INHIBITION OF GM-CSF PRODUCTION IN FIBROBLAST-MONOCYTE COCULTURE BY PREDNISONE AND EFFECTS OF rhGM-CSF ON HUMAN LUNG FIBROBLASTS

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1. ABSTRACT

Fibroblasts play a sentinel role in asthmatic disease. They are the main constituents of connective tissue and are increased in number in the asthmatic lung. They are also capable of secreting a diverse repertoire of cytokines and are able to be activated by pro-inflammatory cytokines and cell-cell contact. Previously we have reported that normal human lung fibroblasts (NHLF) can be activated by monocytes (U937) through cell-cell contact to produce GM-CSF. Here we show that GM-CSF production from NHLF activated by monocyte contact is inhibited by prednisone, a synthetic glucocorticoid used in the treatment of asthma. GM-CSF is an acidic glycoprotein that potentiates development of cells in the granulocyte and macrophage lineage and is secreted at sites of peripheral inflammation. The receptor for GM-CSF was found on NHLF by flow cytometry and was able to be up-regulated by interleukin (IL)-1 beta, tumor necrosis factor (TNF)-alpha and recombinant human (rh) GM-CSF. To test autocrine effects of GM-CSF on fibroblasts, rh GM-CSF was used in proliferation studies and was found to decrease fibroblast proliferation. Prednisone was used to block NF-kappaB activation and GM-CSF gene expression as well. These data indicate mechanism of action and treatment for cell-cell contact mediated inflammation of infiltrating monocytes with fibroblasts as seen in asthma and other diseases like graft versus host disease.

2. INTRODUCTION

Asthma is a chronic lung disease that affects 14-15 million persons in the United States alone (1). Many cytokines potentiate the inflammatory response associated with allergic asthma. Fibroblasts can also secrete some of these pro-inflammatory cytokines when activated. These cytokines cause vasodilation, increased mucus production, and broncho-constriction associated with an asthmatic attack. Upon initial sensitization, antigen presenting cells like macrophages present major histocompatibility complex (MHC) bound antigen to T-cells. The subsequently activated T-cells release interleukin (IL)-4 and IL-13 which
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promote B-cell differentiation into IgE secreting plasma cells. Antigen specific IgE may further cross-link the high affinity IgE receptor (Fc epsilon RI) on the surface of mast cells, causing them to degranulate. Mast cell release potent synthesized and preformed mediators that potentiate airway inflammation (2,3).

Fibroblasts are capable of contributing to host defenses by producing cytokines like IL-6, IL-8, macrophage chemoattractant protein-1 (MCP-1), and granulocyte macrophage-colony stimulating factor (GM-CSF) (4,5). Of interest, GM-CSF is a pluripotent cytokine that recruits and activates eosinophils and has colony stimulating effects on granulocytes and macrophages. Eosinophil number and activation have been implicated in the severity of airway hyperresponsiveness and sensitivity (6,7). Eosinophils themselves are highly granulated and can produce many damaging molecules like eosinophilic cationic protein and major basic protein. These cells play an important role in the late phase response of allergic reactions and help lead to the damage and repair mechanisms that are associated with airway remodeling. GM-CSF, along with IL-3 and IL-5, is a potent growth factor for eosinophils and increases their survival in peripheral tissues by as much as several days (8-10). Fibroblasts may add to this eosinophilic collection of anti-apoptotic cytokines by producing GM-CSF. Monocytes, on the other hand, are potent antigen presenting cells known to infiltrate tissues of the asthmatic lung.

We have previously shown that U937 as well as freshly isolated peripheral blood monocyte contact is a potent activator of GM-CSF production in NHLF and that this is mostly mediated by direct contact of the cells with NHLF. Cellular membrane isolation experiments showed that U937 derived membranes induced GM-CSF production in NHLF while NHLF derived membranes failed to induce GM-CSF production in U937- indicating that NHLF were the primary producers of GM-CSF (11). Here we studied the expression of GM-CSF receptor on NHLF and its regulation by proinflammatory cytokines (IL-1beta, and tumor necrosis factor- alpha) and recombinant human (rh) GM-CSF. We also studied the effects of rhGM-CSF on fibroblast proliferation. For its clinical significance, prednisone was used in studying GM-CSF inhibition in NHLF-U937 cocultures along with nuclear factor-kappa B (NF-kappaB) activation and GM-CSF gene expression. NF-kappaB is a nuclear factor that when freed from an inhibitor, I kappaB, in the cytoplasm, relocates to the nucleus where it induces transcription of pro-inflammatory genes like TNF-alpha, IL-1beta, IL-6, IL-8, intercellular adhesion molecule-1 (ICAM-1), and GM-CSF (1).

3. MATERIALS AND METHODS

3.1. Tissue Culture

Normal Human Lung Fibroblasts (NHLF) (Clonetics-BioWhittaker, Walkersville, MD) were grown in Fibroblast Basal Medium (Clonetics-BioWhittaker, Walkersville, MD) and incubated in 5% CO2 at 37°C. Media was supplemented with 2% fetal bovine serum, human fibroblast growth factor-B (1.0 microgram/mL), insulin (5μg/mL), gentamicin and amphotericin B, according to supplier’s instructions. NHLF were cultured in 12 well culture plates at cell concentrations of 2.0 x 10^5 cells per well and incubated overnight. U937 cells were grown in RPMI 1640 (Gibco BRL, Frederick, MD) supplemented with 11.1% FBS and 1% 1M Hepes buffer solution (Rockville, MD) as previously described (11). Co-culturing of fibroblasts with U937 cells was done in 12 well plates with 2.0 x 10^5 NHLF per well and 1.0 x 10^6 cells/mL of U937 in 2mL cultures of NHLF media for 24 hours. For GM-CSF production studies, prednisone was added at 0.1, 1.0, and 10 micromolar to NHLF cultures and allowed to incubate for 48 hours before addition of fresh media with U937 cells. For NF-kappaB activation studies, NHLF were grown in 100 x 20 mm tissue culture plates (Falcon, Franklin Lakes, NJ) at 1 x 10^6 cells/plate in 5 mL of media overnight. Prednisone was then added at 1.0 micromole and allowed to incubate untouched for 48 hours. After 48 hours, new media with U937 (1 x 10^6 cells/mL) was added and allowed to incubate for two hours. The NHLF were then washed, trypsinized and frozen at -80°C until electromobility shift assay (EMSA) was performed.

3.2. Assay Of Cytokine Production

GM-CSF levels in cell-free supernatants were assayed by enzyme linked immunosorbent assay (ELISA) as previously described using commercially available kits (R&D Systems, Minneapolis, MN) (11-13). A standard curve was plotted with increasing concentrations of GM-CSF. Supernatants were incubated in a 96 well microtiter plate and levels of GM-CSF assayed. Results were analyzed on an ELISA plate reader (Dynatech MR 5000 with supporting software) (12,14,15).

3.3. Flow Cytometry

NHLF were stimulated with IL-1beta (10 ng/mL), TNF-alpha (100 U/mL), and rhGM-CSF (100 ng/mL) and incubated overnight in 5% CO2. After 24 hours the cells were harvested with a rubber policeman, stained in the dark with monoclonal antibody for 30 minutes, washed in Dulbeccos PBS (pH 7.4) and then fixed in a 1% para-formaldehyde solution. Samples were stained with GM-CSF receptor alpha chain (CD 116) antibody conjugated with FITC (PharMingen BD, San Hose, CA) and assayed with a Becton Dickinson flow cytometer (FACScan) (16).

3.4. NHLF proliferation

NHLF treated with rhGM-CSF (0, 10, and 100 ng/mL) were subjected to culture in a 96 well plate for 72 hours. Two hundred microliters of cells at 1.0 x 10^5/mL were added to each well and allowed to incubate undisturbed for 72 hours. Six hours before harvest, tritiated thymidine was added to be incorporated by proliferating cells. At the end of 72 hours cells were trypsinized and aspirated through Mash II glass fiber filter paper #23-985 (grade 394 AH) (Whittaker, Walkersville, MD). Filter papers were suspended in scintillation fluid and read on a beta counter from Beckman Coulter (Irvine, CA) (15).

3.5. Nuclear Factor- Kappa B Assay

Nuclear proteins were extracted from NHLF by a method previously described with modifications (11,17).
NHLF were trypsinized from 100 x 20 mm plates at 2.0 x 10^6 cells per plate, washed three times in PBS, and collected in a 1.5 mL microcentrifuge tube. To add to this was 100 microliters of ice cold hypotonic buffer: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 micromolar aprotinin, 1 micromolar pepstatin, 14 micromolar leupeptin, 50 mM NaF, 30 mM beta-glycerophosphate, 1 mM Na3VO4, and 20 mM p-nitrophenyl phosphate. Cells were incubated on ice for 30 minutes and vortexed after addition of 6.25 microliters of 10% Nonidet P-40. After 2 minutes of centrifugation at 30,000 x g, supernatents were kept at -80°C overnight. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous layer was washed twice with 0.8 mL phenol:chloroform (1:1, v/v), and once with 0.8 mL of chloroform. Each time, the suspension was centrifuged at 12,000 x g for 15 minutes at 4°C. An equal volume of isopropanol was added to the aqueous phase, and the preparation was refrigerated at -20°C overnight. The samples were then centrifuged at 12,000 x g for 30 minutes at 4°C and the RNA pellet washed with 1 mL 75% ethanol. The RNA pellet was air dried and suspended in 20 microliters of DEPC-treated water. RNA was quantitated by optical density readings at 260 nm, and electrophoresed in ethidium bromide-stained agarose gels at 1000 ng per well to determine the integrity of the 28S and 18S RNA bands. First strand cDNA was synthesized in the presence of murine leukemia virus reverse transcriptase (2.5 U/microliter), 1 mM each of the nucleotides dATP, dCTP, dGTP and dTTP; RNase inhibitor (1 U/microliter), 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), and MgCl2 (5 mM), using oligo(dT)19 (2.5 micromolar) as a primer. The preparation was incubated at 42°C for 2 minutes in a DNA thermocycler (Perkin-Elmer Corp., Norwalk, CT) for reverse transcription. PCR amplification was done on aliquots of the cDNA in the presence of MgCl2 (1.8 mM), each of dNTPs (0.2 mM), and AmpliTaq polymerase (1 U/50 microliters), and paired cytokine-specific primers (0.2 mM of each primer) to a total volume of 50 microliters. PCR consisted of 1 cycle of 95°C for 2 min, 28 cycles of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min, and lastly, 1 cycle of 72°C for 10 min. Ten microliters of the amplified products were subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide. GM-CSF bands were compared to predicted base pair migration distances from a PhiX 174 Hae III DNA marker (Promega, Madison, WI).

### 3.7. Statistical Analysis

All values are given as the mean plus or minus (±) standard deviation (SD). Statistical analysis was done using the Student's t-test and Statistica version 5 computer software (StatSoft, Inc Tulsa, OK). A p-value of < 0.05 was considered significant.

### 4. RESULTS

#### 4.1. NHLF-U937 Cocultures Induce GM-CSF Expression

To test the effect of U937 contact on NHLF GM-CSF production, we set up a coculture system to incubate NHLF and U937 together. For comparison, TNF-alpha, a potent activator of GM-CSF in NHLF (11) was added to NHLF alone and then to NHLF-U937 cocultures to see if it had any additional effect on GM-CSF production. As shown in figure 1, GM-CSF production in NHLF and U937 controls was minimal (21.23 ± 1.97 pg/mL and 1.37 ± 0.21 pg/mL respectively.) When NHLF were treated with TNF-alpha or cocultured with U937, GM-CSF production rose significantly (557.70 ± 64.20 pg/mL and 618.60 ± 53.82 pg/mL, respectively).
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Figure 2. GM-CSF receptor expression on resting NHLF (A). NHLF were treated with IL-1beta (10 ng/mL) (B), TNF-alpha (100 U/mL) (C), or rhGM-CSF (100 ng/mL) (D) for 24 hrs before being harvested and assayed by flow cytometry. An isotype antibody was used as a negative control for the GM-CSF FITC labeled antibody.

Figure 3. Effects of rhGM-CSF on NHLF proliferation. NHLF were grown in the presence of 0, 10, or 100 ng/mL rhGM-CSF for 72 hours. Six hours prior to harvest, tritiated thymidine was added to be incorporated by live cells. NHLF were harvested and assayed on a beta counter. rhGM-CSF at 10 ng/mL and 100 ng/mL significantly decreased NHLF proliferation after 72 hours. Data are means ± SD for quadruplicate samples. * p< 0.003 and ** p < 0.0002 compared to the control.

4.2. NHLF Express GM-CSF Receptor

Normal human lung fibroblasts express high amounts of receptor for GM-CSF as seen by flow cytometry. GM-CSF receptor expression on resting NHLF were high (93.5%) (figure 2A). When NHLF were treated with IL-1beta at 10 ng/mL for 24 hours, GM-CSF receptor expression rose slightly (97.2%) (figure 2B). Treatment with TNF-alpha for 24 hours at 100 U/mL also produced an increase, but to a lesser extent (95.2%) (figure 2C). In order to determine whether GM-CSF had any effect on the expression of its own receptor in NHLF, we treated NHLF cells with 100 ng/mL of rhGM-CSF for 24 hours and assayed for GM-CSF receptor expression as before. rhGM-CSF was able to slightly increase the expression of its own receptor on NHLF (94.7%) (figure 2D). GM-CSF receptor expression was also detected on U937 by flow cytometry but was unable to be regulated by IL-1beta, TNF-alpha, or rhGM-CSF (data not shown).

4.3. Autocrine Effects of GM-CSF on NHLF Proliferation

We next sought to determine if the GM-CSF receptor expressed on NHLF was functional. Proliferation studies with tritiated thymidine were conducted with increasing amounts of rhGM-CSF. After 72 hours, NHLF proliferation was decreased with rhGM-CSF treatment. Ten nanograms per milliliter (10 ng/mL) and 100 ng/mL of rhGM-CSF significantly inhibited NHLF proliferation (p < 0.003 and p < 0.0002, respectively) (figure 3), with 100 ng/mL having almost a 50% reduction in cellular uptake of tritiated thymidine.

4.4. Prednisone Down Regulates GM-CSF production in NHLF-U937 Cocultures

NHLF were pretreated with 0.1, 1, and 10 micromolar of prednisone and then subjected to coculture with U937. NHLF and U937 control levels of GM-CSF were low, 2.42 ± 0.28 pg/mL and 2.04 ± 0.28 pg/mL respectively, while NHLF plus U937 produced significantly higher amount s of GM-CSF (690.7 ± 56.2 pg/mL, p < 0.0005 compared to both NHLF and U937 control). Preincubation with prednisone produced a significant dose dependent reduction in GM-CSF expression in NHLF-U937 cocultures. Prednisone at 0.1 micromolar caused a reduction in GM-CSF production however it was not significant (543.51 ± 26.14 pg/mL). Treatment with 1 micromolar and 10 micromolar concentrations of prednisone resulted in a significant decrease in GM-CSF production (478.2 ± 26.7 pg/mL and 283.6 ± 11.6 pg/mL respectively; p < 0.05 and p < 0.01 respectively compared to coculture alone) (figure 4).

4.5. Effects of Prednisone on NF-kappaB Translocation and GM-CSF Gene Expression in NHLF

NF-kappaB is an important transcription factor that mediates the transcription of many proinflammatory genes, including GM-CSF. For this reason we subjected our coculture to NF-kappaB analysis by EMSA and found that coculturing of U937 with NHLF induced NF-kappaB activation (figure 5A). Preincubation of the NHLF for 48 hours prior to the addition of U937 with 1 micromolar prednisone reduced the amount of NF-kappaB that translocated to the nucleus. Preincubation with prednisone also decreased gene expression of GM-CSF as detected by RT-PCR (figure 5B).

5. DISCUSSION

Fibroblasts play a major role in the pathogenesis of fibrotic disease, including asthma and airway
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Figure 4. The effects of prednisone on inhibiting GM-CSF production in NHLF-U937 coculture. NHLF were pretreated with prednisone for 48 hours prior to addition of U937 and then harvested after 24 hours and assayed by ELISA. Prednisone was able to decrease GM-CSF production in cocultures in a dose dependent manner. Data are means ± SD for duplicate samples. * p < 0.0005 compared to NHLF and U937 controls; ** p < 0.04 and *** p < 0.01 compared to coculture.

Figure 5. A) NF-kappaB activation in NHLF-U937 cocultures. NHLF were pretreated with prednisone and then cocultured with U937 for 2 hours before being harvested for NF-kappaB expression by EMSA. B) GM-CSF gene expression in NHLF-U937 cocultures. RT-PCR analysis shows induction of GM-CSF gene expression in NHLF after six hours of coculture with U937. Pretreatment with prednisone reduced GM-CSF gene expression.

remodeling. Their position in the basal lamina of the submucosal lung allows them to mediate collagen deposition, respond to chemical mediators, and contact infiltrating cells. For this reason, we examined the production of a potent cytokine, granulocyte macrophage-colony stimulating factor (GM-CSF), from normal human lung fibroblasts (NHLF) cocultured with the myelomonocytic cell line U937. GM-CSF, as the name implies, stimulates granulocyte and macrophage growth and development, in particular, eosinophils. Eosinophils are associated with epithelial tissue damage and denudation in the airways of allergic persons. Also, Surquin et al. have shown the presence of eosinophil infiltrates in transplanted skin grafts of mice (18). When eosinophil infiltration was blocked with anti IL-4, anti IL-5, and anti CCR3 monoclonal antibodies, skin graft survival was prolonged. The importance of fibroblasts in eosinophil biology has been described as well. Vancheri et al. have reported that in vitro GM-CSF from human lung fibroblasts increased eosinophil survival (19). In addition, Solomon et al. showed similar results with conjunctival fibroblasts and eosinophiles (20). Here, eosinophils were cultured with human conjunctival fibroblasts and viability was assayed by trypan blue exclusion. They found that coculture of eosinophils with conjunctival fibroblasts increased eosinophil survival and that this was partially mediated by GM-CSF. We decided to see if GM-CSF had any effects on NHLF function and if NHLF-U937 coculture-induced GM-CSF production could be inhibited by prednisone. We also sought to derive a mechanism for prednisone mediated down regulation of GM-CSF production in our coculture. As we have previously described, U937 activate NHLF to produce GM-CSF (11). This activation was associated with NF-kappaB translocation and increased GM-CSF gene transcription. Direct cell to cell contact was needed for this activation, for when the NHLF and U937 were separated by a 0.4 micrometer porous membrane, GM-CSF production fell significantly. It was also shown by isolated cellular membrane experiments that NHLF were to main producers of this GM-CSF.

In this report we showed that monocyte (U937) contact is a potent activator of GM-CSF production in fibroblasts (NHLF), comparable to that of TNF-alpha, a known potent activator of NHLF (11). The combination of U937 and TNF-alpha had no additive or synergistic effects on the amount of GM-CSF produced, however (figure 1). A Previous study by Postiglione et al. has shown that human gengival fibroblasts express GM-CSF receptor alpha chain by flow cytometry (21). They also tested the effects of increasing rhGM-CSF concentrations (10 ng/mL to 150 ng/mL) on regulation of GM-CSF receptor in these cells and found that high concentrations of rhGM-CSF (80 ng/mL) increased fibroblasts proliferation. We also examined the expression of GM-CSF receptor on resting NHLF and found that rhGM-CSF at 100 ng/mL increased the expression of GM-CSF receptor. We also found that treatment with IL-1beta (10ng/mL) and TNF-alpha (100 U/mL) increased the expression of GM-CSF receptor over control levels (Figure 2 A, B, C, and D). GM-CSF receptor
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Figure 6. Schematic showing the activation of NHLF by U937 contact. Monocyte infiltrates can contact lung fibroblasts and induce GM-CSF production. IL-1beta, TNF-alpha, and rhGM-CSF upregulate GM-CSF receptor expression on NHLF. GM-CSF can decrease fibroblasts proliferation while having stimulatory effects on eosinophils. Eosinophils can therefore cause epithelial damage and allergic response.

Glucocorticoids, like prednisone, work by stabilizing the NF-kappaB/I-kappaB complex, thus preventing translocation to the nucleus. Prednisone binds to its receptor in the cytoplasm and induces a signal that increases transcription of the I-kappaB gene. Increased numbers of I-kappaB bind free NF-kappaB and prevent its translocation to the nucleus. Prednisone is used in the treatment of moderate to severe persistent asthma, and for this clinical importance, was used to test its effects on GM-CSF inhibition in our NHLF-U937 coculture. Pretreatment of NHLF with three concentrations of prednisone decreased GM-CSF production in a dose dependent manner in our cocultures (figure 4).

NF-kappaB has many important implications in inflammatory diseases as well. The translocation of NF-kappaB into the nucleus leads to the induction of pro-inflammatory genes like TNF-alpha, IL-1beta, IL-6, IL-8, ICAM-1, and GM-CSF. We showed that prednisone can inhibit NF-kappaB activation in NHLF that are activated by U937 contact (figure 5A). This inhibition led to the suppression of GM-CSF protein production. Drugs that work by inhibiting NF-kappaB in different ways may also play a potential role in patient care. Decreases in GM-CSF gene transcripts as viewed by RT-PCR were also inhibited by pretreatment with prednisone (figure 5B). These data suggests that prednisone inhibits GM-CSF production in NHLF-U937 cocultures by blocking NF-kappaB activation and thus blocking GM-CSF gene expression and protein production. Thus, these results provide a mechanism for prednisone treatment in asthma and other inflammatory diseases.

These results present a complex nature of how inflammatory events are regulated in the lung. Fibroblasts may be activated by infiltrating monocytes to produce GM-CSF. GM-CSF can stimulate the production of neutrophilic granulocytes and macrophages as well as feed back to inhibit lung fibroblast proliferation. This decrease in proliferation may help with the role fibroblasts play in collagen deposition by keeping their numbers under control. Higher GM-CSF concentrations may lower NHLF numbers but may also influence eosinophil recruitment and activation. However, many other cytokines and chemokines affect this reaction cycle and the complexity of cell-cell signaling and the cytokine network are still not
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completely understood. These results do, however, give some insight into the mechanisms by which GM-CSF can regulate an inflammatory reaction and how prednisone may inhibit it (figure 6).

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7. REFERENCES


Key words: Fibroblasts, U937 cells, Monocytes, NF-kappaB, Granulocyte macrophage colony stimulating factor, Granulocyte macrophage colony stimulating factor receptor, Prednisone, Asthma, Inflammation

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