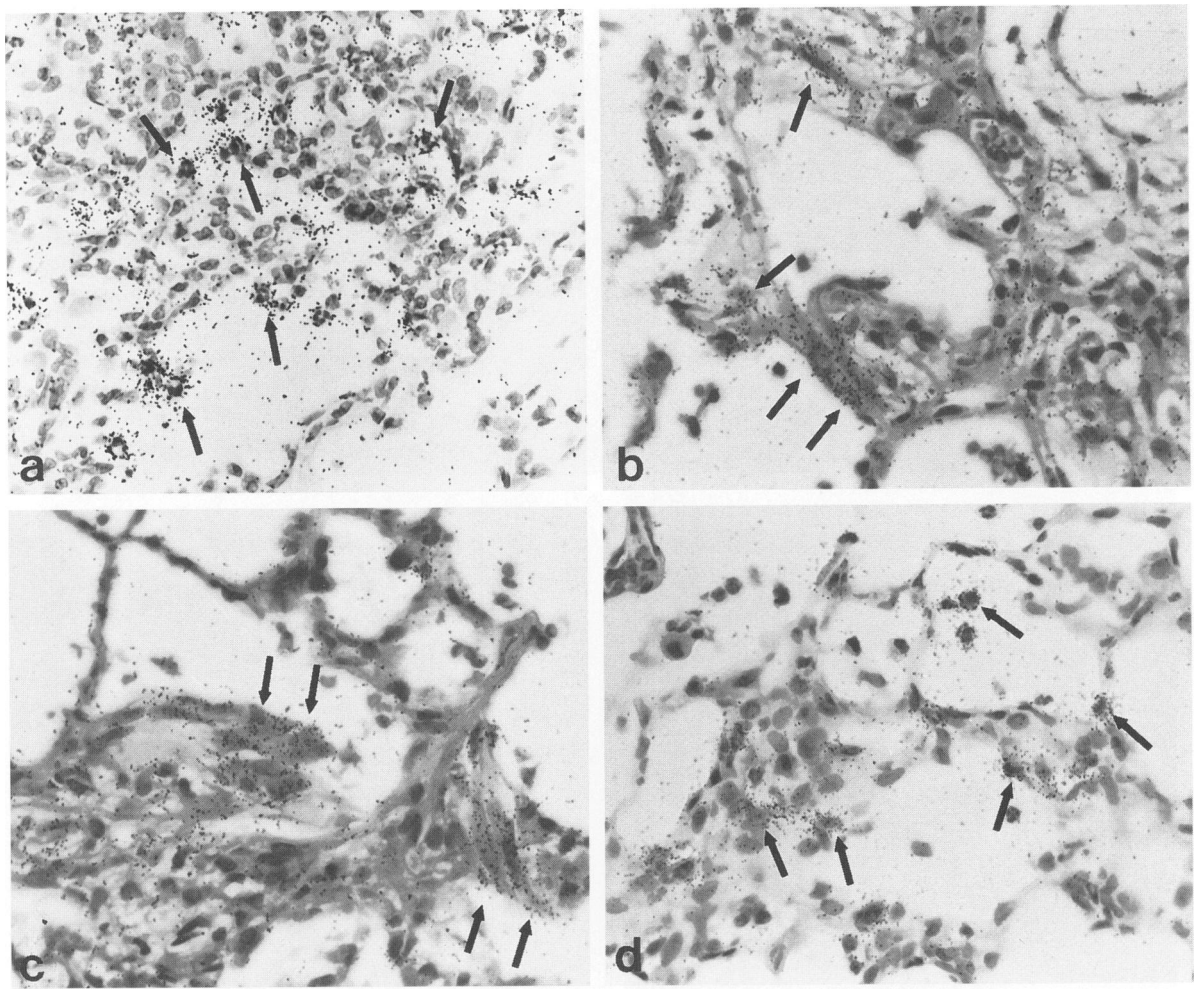


Figure 4. *TGF-β_1 mRNA expression in days 7 and 14 lungs. On day 7, strong expression in eosinophils and mononuclear cells are shown in* a *and* d, *respectively. Expression by myofibroblasts/fibroblasts at days 7 and 14 are shown in* b *and* c, *respectively. All micrographs were photographed at* $\times 400$.

the bronchiolar epithelial cells at these time points. Immunohistochemical staining with the rabbit polyclonal (R&D Systems) and the mouse monoclonal (Celtrix Pharmaceuticals) anti-TGF-β antibodies in serial sections of methacarn-fixed tissues, revealed strong TGF-β protein expression in these newly reactive myofibroblasts and eosinophils within the fibrotic lesions (Figure 3, b and d). Immunohistochemical staining with the anti-LC antibody at even lower dilutions exhibited much weaker or no staining of these same cells in methacarn-fixed tissues, whereas essentially no staining of these cells was apparent in formalin-fixed tissues, as was the case with the other two antibodies. Most of the bronchiolar epithelial cells, especially some appearing to be regenerating, and a number of mononuclear cells (mostly macrophages as determined by ED-1 immunohistochemical staining) also showed essentially equally strong positive staining with all three anti-TGF-β antibodies used in this study, independent of the method of tissue fixation (Figure 3f).

Days 21 and 28 After Bleomycin Treatment

TGF-β protein and mRNA expression at these time points were substantially diminished (data not shown). This reduction in TGF-β expression was associated with maturation of the fibrotic lesions, which appeared less inflamed and consisted primarily of aggregations of fibroblasts with markedly diminished staining with anti-α-SM actin antibody.[20] Toluidine blue staining revealed that an increased number of mast cells were present surrounding and within the fibrotic lesions, but immunostaining with anti-TGF-β antibodies or *in situ* hybridization analysis for TGF-β_1 mRNA failed to show expression of this cytokine by

these late appearing cells. Although significant numbers of ED-1- and anti-CD3-positive cells were stillevident, these cells now expressed substantially lower levels of TGF-β. Chromotrope 2R- and BMK-13-positive eosinophils were markedly diminished at these time points and could be identified only in a few sections, consistent with previous observations.[19]

Discussion

The cellular source for TGF-β gene expression *in vivo* has not been identified unequivocally in inflammation and fibrosis.[10–13] Previous studies have suggested the lung epithelial cells and alveolar macrophages as primary sources of TGF-β in human idiopathic and rodent bleomycin-induced pulmonary fibrosis.[10–13] The immunohistochemical studies in these previous reports used standard methods of tissue fixation and primarily relied on the anti-LC antibodies. In this study, methacarn tissue fixation was found to enhance specific staining for TGF-β with three different antibodies, which yielded somewhat different patterns of staining relative to these previous studies. These immunohistochemical results in conjunction with the *in situ* hybridization study showed that early infiltrating eosinophils and, at later time points, newly reactive myofibroblasts and fibroblast-like cells served as important sources of TGF-β gene expression, in addition to bronchiolar epithelial cells and macrophages during the period of active fibrosis (days 7 and 14 after bleomycin injection). None of the sections revealed significant expression of TGF-β by identifiable alveolar epithelial cells at all time points examined. With the notable exception of bronchiolar epithelial cells (which showed an increase only in TGF-β protein by immunostaining), increases in TGF-β cellular expression were consistently seen at both mRNA and protein levels. The immunohistochemical results regarding TGF-β expression differs from that of previous studies showing epithelial cells and alveolar macrophages as the major sources of increased TGF-β protein expression in the same animal model.[10] The difference appeared to be a result of the different fixation methods (formaldehyde *versus* methacarn) and anti-TGF-β antibodies (anti-LC *versus* anti-TGF-β antibodies from R&D Systems and Celtrix Pharmaceuticals) used in this study. This conclusion is based on the lack of staining of myofibroblasts/fibroblasts and eosinophils by (1) the anti-LC antibody in tissues fixed with either formalin or methacarn and (2) all three antibodies in formaldehyde-fixed tissues. Although the exact reason is undetermined, part of the explanation may be that the methacarn fixation allows for greater preservation of antigenic properties and thus enhanced sensitivity for immunochemical studies,[19] whereas the differences with the anti-LC antibody may be because of its more selected and/or differently targeted epitope(s).

During the fibrotic process in this animal model, lung mesenchymal cells proliferate and differentiate into myofibroblasts and/or fibroblast-like cells, which is characterized by high expression of collagen and α-smooth muscle actin as well as hypertrophy.[20,21] At days 7 and 14 after bleomycin treatment, these cells organized and formed patchily distributed and differently sized fibrogenic foci containing newly deposited extracellular matrix and a variety of inflammatory cells, located mainly within the alveolar space, around bronchioles and blood vessels, and within thickened septa.[20,21] Apparent correlation between the increased expression of TGF-β mRNA and protein within these foci in myofibroblasts/fibroblasts as well as selected associated inflammatory cells in this study is consistent with the previous suggestion that TGF-β is associated with the altered phenotype of resident fibroblasts found in human idiopathic pulmonary fibrosis.[12] A remarkable finding in the present study supporting such a conclusion is the demonstration that almost all of the TGF-β_1 mRNA expression by day 14 was in myofibroblasts that were shown in a previous study to be the primary cell responsible for increased collagen expression in fibrotic lungs.[20] This further suggests that they can synthesize TGF-β_1 protein *de novo* instead of exclusive reliance on internalization of TGF-β secreted by adjacent activated macrophages and/or released from surrounding degraded extracellular matrix.[10,12,13] Furthermore, there is some evidence to indicate that cytokines may not diffuse well through cellular barriers and even extracellular matrix.[28–30] Thus it is reasonable to propose that the presence of high amounts of TGF-β protein within active fibrotic foci may be a result, at least in part, of increased gene expression by eosinophils and macrophages early on and by myofibroblasts/fibroblasts at later time points in this animal model. In contrast to the concomitant increased expression of mRNA and protein in these cells, the increased protein expression in bronchiolar epithelial cells was unaccompanied by increased mRNA expression, suggesting in this instance the possibility of uptake by the epithelial cells of TGF-β secreted by adjacent cells and/or released by subjacent degraded extracellular matrix of the basement membrane.

Although the basis and significance for increased TGF-β expression by the newly reactive myofibroblasts/fibroblasts remains to be determined, given the findings of the present and previous studies, it is tempting to speculate that TGF-β produced by resident and infiltrating macrophages and eosinophils at early time points (before day 7 after bleo-

mycin treatment) may be responsible for altering the phenotype of adjacent lung mesenchymal cells, including the auto-induction of TGF-β.[28] In this manner, increased TGF-β production by these mesenchymal cells is expected to have autocrine regulatory consequences on extracellular matrix gene expression and other functional effects of stimulation by this cytokine, including paracrine influences on inflammation.

The key novel finding in the present study is the identification of eosinophils as an important source of TGF-β expression in injured lungs undergoing fibrosis. There is growing evidence that suggests participation of eosinophils in the remodeling of connective tissue of fibrosis in terms of collagenase activity and cytokine and major basic protein production.[17–19,30] A variety of cytokines, including TGF-β, TGF-α, and monocyte chemotactic protein-1 are now known to be produced by eosinophils.[16,19] Recent studies demonstrate prominent up-regulation of TGF-β mRNA by eosinophils in the granulation tissue of healing wounds and other diseased tissues *in vivo*,[17] whereas lung eosinophil monocyte chemotactic protein-1 expression has been demonstrated in this same model of bleomycin-induced pulmonary fibrosis.[19] The direct demonstration in the present study of the increased expression of TGF-β protein and mRNA by eosinophils at the site of active fibrosis suggests that functional interaction between eosinophils and fibroblasts may be present during the fibrotic process. A recent study indicates that the synergistic effect of major basic protein from eosinophils and interleukin-1α or TGF-β on fibroblast cytokine production may be part of a mechanism by which eosinophils influence lung inflammation and fibrosis.[30]

Acknowledgments

We express our gratitude to Bristol-Myers for providing the gift of bleomycin and to Celtrix Pharmaceuticals for the gift of monoclonal anti-TGF-β antibody. We also acknowledge the excellent technical assistance of Bridget M. McGarry and Lisa K. Riggs.

References

1. Sporn MB, Roberts AB: Transforming growth factor-β: recent progress and new challenge. J Cell Biol 1992, 119:1017–1021
2. Nakatsukasa H, Nagy P, Everts RP, Hisa CC, Marsden E, Thorgeirsson SS: Cellular distribution of transforming growth factor-β1 and procollagen type I, III and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. J Clin Invest 1990, 85:1833–1843
3. Smith EA, Leroy EC: A possible role for transforming growth factor-β in systemic sclerosis. J Invest Dermatol 1990, 95:1255–1275
4. Peltonen J, Hsiao LL, Jaakola S, Sollberg S, Aumailley M, Timpel R, Chu ML, Uitto J: Activation of collagen gene expression in keloids: co-localization of type I and VI collagen and transforming growth factor-β1 mRNA. J Invest Dermatol 1991, 97:240–248
5. Yoshioka K, Takemura T, Murakami K, Okada M, Hino S, Miyamoto H, Maki S: Transforming growth factor-β protein and mRNA in glomeruli in normal and diseased human kidneys. Lab Invest 1993, 68:154–163
6. Waltenberger J, Lundin L, Oberg K, Wilander E, Miyazono K, Heldin CH, Funa K: Involvement of transforming growth factor-β in the formation of fibrotic lesions in carcinoid heart disease. Am J Pathol 1993, 142: 71–78
7. Hoyt DG, Lazo JS: Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-β precede bleomycin-induced pulmonary fibrosis in mice. J Pharmacol Exp Ther 1988, 246: 765–770
8. Raghow R, Irish P, Kang AH: Coordinate regulation of transforming growth factor β gene expression and cell proliferation in hamster lungs undergoing bleomycin-induced pulmonary fibrosis. J Clin Invest 1989, 84: 1836–1842
9. Phan SH, Kunkel SL: Lung cytokine production in bleomycin-induced pulmonary fibrosis. Exp Lung Res 1992, 18:29–43
10. Khalil N, Bereznay O, Sporn M, Greenberg AH: Macrophage production of transforming growth factor β and fibroblast collagen synthesis in chronic pulmonary inflammation. J Exp Med 1989, 170:727–737
11. Khalil N, O' Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Bereznay OH, Greenberg AH: Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol 1991, 5:155–162
12. Broekelmann TJ, Limper AH, Colby TV, McDonald JA: Transforming growth factor β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci USA 1991, 88:6642–6646
13. Limper AH, Broekelmann TJ, Colby TV, Malizia G, McDonald JA: Analysis of local mRNA expression for extracellular matrix proteins and growth factors using *in situ* hybridization in fibroproliferative lung disorders. Chest 1991, Suppl:55S-56S
14. Pierce GF, Tarpley JE, Yanagihara D, Mustoe TA, Fox GM, Thomason A: Platelet-derived growth factor (BB homodimer), transforming growth factor-β1, and basic fibroblast growth factor in dermal wound healing. Am J Pathol 1992, 140:1375–1388

15. Shah M, Foreman D, Ferguson MWJ: Control of scarring in adult wounds by neutralizing antibody to transforming growth factor β. Lancet 1992, 339:213–214
16. Spry, CJF, Karry AB, Gleich GJ: Trend: eosinophils. Immunol Today 1992, 12:384–391
17. Wong DTW, Donoff RB, Yang J, Song BZ, Matossian K, Nagura N, Elovic A, Mcbride J, Gallagher G, Todd R, Chiang T, Chou LSS, Yung CM, Galli SJ, Weller PF: Sequential expression of transforming growth factor α and β1 by eosinophils during cutaneous wound healing in the hamster. Am J Pathol 1993, 143:130–140
18. Ohno I, Lea R, Finotto S, Marshall J, Denbury J, Dolovich J, Gauldie J, Jordana M: Granulocyte/macrophage colony-stimulating factor (GM-CSF) gene expression by eosinophils in nasal polyposis. Am J Respir Cell Mol Biol 1993, 5:505–512
19. Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH: Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol 1994, 153:4733–4741
20. Zhang K, Rekhter MD, Gordon D, Phan SH.: Localization of myofibroblasts in the lung, and their role in collagen gene expression in a model of pulmonary fibrosis. Am J Pathol 1994, 145:114–125
21. Barral-Neto M, Barral A, Brownell CE, Yaw S, Ellingsworth LR, Twardzik DR, Reed SG: Transforming growth factor β in leishmanial infection: a parasite escape mechanism. Science 1992, 257:545–548
22. Mitchell J, Woodcock-Mitchell J, Reynolds S, Low RB, Leslie KO, Alder KB, Gabbiani G, Skalli O: α-Smooth muscle actin in parenchymal cells of bleomycin-injured rat lung. Lab Invest 1989, 60:643–650
23. Landrum AC: The staining of eosinophil polymorphs and enterochromaffin cells in histological sections. J Pathol Bacteriol 1944, 56:441–445
24. Barkans JR, Mogbel R, Bradleg B, Fraw AJ, Kay AB: Immunohistological comparison of an anti-MBP monoclonal antibody (mAB) with other anti-eosinophils antibodies. Clin Exp Allergy 1990, 20-(suppl):1–9
25. Breen E, Shull S, Burne S, Absher M, Kelly J, Phan S, Cutroneo KR: Bleomycin regulation of transforming growth factor-β mRNA in rat lung fibroblasts. Am J Respir Cell Mol Biol 1992, 6:146–152
26. Isik FF, McDonald TO, Ferguson T, Yamanaka E, Gordon D: Transplant arteriosclerosis in a rat aortic model. Am J Pathol 1992, 141:1139–1146
27. Mangum JB, Everitt JI, Bonner JC, Moore LR: Coculture of primary pulmonary cells to model alveolar injury and translocation of proteins. In Vitro Cell Dev Biol 1990, 26:1135–1143
28. Kelley J, Shull S, Walsh JJ, Cutroneo KR, Absher M, Baldor L: Auto-induction of transforming growth factor-β in human lung fibroblasts. Am J Respir Cell Mol Biol 1993, 8:417–424
29. Fava RA, McClure DB: Fibronectin-associated transforming growth factor. J Cell Physiol 1987, 131:184–189
30. Rochester CL, Ackerman SJ, Zheng T, Elias JA: Major basic protein regulation of cytokine production by human lung fibroblasts. Am Rev Respir Dis 1994, 149:A13