Transfer of Granulocyte-Macrophage Colony-stimulating Factor Gene to Rat Lung Induces Eosinophilia, Monocytosis, and Fibrotic Reactions

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine whose expression is increased in numerous respiratory diseases, particularly in asthma. However, the role of GM-CSF in the pathogenesis of these conditions in vivo remains unclear. Here, we report the functional activities of GM-CSF highly expressed in rat lung after intrapulmonary transfer of the gene coding for murine GM-CSF by using an adenoviral vector. This high, transient expression of GM-CSF led to the sustained but self-limiting accumulation of eosinophils and macrophages associated with tissue injury in the lung followed by varying degrees of irreversible fibrotic reactions observed in later stages. These results suggest that GM-CSF plays a previously unrealized role in the development of respiratory conditions characterized by eosinophilia, granuloma and/or fibrosis and provide the rationale for targeting this molecule in these diseases. (*J. Clin. Invest.* 1996. 97:1102–1110.) **Key words:** cytokine • eosinophil • macrophage • fibrosis • adenoviral vectors

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF)¹ is a member of the hematopoietic cytokine family which also includes interleukin (IL)-3 and IL-5. GM-CSF differs from IL-3 and IL-5 in that it can be produced by and act upon a much wider spectrum of cell types (1, 2). Abundant in vitro evidence has shown that GM-CSF stimulates hematopoiesis of both granulocyte and monocyte lineages from the bone marrow and causes activation and prolonged survival of mature

granulocytes and monocytes (1, 2). These activities of GM-CSF, particularly those on eosinophils and macrophages, are speculated to contribute to the molecular mechanisms underlying numerous pulmonary disorders including asthma characterized by eosinophilic infiltration, macrophage activation and elevated tissue expression of hematopoietic cytokines including GM-CSF (3–5). However, the functional aspects of GM-CSF have not been fully studied in the context of local lung tissue. Overexpressing GM-CSF in hematopoietic cells in genetic or retroviral-mediated transgenic mice failed to elicit eosinophilia in the lung, yet both developed a lethal syndrome primarily mediated by macrophage or macrophage/neutrophil expansion (6, 7). These data strongly suggest a necessity for developing a model to investigate the function of GM-CSF expressed locally in the lung.

The advent of adenoviral-mediated gene transfer techniques provides us a unique opportunity to study pulmonary activities of GM-CSF (8). Using an animal model previously well characterized by us (9, 10), the cytokine transgene carried by adenoviral vectors can be distinctly targeted to and expressed by bronchial and alveolar epithelial cells and, to some degree, alveolar macrophages in the lung. This gene transfer approach differs from genetic transgenics in that it results in an efficient, transient but sustained, and lung tissue-directed expression of transgene protein (9–12). These features closely mimic events occurring locally in the lung during inflammation.

We have provided here the first in vivo evidence that overexpression of GM-CSF in rat lung by using a recombinant replication-deficient adenoviral vector elicits a significant eosinophilic response and macrophage granuloma formation accompanied with tissue damage. We have also provided evidence suggesting a previously yet unrecognized role for GM-CSF in the development of pulmonary fibrotic reactions.

Methods

Construction of recombinant adenovirus vectors. An 800-bp fragment of murine GM-CSF cDNA was isolated from pCDSR α by digestion with BamHI and DraI. The shuttle plasmid pACCMV containing 0 to 17 mu human type 5 adenovirus genome with a CMV promoter (760 bp), multicloning sites and SV40 splicing junction/polyA signal (430 bp) inserted in the E1 region of viral genome (kindly provided by Dr. Bob Gerrard at the Department of Cardiology, Howard Hughes Medical Institute, University of Texas), was first digested with SalI, and the ends were repaired using T4 kinase and dNTPs (New England Biolabs, Inc., Beverly, MA), followed by a secondary digestion with BamHI to generate the 3' complementary ends. The GM-CSF fragment was then subcloned into the BamHI/SalI site in PACCMV using T4 ligase (New England Biolabs) to generate the recombinant plasmid PACCMVmGM-CSF (see Fig. 1 a). The presence of GM-

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1. **Abbreviations used in this paper:** BAL, bronchoalveolar lavage; GM-CSF, granulocyte-macrophage colony stimulating factor.

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CSF insert was confirmed by restriction digestions. The PACCM-VmGM-CSF was co-transfected, following a standard procedure previously described by us (13), into 293 cells along with a plasmid pAdBHG10 which contained the most rightward sequences (3.7 to 100 mu) of human type 5 adenovirus genome with a partial deletion in the E3 region (14). The recombinant replication-deficient adenovirus Ad5E1PACCMVmGM-CSF (Ad5E1GM-CSF) was rescued by homologous recombination (see Fig. 1 a). The presence of GM-CSF cDNA in the viral genome was verified by analyzing viral genome fragments upon HindIII digestion and by Southern hybridization (13). The control virus Ad5dl70-3 was constructed and characterized as previously described (14), and similar to Ad5E1GM-CSF, this virus had the E1 region crippled, hence incapable of replication.

High titers of the above viruses were generated as previously described with some modifications (13). Briefly, viruses purified by two rounds of CsCl gradient centrifugation were subjected to chromatography using PD-10 Sephadex columns (Pharmacia Biotech Inc., Baie d'UrFe, Quebec, Canada) to remove CsCl. The virus fractions were collected in PBS containing 10% glycerol, measured for conductance to ensure a complete removal of CsCl, pooled, titrated, aliquoted and stored in -70°C until use.

Characterization of recombinant adenovirus vector expressing GM-CSF in vitro. GM-CSF transgene mRNA was examined by Northern hybridization analysis (15) using total RNA from 293 cells infected with 10 plaque-forming units (pfu)/cell of Ad5E1GM-CSF for 24 and 48 h. The supernatants from these cells and from infected rat alveolar macrophages were assayed for GM-CSF by using an ELISA kit (Endogene Inc., Cambridge, MA). This ELISA was specific for mouse GM-CSF without crossreactivity with rat GM-CSF, with the sensitivity of 4 pg/ml.

Delivery of recombinant adenovirus vectors to the lung. Following a standard procedure previously described by us (10), 300 μl of Ad5E1GM-CSF or control virus Ad5dl70-3 diluted in PBS to a concentration of 1×10^9 pfu, was instilled intratracheally to the lung of Sprague-Dawley male rats weighing 220–280 grams (Charles River Laboratories, Ottawa, Canada). At the end of 1, 2, 4, 7, 12, 18, and 24 d after gene transfer, rats were anesthetized, blood samples were taken from the abdominal aorta, and serum preparation and bronchoalveolar lavage (BAL) were performed as previously described (10).

Transgene and transgene protein expression in the lung. The left lung of rats obtained at each time point was snap-frozen in liquid nitrogen. Total lung RNA extraction and Northern hybridization were performed as previously detailed (15). Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine transgene mRNA expression with total lung RNA using PCR reagents from Promega Corp. (Madison, WI) following the standard protocol. The specific primers for PCR were chosen to ensure the amplification of the transgene-specific GM-CSF mRNA but not the endogenous rat GM-CSF mRNA (16, 17). The sense and anti-sense primer sequences were 5'-GTCTCTAACGAGTTCTCCTTCAAG-3' and 5'-TTCAGAGGGCTATACTGCCTTCCA-3', respectively. The primers for rat GAPDH were designed as described (18). BAL samples were collected at various times and assayed for transgene protein GM-CSF by ELISA as described above or for TNF α by ELISA specific for both murine and rat TNF α (Genzyme, Cambridge, MA). Serum samples collected from the same animals were also assayed for circulating levels of GM-CSF by ELISA.

Cytologic examination of BAL and blood samples. Total cell numbers in BAL were determined using a hemacytometer. Differential cell types were determined on cytopspins stained with Diff-Quik (Baxter, McGaw Park, IL) by randomly counting 300–400 cells/cytopsin. To analyze the total peripheral blood leukocyte counts and differentials, blood samples were collected into heparin-coated tubes. Total leukocyte numbers were counted on a hemacytometer after lysing red blood cells with a lysis buffer containing 94% H_2O , 3% acetic acid, and 3% Diff-Quik purple stain. Differential leukocytes were determined on blood smears stained with Diff-Quik by counting 500 to 700 cells/blood smear.

Histopathologic examination of lung and other tissues. The right lung and in some instances, the whole lung of each animal were fixed by perfusion with 10% formalin (Fisher Scientific, Fairlawn, NJ). Tissues from heart, liver, spleen, and kidney were also fixed in 10% formalin. Multiple sections from different lobes of the lung or from other organs were stained with hematoxylin/eosin for routine histopathology, with Congo Red for identification of tissue eosinophils (19, 20), or with Elastic van Gieson for collagen and elastin.

Results

Adenoviral vector expressing GM-CSF. To characterize the recombinant viral vector containing GM-CSF cDNA (Fig. 1 a), the HindIII-digested fragments from the viral genomic DNA of Ad5E1GM-CSF were hybridized to a cDNA probe for murine GM-CSF. The GM-CSF transgene in the genome was detected in a 2-kb fragment as expected (Fig. 1 b). To verify that this GM-CSF transgene could be efficiently transcribed, total cellular RNA from 293 cells infected for 24 or 48 h with Ad5E1GM-CSF or a control vector was hybridized with the cDNA probe. Only cells infected with Ad5E1GM-CSF displayed a strong transgene-derived message for GM-CSF with the predicted size being about 1.7 kb which was different from the endogenous 0.9 kb-sized mRNA species for GM-CSF (Fig. 1 c). The message was stronger at 24 h than at 48 h due to viral lysis of 293 cells. The transgene protein GM-CSF was markedly produced and released into the supernatant as detected by ELISA (775 ng/ 10^6 cells at 48 h). Infected primary rat alveolar macrophages also released significant amounts of GM-CSF for up to 3 wk in vitro (7–128 ng/ml). This transgene protein was bioactive on both murine and rat macrophages inducing proliferation and/or activation in vitro (data not shown).

Transgene and protein expression post-GM-CSF gene transfer to the lung. To study the function of GM-CSF expressed in the lung, Ad5E1GM-CSF or the control vector Ad5dl70-3 was intratracheally delivered into rat lung. Expression of transgene and transgene protein in the lung was examined at various times post-gene transfer. To examine transgene mRNA expression, the reverse transcription-polymerase chain reaction (RT-PCR) technique was employed using total RNA from lung tissues. A predicted 401-bp-sized PCR product for GM-CSF was detected only in the lung receiving Ad5E1GM-CSF but not the control vector (Fig. 2 a). Expression was strong between days 1 and 4, started to decline thereafter and became hardly detectable by day 24. By Northern gel analysis with the same RNA samples, a strong GM-CSF message was detected only at days 1 and 4 which was confirmed to be transgene-derived by its differential size, and there was no endogenous 0.9 kb GM-CSF message detected (data not shown).

To assess the transgene protein secretion in the lung, bronchoalveolar lavage (BAL) fluids were assayed for GM-CSF by ELISA. Consistent with the kinetics of transgene expression, high levels of GM-CSF (8–28 ng/ml) were detected in BAL for at least 4 d after GM-CSF gene transfer (Fig. 2 b), declined by day 7 and became undetectable by day 24. There were no detectable levels of transgene protein in BAL from rats receiving the control vector. To examine whether other proinflammatory cytokines were enhanced, rat tumor necrosis factor α (TNF α) in BAL was measured by ELISA. Small amounts of TNF α were detected only in some of BAL samples from either Ad5E1GM-CSF or control vector-treated animals at various

Figure 1. Construction and characterization of recombinant adenovirus Ad5E1PACCMVmGM-CSF (Ad5E1GM-CSF) expressing GM-CSF. (a) The recombinant plasmid PACCMMVmGM-CSF was constructed by inserting murine GM-CSF cDNA into the multicloning site of a shuttle vector pACCMV which harbored a cytomegalovirus promoter (CMV) and a SV40 splicing junction/poly A signal (polyA). The recombinant adenovirus Ad5E1GM-CSF was generated by homologous recombination after co-transfecting 293 cells with PACCMMVmGM-CSF and a virus-rescuing vector pAdBHG10. (b) The genomic DNA was extracted from purified Ad5E1GM-CSF and digested with HindIII. The resultant fragments were gel-separated and hybridized to a GM-CSF cDNA probe. Lanes 1 and 2, 5 and 10 μ g of viral genomic DNA from Ad5E1GM-CSF, respectively; lane 3, 5 μ g of viral genomic DNA from Ad5dl70-3 as a control. (c) 293 cells were infected with 10 pfu/cell of Ad5E1GM-CSF or Ad5dl70-3 as a control and total RNA was extracted and hybridized to a GM-CSF cDNA probe. Lanes 1 and 2, Ad5E1GM-CSF-infected cells at 24 and 48 h, respectively; lane 3, Ad5dl70-3-infected at 24 h.

times (0–46 pg/ml) and the differences, if any, were small between two treatments.

Transgene protein levels in serum post-GM-CSF gene transfer to the lung. To confirm that after intratracheal transgene delivery, the transgene product was compartmentalized within the lung as documented by us and others (9–12), sera taken at various times were assayed for GM-CSF by ELISA. Relatively low levels of GM-CSF (40–72 pg/ml) were detected only in sera from animals receiving Ad5E1GM-CSF (Fig. 2b). These levels of GM-CSF were about 200 to 400 times lower than those found in BAL and became undetectable by 12 d.

Changes in cellular components in BAL post-GM-CSF gene transfer to the lung. To assess the cellular responses to GM-CSF in the lung, total and differential cell counts in BAL were determined. Both Ad5E1GM-CSF and control vectors brought

about similarly low levels of neutrophilic and lymphocytic accumulation in BAL within the initial 4 d (data not shown). Moderately higher counts were noticed only at day 7 in animals receiving Ad5E1GM-CSF. These changes all declined thereafter towards background levels. These changes likely reflected the tissue response to the viral vectors, consistent with previous findings by us and others (10, 12). In contrast, an eosinophilic accumulation was seen only in BAL from animals overexpressing GM-CSF (Fig. 3a). This eosinophilic accumulation was apparent as early as 2 d after GM-CSF gene transfer, peaked between days 4 and 7 and declined towards background levels by day 18. The number of macrophages also significantly increased in BAL of these animals compared to controls (Fig. 3b). These increases were not prominent until day 4, became maximal at day 7, and were sustained for $\sim$ 2 wk.

Tissue eosinophilia and monocytosis post-GM-CSF gene transfer to the lung. To assess the effect of GM-CSF on eosinophil and macrophage accumulation in situ, lung tissues were histopathologically examined. Consistent with findings in

Figure 2. GM-CSF transgene and protein expression in the lung. (a) Ad5E1GM-CSF or Ad5dl70-3 as control was delivered intratracheally into rat lung and total lung RNA was extracted at days 1, 2, 4, 7, 12, 18, and 24 after gene transfer and amplified by PCR using specific primers for murine GM-CSF. The PCR products were hybridized to a GM-CSF cDNA probe. The same total RNA samples were also amplified by PCR for rat GAPDH as an internal control and the PCR products (555 bp) were visualized by ethidium bromide staining. (b) BAL fluids or sera were recovered at various times post-Ad5E1GM-CSF and assayed by ELISA for murine GM-CSF transgene protein. All samples from control vector-treated rats contained undetectable levels of GM-CSF (not graphed). Results are expressed as mean $\pm$ SEM from 3 to 5 animals/time point on logarithmic scale.

Figure 3. Effects of GM-CSF on eosinophils (a) and macrophages (b) accumulation in BAL. Rats were treated and BAL fluids recovered as above. Differential counts of cells in BAL were determined on cytospins stained with Diff-Quik. Results are expressed as mean $\pm$ SEM from 3 to 5 animals/time point.

BAL, the control vector elicited only some background infiltrates composed mainly of mononuclear cells mixed with some neutrophils in the perivascular and peribronchial areas, and eosinophils could be hardly seen throughout the lung at all times (Fig. 4 a and b). In contrast, significant tissue eosinophilia was observed in the lung of animals overexpressing GM-CSF. Using Congo Red specific staining, eosinophilic accumulation started to be seen as early as 1 d after GM-CSF gene transfer and became marked between days 2 and 12 in the perivascular and peribronchial regions (Fig. 4 c and e) and/or within the lesions of macrophage granuloma (Fig. 4 d). Often intravascular eosinophils could be seen. In comparison, few neutrophils were seen in these regions. From day 18 onward, much fewer eosinophils were seen.

In addition to eosinophilia, a massive parenchymal accumulation of macrophages was noticed from day 2 and became most striking at days 4 and 7 after GM-CSF gene transfer, in contrast to controls (Fig. 4 b). Microscopically, different-sized

granulomatous foci were seen throughout the lung, composed mainly of epithelioid cells and macrophages with infiltrating eosinophils and scattered multinuclear giant cells (Fig. 4 d, Fig. 5, a–c). Lymphocytes were only occasionally seen in areas near the vessel. Cells undergoing mitosis of different stages within granuloma were noticed, particularly at day 7 (Fig. 5 c). The alveolar septa largely disappeared in granuloma usually with some hyperplastic residual bronchial structures (Fig. 5, b and c). These macrophage granulomatous foci subsided largely with only some loose macrophage aggregates seen from day 12 onward.

Tissue remodeling after GM-CSF gene transfer to the lung. Fibrotic reactions following severe lung injury were examined macroscopically and microscopically in sections stained with hematoxylin/eosin or with Elastic van Gieson (EVG) specific staining for collagen and elastin. There were no apparent signs of fibrotic reactions in the lung of animals receiving the control vector throughout the study (Fig. 6 a). In the lung of animals overexpressing GM-CSF, up to day 7, there was no overt fibrotic disorganization of lung structure even within granulomas (Fig. 6 b). However, from day 12 onwards, the shrinkage of lung lobes, particularly the right upper lobe, was noticed in most animals examined for each time point. The shrinkage appeared more prominent near the hila and worsened with time. Microscopically, at day 12, fibrous meshworks were seen in the perivascular and peribronchial areas or in the parenchyma, morphologically reminiscent of granulation tissue containing fibroblast-like cells, capillaries, mononuclear cells and eosinophils as well as collagen and elastic fibrils (Fig. 6, c and d). The normal alveolar architecture disappeared in these regions. By days 18 and 24, the previous granulation tissue appeared to be replaced by an advanced fibrous meshwork with disorganized collagen and elastic fibers (Fig. 6, e–g), particularly in areas underneath the shrunk lung surface (Fig. 6, f and g). In some areas, particularly at day 24, there were bundles or patches of fibrous tissue rich in fibroblast-like cells, collagen and elastin with pigmented macrophages, and the adjacent air spaces were often seen with thickened fibrous septa (Fig. 6, h–j). Table I summarizes the above GM-CSF-induced morphologic changes.

Changes in peripheral blood leukocyte counts and other organs post-GM-CSF gene transfer to the lung. The effect of circulating GM-CSF on blood leukocyte counts and other organs was examined. Total leukocyte counts were not significantly different between controls and animals expressing GM-CSF. Neither were numbers of monocytes and lymphocytes. There was about onefold increase in the number of neutrophils noticed only between days 1 and 4 after GM-CSF gene transfer. The number of blood eosinophils was markedly increased. The increases were most evident at days 4 and 7 being 12 and 8 times higher than controls, respectively. Upon examination of histopathology of the spleen, heart, liver and kidneys, increased numbers of eosinophils were found only in the spleen, mainly in the perifollicular zones, at day 4 (data not shown). The other organs had no significant histopathologic alterations.

Discussion

We have created a model to study the pulmonary activities of GM-CSF by highly expressing this molecule within the lung of

Figure 4. Lung tissue eosinophilia induced by GM-CSF. Lung tissues obtained at various times were fixed by perfusion with 10% formalin. (*a* and *b*) Days 2 and 4 after control vector Ad5dl70-3, Congo red stained and hematoxylin counterstained; (*c*, *d*, and *e*) Days 2, 4, and 12 after Ad5E1GM-CSF, Congo red-stained and hematoxylin counterstained. (*Arrows*) Some of eosinophils stained red. (*B*) bronchial epithelium. *a*, $\times 590$; *b* and *e*, $\times 750$; *c* and *d*, $\times 470$.

normal adult animals using a viral-mediated transgene approach. This expression was characterized by its transient but sustained nature. GM-CSF was shown to induce a marked tissue eosinophilia and macrophage granulomatous reaction which correlated with the kinetics of GM-CSF transgene expression in the lung. These dramatic cellular events were fol-

lowed by a tissue repair process comprising early granulation tissue formation and later fibrotic remodeling.

GM-CSF has been shown *in vitro* to activate eosinophil functions including differentiation, chemotaxis, chemotaxis priming, survival, transendothelial migration, expression of CD11b, CD4 and HLA-DR, and mediator release (21–23).

Figure 5. Lung macrophage granuloma formation induced by GM-CSF. (*a* and *b*) Day 4; (*c*) Day 7 after Ad5E1GM-CSF, hematoxylin/eosin (HE) stained. (*B*) Residual hyperplastic bronchial structure within a granuloma. (*Arrowhead*) Multinuclear giant cell within a granuloma. (*Open arrow*) Mitotic figure of anaphase. *a*, $\times 250$; *b*, $\times 320$; *c*, $\times 630$.

Figure 6. Lung fibrotic reactions induced by GM-CSF. (a) Day 12 after control vector Ad5dl70-3, EVG staining collagen in red and elastin in black. (B) Bronchial structure. (V) Vascular structure; (b) Day 4 after Ad5E1GM-CSF, EVG stained. (B) Bronchial epithelium. (V) Vascular structure; (c) Day 12 after Ad5E1GM-CSF, HE stained. (Arrowheads) Capillaries. (Open arrows) Fibroblast stromal cells. (Double arrow) Pleural surface; (d) Day 12 after Ad5E1GM-CSF, EVG stained; (e) Day 18, EVG stained. (f) Day 24, HE stained. (B) Residual hyperplastic bronchial structure in fibrous tissue. (Arrow) Shrunken lung surface. (g) Day 24 after Ad5E1GM-CSF, EVG stained. (Arrow) Shrunken lung surface. (h-j) Day 24 after Ad5E1GM-CSF, HE stained (h) or EVG stained (i and j). (Arrowheads) Pigmented macrophages. f and g, $\times 150$; h, $\times 250$; a, c, d, e, and j, $\times 315$; b, $\times 400$; i, $\times 630$.

Table I. Highlights of Histopathology in the Lung Overexpressing GM-CSF

Day	Eosinophilia	Monocytosis	Epithelium/alveolar septum injury	Granulation tissue formation	Collagen deposition	Elastin deposition	Lung shrinkage
2	++	+					
4	+++	+++	+++				
7	+++	+++	+++				
12	++	+	+	+++	++	++	+
24		+	+	+	++	+++	+++

Morphologic observation was made on multiple sections from each lung specimen. A total of 3–6 animals were used for each time point. The extent of each histologic change is expressed as significant (+++), moderate (++), mild (+), or insignificant (blank). Eosinophils and deposition of collagen or elastin were identified using specific staining procedures (see Methods).

Significantly increased levels of this cytokine are detected in sera, BAL and lung tissues associated with increased numbers of blood and tissue eosinophils and other inflammatory cell types in asthmatic patients (23–25). Increased amounts of GM-CSF are also found in BAL of patients with chronic lung diseases of nonallergic nature but associated with eosinophilia (26). However, the *in vivo* role of GM-CSF in the pathogenesis of eosinophilia in these conditions has remained speculative. Here we provide *in vivo* evidence directly linking GM-CSF to the occurrence of eosinophilia in the lung. The intensity of eosinophil accumulation in the lung was markedly underestimated by the information gained from BAL since most eosinophils were found present in the perivascular and peribronchial connective tissues and granulomas. In addition, since the stain used may largely identify granulated cells in the tissue, the degree of lung eosinophilia may also be underestimated. The early onset and the maintenance of eosinophilia in the lung correlated with the kinetics of GM-CSF levels in the lung and of elevated numbers of blood eosinophils. The latter likely represented a bone marrow response to low circulating levels of GM-CSF leaking out of the lung. The lung was the only tissue site accumulating eosinophils. The transient increase of eosinophils in the perifollicular zones of the spleen likely mirrored increased numbers of blood eosinophils as the cells residing in these zones are normally proportionate to those in the blood. Thus, the increased number of blood eosinophils and the migration of these cells towards high concentrations of GM-CSF within the lung, perhaps in conjunction with increased local survival, may be key components in the mechanisms underlying sustained pulmonary eosinophilia. The fact that GM-CSF only stimulates neutrophil hematopoiesis and activation but not chemotaxis (27) may explain why in our case there was a mild neutrophilia in the blood but not in the lung.

The contribution of other relevant molecules to eosinophilia in our model seems unlikely. The levels of TNF α in the lung were low and the increases over control were small. IL-5 is the other hematopoietic cytokine capable of activating eosinophils *in vitro* and has been detected in blood, BAL and lung tissues of asthmatics, similar to GM-CSF (3, 4). Transgenic mice overexpressing IL-5 display a marked peripheral blood eosinophilia with some degrees of eosinophilic infiltration in the lung interstitium (19). We have recently found that adenoviral-mediated overexpression of IL-5 in the lung induces a pulmonary eosinophilia. However, the participation of IL-5 in GM-CSF-induced eosinophilia was unlikely since IL-5 mRNA expression was not detected at any time point in our

model (data not shown). The cytokine specificity for the development of eosinophilia in the lung is further supported by our findings that overexpressing IL-6 or macrophage inflammatory protein 2 (MIP-2) in the lung does not induce eosinophilia.

The potent effect of GM-CSF on macrophage granuloma formation we observed reveals the functional diversity of GM-CSF in the lung. Like eosinophilia, the information obtained from BAL analysis far underestimated the potency of GM-CSF on macrophage expansion because of granuloma formation in the tissue. The GM-CSF-induced granuloma was predominantly composed of macrophages, macrophage-derived epithelioid cells and giant multinuclear cells, and eosinophils, morphologically similar to the nonimmune type of granuloma (28, 29). The lack of lymphocytic infiltration suggests a direct effect of GM-CSF on granuloma formation. The findings that GM-CSF caused little change in blood monocyte counts, and only a small number of monocytes but marked mitotic activities were found in granulomas, suggest that granuloma formation resulted primarily from local expansion of macrophages with a contribution from enhanced monocyte recruitment and local differentiation of monocytes to macrophages. Indeed, we and others have shown that *in vitro* GM-CSF promotes monocyte survival and differentiation to macrophages and macrophage proliferation (2). The effect of GM-CSF on macrophage expansion has also been shown in GM-CSF transgenics (6, 7). Significantly elevated amounts of GM-CSF have been found in certain forms of lung granulomas (30). While the molecules involved in lung granuloma formation still remain largely unknown, our findings suggest that GM-CSF may be one of key molecules involved in the pathogenesis of pulmonary granulomas characterized by macrophage and eosinophil accumulation such as eosinophilic granuloma (histiocytosis X), Wegener's granulomatosis and parasitic granulomatous inflammation (31).

Unexpectedly, a lung tissue remodeling process followed GM-CSF-induced eosinophilia, macrophage hyperplasia and lung injury. This process proceeded from an early granulation tissue formation to progressive fibrotic derangements of lung architecture, indicating the irreversible nature of the response. This sequence of events suggests that GM-CSF may induce fibrotic reactions at least in part through causing lung parenchymal injury subsequent to macrophage and eosinophil activation, thus supporting an "injury-fibrosis" theory (32). In addition, GM-CSF may directly stimulate lung stromal cell types to undergo migration and proliferation in this process (33, 34). Indeed, by using immunohistochemistry, we have identified

accumulation of myofibroblast-like cells positive for α -smooth muscle actin in the fibrosing areas in the lung 12 and 24 d after GM-CSF gene transfer (data not shown). The minipump-delivered GM-CSF has also been shown to elicit emergence of myofibroblasts in the peritoneal cavity of rats (35). Peritoneal fibrous tissue formation is also noticed in GM-CSF transgenics (6). Currently, we are investigating whether there is increased production of fibrogenic cytokines such as TGF β in response to GM-CSF overexpression in the lung. It is noteworthy that the fibrotic response we observed seems to be unique to GM-CSF lung overexpression since there is no fibrotic sequela observed by us associated with lung overexpression of IL-6, MIP-2, or IL-5 which induces a specific expansion in rat lung of lymphocytes, neutrophils, or eosinophils, respectively. Of particular interest is that IL-5 overexpression in the lung only induces eosinophilia and mild lymphocytic accumulation but not macrophage expansion and fibrosis, suggesting that IL-5 has rather restricted biologic effects in the lung. In contrast, GM-CSF represents a potent proinflammatory cytokine capable of activating a wide spectrum of inflammatory mechanisms in the lung. And the final tissue remodeling response to GM-CSF may involve coordinated signals from both macrophages and eosinophils. Indeed, both eosinophils and macrophages are found to be temporally linked to a number of lung diseases with fibrotic sequela including asthma (36), some forms of granulomas and interstitial lung diseases associated with eosinophilic pneumonia (31). Recently, Zhang and his colleagues in Phan's laboratory have identified eosinophils as a source of TGF β in bleomycin-induced pulmonary fibrosis (37). Thus, GM-CSF may be now considered as a fibrogenic cytokine along with TGF β , FGF, PDGF, and TNF α (35, 38). Having discussed all of these, it is worth noting that the adenoviral-mediated gene transfer approach, like any other gene transfer technologies, is by no means perfect since transgene expression is achieved via viral infection. Thus, it cannot be completely ruled out that the GM-CSF-mediated effects occurred over a background of viral infection.

Taken together, we have shown that GM-CSF represents a potent cytokine whose overexpression in the lung of adult normal animals induces a pulmonary triad: eosinophilia, macrophage granuloma, and fibrotic reaction. These findings have implications in designing therapeutic interventions for lung diseases such as asthma and call for attention to the role of GM-CSF in the pathogenesis of those pulmonary granulomas with yet unknown causes and mechanisms. On the other hand, GM-CSF gene transfer may be useful in combating cancers via its effects on macrophages and eosinophils and to treat genetic lung diseases with GM-CSF deficiency such as pulmonary alveolar proteinosis.

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