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The Effects of Bleomycin on Alveolar Macrophage Growth Factor Secretion

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Previous work in this laboratory has demonstrated increased secretion of fibroblast growth factor (MDGF) activity by alveolar macrophages obtained from mice with bleomycin-induced pulmonary fibrosis. The mechanism by which bleomycin promotes this increase in MDGF secretion is not clear, however. The purpose of this study was to determine the direct effects of bleomycin on alveolar macrophages. Normal rat alveolar macrophages obtained by lavage were cultured in the presence or absence of bleomycin; conditioned media from these cultures were dialyzed to remove bleomycin and then assayed in vitro ***for MDGF activity. Alveolar macrophages incubated with 0.01 μg to 1 μg/ml bleomycin for 18 hours secreted significantly more MDGF than macrophages incubated without bleomycin. Viability of macrophages as determined by exclusion of trypan blue and release of LDH was unaffected by any dose tested. Maximal MDGF production was seen with bleomycin doses of ≥0.1 μg/ml. When alveolar macrophages were incubated with 0.1 μg/ml bleomycin for 0.5–18 hours, MDGF activity was detected as early as 1 hour, with peak responses found at 4–8 hours. Macrophages stimulated with bleomycin continued to produce significant amounts of MDGF even after bleomycin was removed and replaced with fresh (bleomycin-free) media. MDGF secretion by bleomycin-stimulated alveolar macrophages was inhibited by cycloheximide, and the 5-lipoxygenase inhibitors NDGA (nordihydroguairetic acid) and BW755c, indicating not only a requirement for protein synthesis but also for metabolites of the 5-lipoxygenase pathway of arachidonic acid metabolism for full expression of activity. These experiments demonstrate that bleomycin can stimulate alveolar macrophages to produce MDGF* in vitro *at concentrations comparable with those found to induce pulmonary fibrosis in rats. This direct stimulation of MDGF production by bleomycin may be an important mechanism by which this drug initiates a fibrotic response in the lungs. (Am J Pathol 1989, 134: 355–363)***

Bleomycin-induced pulmonary fibrosis in laboratory animals is morphologically and biochemically similar to interstitial pulmonary fibrosis in humans.[1–5] In animals and humans there is increased cellularity in the lungs; in the early stages this consists mostly of neutrophils, lymphocytes and macrophages, followed later by fibroblasts with accompanying increases in collagen. Many studies of late have focussed on the role of the alveolar macrophage in this fibrotic process.[6–11] This focus on the macrophage is the result of the cumulative work of many laboratories that have demonstrated that macrophages are extremely versatile secretory cells capable of producing a variety of proteins and lipids, which may in turn enhance or inhibit the activation and proliferation of other cells.[12–16] Of particular interest in fibrosis are mediators which regulate the proliferation of fibroblasts and their synthesis of collagen. A firm connection between macrophage-derived mediators and pulmonary fibrosis has been established by the study of macrophages isolated from fibrotic human and animal subjects.

Alveolar macrophages from patients with interstitial pulmonary fibrosis secrete increased amounts of fibroblast growth factor activity. This activity has been referred to as alveolar macrophage-derived growth factor (AMDGF), but a portion of this activity is now known to be due to a platelet-derived growth factor (PDGF)-like protein and fibronectin.[6,9,11] Likewise alveolar macrophages isolated from rats and mice with bleomycin- or asbestos-induced fibrosis secrete greater quantities of growth factor activity for fibroblasts.[8,17–19] Throughout this paper the more general abbreviation MDGF (macrophage-derived growth factor) will be used to represent this secreted ac-

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tivity, because it does not imply or assume the predominance of any one factor. Until the precise composition, in terms of known cytokines, is determined, it would be premature to use a more specific term to refer to this activity in macrophage conditioned media.

Agents known to cause pulmonary fibrosis also have been shown to be capable of directly affecting macrophage production of fibrogenic mediators. Asbestos[20] and silica particles[21] activate macrophages *in vitro* to produce substances that increase fibroblast collagen synthesis. Also, other particulate stimuli such as immune complexes, which have been found in association with idiopathic pulmonary fibrosis,[22] can provoke enhanced MDGF release by normal human alveolar macrophages when cocultured.[2]

The purpose of this study was to determine whether a similar interaction between bleomycin and alveolar macrophages might be one mechanism by which this drug induces pulmonary fibrosis.

Materials and Methods

Reagents

Bovine serum albumin (BSA, immunoglobulin-free), cycloheximide, nordihydroguaiaretic acid (NDGA), indomethacin, insulin, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, Dulbecco's modified Eagles medium (DMEM), penicillin-streptomycin stock solution and fetal calf serum (FCS) were obtained from GIBCO (Grand Island Biological, Grand Island, NY). BW755c and PHA (phytohemagglutinin) were from Wellcome Research Laboratories (Beckenham, England). Trypsin (1:250) for isolation and passaging of fibroblasts was from Difco Laboratories (Detroit, MI). Outdated bleomycin (Blenoxane) was a gift of Bristol Laboratories Division of Bristol-Myers Co. (Syracuse, NY). Ketamine (100 mg/ml) was purchased from Parke-Davis (Detroit, MI). [Methyl-^{3}H]thymidine (6.7 Ci/mmole) was from New England Nuclear (Boston, MA).

Animals

Male Fischer 344 rats, specific-pathogen free, weighing 150–200 g, were used for all studies and were obtained from Charles River (Portage, MI). These rats arrived in filtered cages and were maintained in clean animal quarters separate from other laboratory animals. Female CBA/J mice (Charles River), specific-pathogen free, were used as a source of thymocytes for interleukin-1 (Il-1) assays.

Macrophages and Preparation of Conditioned Media

Alveolar macrophages were obtained essentially as described.[19] Rats were given a lethal intraperitoneal injection of ketamine (0.75 ml/rat); the lungs were then perfused with 30–40 ml phosphate-buffered saline (PBS, 140 mM NaCl, 10 mM NaH_2PO_4, 8 mM NaOH), in distilled water, pH 7.2) via the right ventricle to flush blood from the lungs. The lungs were lavaged *in situ* with ten 10 ml washes of sterile 37 C PBS containing 1 mM EDTA and 0.1% (wt/vol) glucose. Lavage fluid was pooled and centrifuged (400*g*, 5 minutes), the supernatant was discarded, and cells were resuspended in RPMI + 10% fetal calf serum (FCS) and 1% antibiotics. All FCS used was first heat inactivated at 56 C for 30 minutes. Cells were plated at a density of 2–3 $\times$ 10^6/35 mm diameter well of tissue culture dishes (Corning). After 2 hours (37 C) the nonadherent cells were removed and adherent cells washed twice with serum-free RPMI. RPMI + 0.2% BSA (wt/vol) with or without bleomycin was then added and incubation was continued for 16–18 hours unless otherwise indicated. For each experiment no-cell control medium ± bleomycin was incubated in empty wells in parallel with cells (No Cell CM). All conditioned media (CM) were dialyzed exhaustively using 6–8 kd MW cutoff dialysis membranes (Spectrum Medical Industries, Los Angeles, CA) against two changes of RPMI at 4 C.

In some experiments macrophages were preincubated for 30 minutes with NDGA (0.5 μM), BW755c (1 μM), cycloheximide (1 μg/ml) or indomethacin (1 μM) before the addition of bleomycin. At the end of the incubation period, CM was collected and dialyzed as described above, then filter sterilized through 0.2 μ pore filters (Sartorius, East Granby, CT) and stored at −70 C until assay. Macrophages were scraped from the wells in the presence of trypsin/EDTA (0.5%, 5 mM) and counted. The final volume of medium/well was adjusted with RPMI + 0.2% BSA so that the final equivalent cell concentration was 2 $\times$ 10^5 cells/ml for all CM. Viability as determined by the exclusion of trypan blue was >95% in all cases. Adherent cells were typically 95% macrophages by analysis of cytocentrifuge smears stained with Wright's Giemsa stain.

MDGF Assay

MDGF activity in the CM was assayed exactly as described previously.[19] Fibroblasts were isolated from normal, specific-pathogen-free rat lungs as described previously,[19] and were used between passages 3 and 12. Briefly, fibroblasts in DMEM + 10% FCS + 1% antibiotics were plated at a density of 5 $\times$ 10^3 cells/well into 96-well tissue culture dishes and incubated for 24 hours. Media

were then removed and replaced with serum-free DMEM for an additional 24 hours, after which the media were replaced with dilutions of macrophage CM. CM were added at a concentration range of 0.5–50%; dilutions were in DMEM + FCS + BSA so that the final concentration in all wells was 0.4% FCS and 0.1% BSA. Fibroblasts were incubated for 48 hours with CM; during the last 6 hours 1 μCi of ^{3}H-thymidine was added per well. Cells were then harvested, washed, and incorporated radioactivity counted as before.[19] Positive controls were always included in each assay, and consisted of 10% FCS, usually resulting in >100000 dpm incorporated. All assays were undertaken at a dose of conditioned media that would not cause incorporation of this magnitude (usually <100,000), to avoid exceeding the capacity of the fibroblast to incorporate thymidine. However, this cannot be used as a normalizing factor for calculation of standardized units of activity due to the extensive variability in the growth stimulatory properties of FCS from lot to lot. Furthermore, the macrophage conditioned media are likely to consist of complex mixtures of growth stimulatory and inhibitory substances with different individual dose response curves, thus making it impossible to precisely standardize the expression of growth factor activity. Each preparation of conditioned media usually results in a different dose–response curve due to the variability in the cellular response from one macrophage preparation to the next. Nevertheless, valid comparisons could still be made by comparing control and experimental mean values for the same preparation of macrophages in any particular experiment. Comparisons between the results of different experiments done on different days were attempted only after normalization of the data to their respective control values for that day, and obtained using the same batch of macrophages.

Because bleomycin has antimitogenic properties, all conditioned media were dialyzed exhaustively before assay as described above. However, conditioned media, especially at high bleomycin concentrations (>1 μg/ml), contained inhibitory activity even after dialysis. To determine if this inhibitory activity at high concentrations was due to residual bleomycin in the media after dialysis, No Cell CM were prepared by incubating bleomycin-containing media in the absence of cells. When these No Cell CM were assayed for MDGF activity, significant inhibition of thymidine incorporation by fibroblasts was seen compared with media without bleomycin or to baseline incorporation in the absence of added conditioned media. The amount of incorporation in bleomycin-containing medium was inversely related to the concentration of bleomycin originally present (0 bleomycin = 9254 ± 4365 dpm and 10 μg/ml bleomycin = 1050 ± 570 dpm). This concentration-dependent response with the No Cell CM was a consistent finding in all experiments. The decreased thymidine uptake in CM containing high concentrations (10 μg/ml) bleomycin is therefore probably due to residual bleomycin. Based on the analysis of dialyzed samples of ^{3}H bleomycin (data not shown) less than 5% of the bleomycin remained in the media after dialysis; however, an original concentration of 10 μg/ml could still be inhibitory after dialysis, because bleomycin at concentrations above 0.1 μg/ml have been shown to inhibit fibroblast DNA synthesis.[25,26] Inhibition of fibroblast DNA synthesis by residual bleomycin also is consistent with the observation that thymidine incorporation decreased as the dose (%) of bleomycin No Cell CM being assayed was increased. In contrast, control No Cell CM (without bleomycin) did not inhibit thymidine uptake when compared with that of DMEM + 0.4% FCS alone (10360 ± 4355 dpm). These findings emphasize the importance of comparing the activity of the macrophage CM with No Cell CM controls containing equivalent concentrations of bleomycin. Hence, all MDGF activity was expressed as follows to correct for the residual inhibitory effect of the bleomycin itself: MDGF activity = (dpm in macrophage CM) − (dpm in No Cell CM).

No Cell CM used in this calculation were prepared and assayed in parallel with the macrophage CM for each experiment so that slight differences in lots of RPMI or BSA would not artifactually influence the results.

Monokine Assay

Il-1 levels in macrophage CM was assayed by the enhancement of thymocyte proliferation to phytohemagglutinin (PHA), exactly as described previously.[23] Tumor necrosis factor (TNF) levels in CM were assayed by cytotoxicity of CM to L929 cells (ATCC), as described.[24] In each assay purified human TNF-α or IL-1α (recombinant; Collaborative Research, Bedford, MA) was used as positive controls and for standardization purposes.[23,24]

Endotoxin Assay

Aliquots of bleomycin at concentrations of 0, 0.01, 0.1, 1, and 10 μg/ml, PBS, RPMI + 0.2% BSA, and conditioned media were tested for the presence of endotoxin using the *Limulus* amebocyte assay with a sensitivity of 0.025 to 0.1 endotoxin units/ml. E-Toxate kit for this assay was from Sigma Chemical Co.

Statistical Analysis

Student's *t*-test was used for comparison of experimental and control groups, with $P < 0.05$ being statistically different. When multiple comparisons were made, analy-

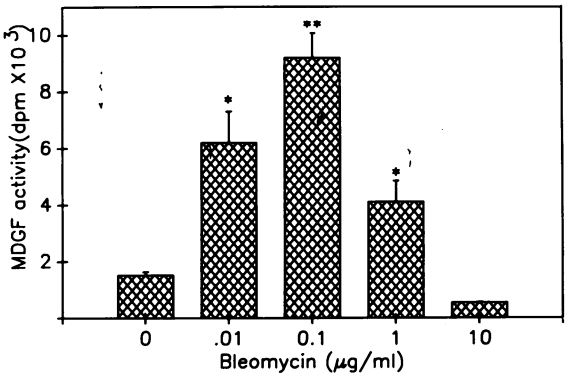

Figure 1. *Effect of bleomycin on MDGF production. Macrophages were incubated with the indicated concentrations of bleomycin for 18 hours, after which CM were assayed for MDGF activity. MDGF activity was expressed as described in the Methods section. Data represents the MDGF activity of 50% CM (mean ± SEM) from a representative experiment with N = 6. The asterisks indicate activity significantly greater than control cell CM (*P < 0.05 and **P < 0.001).*

sis of variance was used employing Scheffé's test for making internal comparisons.

Results

Alveolar macrophages incubated for 18 hours in media alone secreted minimal amounts of MDGF activity. In contrast, macrophages incubated with bleomycin produced significantly greater amounts of MDGF in a dose-dependent manner, with optimal secretion at a dose of 0.1 μg/ml (Figure 1). However, conditioned medium from the highest concentration of bleomycin tested (10 μg/ml) either contained minimal amounts of MDGF or inhibited fibroblast ^{3}H thymidine incorporation relative to the peak of MDGF secretion. The lack of activity at this concentration

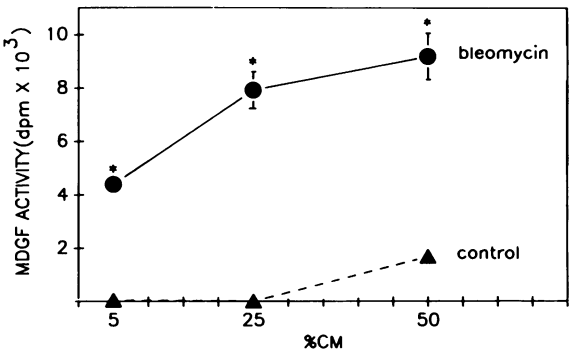

Figure 2. *Dose dependence of MDGF activity in CM. Macrophages were incubated with 0.1 μg/ml bleomycin for 18 hours after which CM was assayed for MDGF activity. Data are expressed as mean dpm ± SEM of a representative experiment with N = 6. MDGF activity was calculated as described in the Methods section. The asterisks indicate activity significantly greater than control cell CM (P < 0.001).*

was not due to toxicity to the macrophages, whose viability by trypan blue exclusion was 95–100% at all concentrations. Furthermore, there was no detectable release of the cytosolic enzyme lactate dehydrogenase (LDH). As discussed in the Methods section, this inhibitory effect was probably due to residual bleomycin in the conditioned media.

In four separate preparations of CM, a concentration-dependent response to bleomycin was evident. It is important to note here that MDGF activity in bleomycin macrophage CM was significantly higher than that of control cells in spite of any inhibition of fibroblast proliferation produced by residual bleomycin in the media (because the manner of expressing MDGF activity has corrected for the inhibitory effects of residual bleomycin). The range of MDGF activity at 0.1 μg/ml of bleomycin was 2300–19,000 dpm above that of control macrophage CM (no bleomycin). Hence, the effect of bleomycin on macrophages cannot be accounted for merely by the reduction of the inhibitory effect of residual bleomycin, but instead suggests secretion of a fibroblast growth-promoting substance. This range of activities may be due to the inherent biologic variability between the populations of macrophages used in each experiment, the particular preparation of fibroblasts used in the MDGF assay, and differences in the growth factor content of separate lots of FCS. Variability was seen in both control (untreated) and bleomycin-treated conditioned media. Similar ranges of variability in MDGF activity between experiments have been reported by others.[15] Nevertheless in each experiment CM from macrophages incubated with 0.1 μg or 1 μg/ml bleomycin were significantly more active than control cells incubated in media alone ($P < 0.05$). The response to 0.01 μg/ml bleomycin macrophage CM was also statistically above control in three of four experiments. MDGF activity of all cell CM was dependent on the dose of CM added (Figure 2). The lack of a direct (one-to-one) linear relationship between dose and magnitude of response is consistent with the complex composition of cellular conditioned media which are likely to contain inhibitory as well as stimulatory factors with differing dose response curves.

Kinetics of MDGF Production

When bleomycin is administered *in vivo* it is almost completely cleared from the lungs in 24 hours[27]; this would suggest that any direct interaction between bleomycin and alveolar macrophages or other lung cells takes place in a relatively short time period or at relatively low concentrations of bleomycin. To test this hypothesis, macrophages were exposed to 0.1 μg/ml bleomycin for variable lengths of time, after which CM were removed for assay.

The secretion of MDGF into the media was found to increase over the first 4 hours (Figure 3); longer incubation periods did not significantly alter this activity. In three experiments, MDGF activity of 4 hours CM, at a concentration of 25%, ranged from 1869 to 3369 dpm above that of control macrophage CM (significant at $P < 0.05$).

To determine if bleomycin-stimulated macrophages would continue to produce MDGF in the absence of the drug, bleomycin-containing media were removed at each time point (as indicated in Figure 3) and replaced with fresh RPMI + BSA without bleomycin. Incubation was then continued for an additional 24 hours. The MDGF activity in these media was also dependent on the length of the initial exposure to bleomycin (Figure 4). Although activity continued to increase over the entire time, only those CM from cultures exposed to bleomycin 4 hours or less contained activity that was significantly higher than that of control CM. This lack of statistical significance at time points ≥8 hours (Figure 4) was not due to significant reductions in activity in the CM of bleomycin-treated macrophages at times ≥8 hours, but was due to significant increases in growth factor activity in control CM when macrophages were incubated for times ≥32 (8 + 24) hours. CM from unstimulated macrophages incubated for a total of 32–42 hours (Figure 4, 8–18 hour time points) contained more MDGF than that of CM collected after the usual 16–18 hours. It was evident from these experiments that bleomycin could interact with alveolar macrophages in a relatively short time (1–4 hours) to induce MDGF secretion and that the MDGF activity remained elevated for at least 24 hours after the cells were first stimulated.

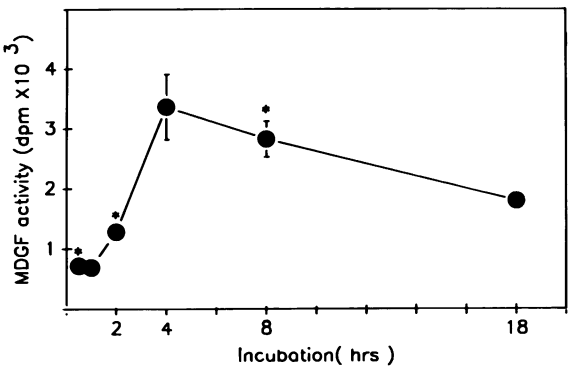

Figure 3. *Kinetics of MDGF release. Macrophages were incubated with 0.1 μg/ml bleomycin. At the indicated times CM were collected and assayed for MDGF activity. Data are the activity in 25% CM (mean ± SEM, N = 3) of a representative experiment assayed in triplicate. MDGF activity was calculated as described in the Methods section. The asterisk indicates activity significantly greater than control cell CM at* $P < 0.002$; *for all other points* $P < 0.05$.

Effect of Metabolic Inhibitors on MDGF Activity

The fibroblast growth-promoting substance in the bleomycin macrophage CM was nondialyzable using tubing with nominal 6–8 kd cut-off. It was stable upon storage and freezing and thawing. To ascertain the chemical nature of the MDGF activity we investigated the effects of several metabolic inhibitors. In decreasing order of effectiveness, MDGF secretion was inhibited by NDGA, cycloheximide, and BW755c (Figure 5). Indomethacin did not affect activity whether it was added to the macrophages before stimulation or added to the fibroblasts when CM was assayed (data not shown). The effects of these inhibitors on MDGF activity imply that protein synthesis is required for MDGF secretion, and that there may be both protein and lipid components to the growth promoters in this CM. Both NDGA and BW755c at these doses are known inhibitors of arachidonic acid metabolism by the 5-lipoxygenase pathway, which produces leukotrienes and hydroxyeicosatetraenoic acids (HETEs).[28]

Effect of LPS on Bleomycin-Stimulated Macrophages

Lipopolysaccharide (LPS) added to macrophages *in vitro* elicits the production of Il-1, prostaglandin E_2, and LtC_4 as well as MDGF.[10,23] Although LPS stimulates MDGF secretion *in vitro*, it is not known to produce a fibrotic response *in vivo*. LPS has actually been shown to inhibit bleomycin-induced pulmonary fibrosis in rats.[18]

To investigate the possibility that LPS might inhibit bleomycin-induced fibrosis at the level of its interactions with alveolar macrophages, the combined effects of LPS

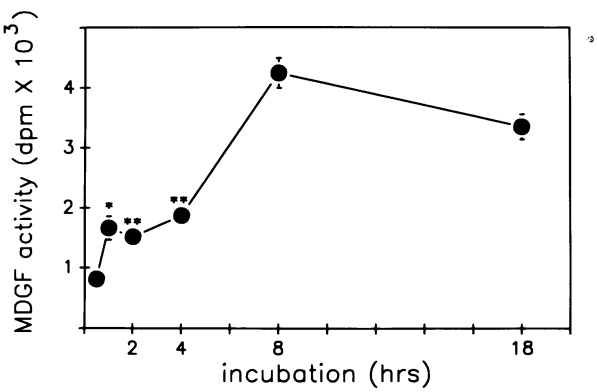

Figure 4. *Release of MDGF over a 24-hour period following bleomycin stimulation. Macrophages were incubated with 0.1 μg/ml bleomycin and CM collected as indicated in Figure 3. After this initial exposure to bleomycin (times indicated on the x-axis), the media were replaced by fresh medium without bleomycin and incubation was continued for 24 hours more. After this second incubation period, CM were collected and assayed as described in the Methods section. MDGF activity was expressed as indicated. Data represent the activity of 25% CM (mean ± SEM, N = 3) of a representative experiment. The asterisk indicates activity greater than the corresponding control cell CM (*$P < 0.05$*), whereas the ** indicates* $P < 0.001$.

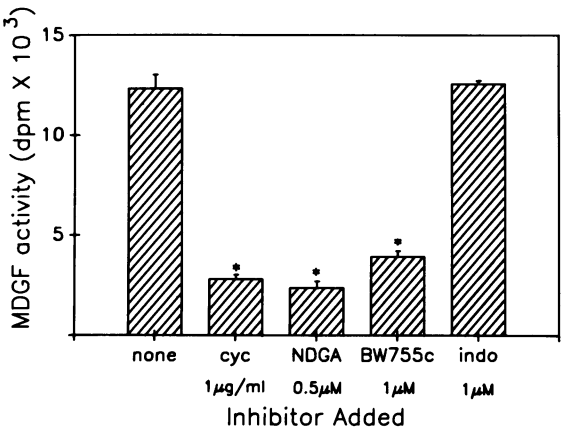

Figure 5. *Effects of metabolic inhibitors on MDGF production. Macrophages were incubated with 0.1 μg/ml bleomycin with or without to indicated inhibitor for 4 hours, after which CM was collected and assayed for MDGF activity at a concentration of 25% CM. MDGF activity was calculated as described in Methods. Data represents mean ± SEM (N = 3) of a representative experiment. The asterisk indicates activity significantly different from that of CM with bleomycin alone, (P < 0.01).*

and bleomycin were examined. As would be predicted from previous findings,[10,23] LPS and bleomycin separately stimulated MDGF secretion (Table 1). It is unlikely that MDGF secretion stimulated by bleomycin was due to endotoxin contamination because no detectable endotoxin was found in either the bleomycin, PBS, or medium used in these experiments. In addition, indomethacin had no effect on MDGF secretion by bleomycin-stimulated macrophages whereas it has been demonstrated previously to increase LPS-stimulated macrophages MDGF secretion.[10]

When the activity secreted by macrophages incubated with LPS alone was compared with that from cells that were also given bleomycin (0.1 μg/ml), the two substances were seen to have a synergistic effect on MDGF secretion only at the lowest concentration of LPS (0.1 μg/ml). A simple additive effect of 0.1 μg/ml LPS and bleomycin would have resulted in activity of approximately 5000 dpm in MDGF activity; the activity actually observed under these conditions was 8775 dpm, suggesting synergy between the two substances at this dose. In contrast, a significant reduction in MDGF secretion was seen with higher doses of LPS (>1 μg/ml). The MDGF activity of 10 μg/ml LPS alone was reduced by 79% in the presence of 0.1 μg/ml of bleomycin (Table 1).

Table 1. *Effects of LPS on MDGF Secretion*

LPS dose (μg/ml)	Bleomycin (0.1 μg/ml)	
	−	+
0	0*	1203 ± 223†
0.1	3809 ± 329	8775 ± 720†
1.0	14142 ± 1028	10258 ± 1769
10.0	13950 ± 2092	2916 ± 434‡

Macrophages were incubated with the indicated dose of LPS ± 0.1 μg/ml bleomycin for 4 hours, after which CM was collected and assayed for MDGF activity, calculated as described in the Methods section. Results are expressed as the mean dpm ± SEM, N = 3.

* Control macrophage CM was equal to No Cell Control CM, therefore MDGF activity of control cell CM (no LPS, no bleomycin) = 0.

† Significantly greater than (−) bleomycin, $P < 0.05$.

‡ Significantly lower than (−) bleomycin, $P < 0.01$.

Effects of Bleomycin on Macrophage Il-1 and TNF Production

Because stimulated macrophages are known to secrete Il-1 and tumor necrosis factor (TNF), both of which have been reported to affect fibroblast proliferation,[10,29,30] macrophage CM was assayed to determine if bleomycin might stimulate secretion of these monokines as well. CM from macrophages stimulated with 0.1 μg/ml bleomycin for 18 hours contained 1.2 ± 0.3 units of Il-1 whereas control macrophage CM contained 1.1 ± 0.3 units (N = 5). TNF activity in this same CM was also negligible, with no differences being found between bleomycin-stimulated or control CM.

Discussion

A large variety of substances can cause pulmonary fibrosis; these include drugs, inorganic and organic particulates, chemicals, and viruses.[3] Although these fibrogenic agents have a common end point, it is not known if there are common mechanisms through which they produce the disease. Work with human material and animal models suggests that stimulation of the alveolar macrophage may be one such shared mechanism.

Alveolar macrophages from healthy animals, when compared with bleomycin-stimulated cells, produce little if any fibroblast growth factors, which has been found by culturing them and assaying for MDGF. This is a common finding of the present study, previous work in this laboratory with mice,[19] and work by other investigators studying human,[2,6,9] rat,[8] and hamster[7] cells. When these cells are obtained from patients with idiopathic pulmonary fibrosis[6] or rats and mice[8,17,19] with induced fibrosis, substantial amounts of MDGF are produced and in humans PDGF secretion is also enhanced.[9] Macrophages from bleomycin-treated hamsters and rats have also been reported to produce factors that inhibit rather than stimulate fibroblast proliferation.[7,31] These contrasting findings seem to be partly due to the conditions under which MDGF activity has been assayed. Macrophage CM incubated with fibroblasts and 0.4–0.5% serum stimulate proliferation in the experiments reported here and in other studies,[6–8,17,19] whereas macrophage CM collected and assayed in the

presence of 10% serum have been found to inhibit proliferation.[7,31]

The MDGF activity produced by the *in vitro* stimulation of alveolar macrophages with bleomycin closely parallels that recovered from alveolar macrophages of bleomycin-treated mice[19] and rats.[8] Macrophages exposed to bleomycin either in culture or *in vivo* produce substantially more MDGF than do parallel control cells. For *in vivo* studies rats are given 0.5–1.5 mg of bleomycin intratracheally[5,8,18]; with an approximate bronchial and intraalveolar lung volume of 10 mls, initial concentrations of 50–150 μg/ml may be achieved. By comparison, *in vitro* doses of 0.01 μg to 1 μg/ml bleomycin substantially increased MDGF production (Figure 1). It seems likely that these effective *in vitro* concentrations may also be found *in vivo* for substantial lengths of time even with relatively rapid clearance of bleomycin from the lungs.[27] Analysis of the kinetics of MDGF secretion suggests that a relatively brief exposure to bleomycin is sufficient to promote elevated and prolonged MDGF production (Figures 3 and 4). Maximal MDGF activity was found in CM of macrophages incubated with bleomycin for 4 hours; the lag period between stimulation and secretion suggests that most of the MDGF in the media is likely to be newly synthesized and not merely preformed MDGF that was released. This is further supported by the finding of substantial MDGF in CM 24 hours after the removal of the drug (Figure 4); and by the fact that protein synthesis is required for MDGF secretion.

A further similarity between the *in vitro* and *in vivo* data[19] was seen when the effects of the metabolic inhibitors on MDGF activity were compared. The relative effects of these inhibitors on activity of rat macrophages cultured with bleomycin and mouse macrophages from fibrotic mice were similar. In both instances, MDGF secretion was reduced by NDGA and BW755c but not by indomethacin and ibuprofen (Figure 5).[19] The inhibition by cycloheximide further confirms the conclusion made from the kinetic studies, that increased activity is dependent on protein synthesis because this dose of cycloheximide has been reported to inhibit protein synthesis in macrophages.[32] NDGA and BW755c inhibit the production of leukotrienes and HETEs (hydroxyeicosatetraenoic acids) via the 5-lipoxygenase pathway of arachidonic acid.[33] The observation that NDGA is a more effective inhibitor of activity than BW755c is probably related to their relative specificity as lipoxygenase inhibitors, with NDGA being more specific. The doses of arachidonate metabolic inhibitors used here have been reported to selectively inhibit the 5-lipoxygenase (NDGA and BW755c) and cyclooxygenase (indomethacin) enzymes of macrophages.[34] Recent work in this laboratory has indicated that the 5-lipoxygenase pathway product LtC_4 does not have a direct effect on fibroblast proliferation,[35] although LtC_4 and LtB_4 have been shown to increase the release of MDGF by macrophages *in vitro*.[10] NDGA-induced reduction of activity implies that bleomycin may stimulate endogenous or macrophage production of LtC_4 and LtB_4 and create a positive feedback mechanism that increases MDGF release. Hence, the NDGA-induced reduction in MDGF activity in the bleomycin-stimulated macrophage CM is probably mediated by abrogating this positive feedback loop. Possible direct or indirect effects of the HETEs or other lipoxygenase products have not yet been investigated.

The results of the inhibitor studies further suggest that endogenous prostaglandins do not play a significant role in the regulation of MDGF production by bleomycin-stimulated macrophages. Ibuprofen in the *in vivo* study,[19] and indomethacin here, had no effect on MDGF activity. This is in contrast to studies of LPS-stimulated mouse macrophages,[10] in which indomethacin was shown to increase MDGF activity. These results also confirm that the bleomycin effect is not due to endotoxin contamination.

The *in vitro* stimulation of macrophages by bleomycin demonstrated here disagrees in part with an earlier study by another laboratory,[8] which concluded that there was no direct effect of bleomycin on rat alveolar macrophage MDGF production. In these experiments by Kovacs and Kelley,[8] MDGF activity in bleomycin-macrophage CM was expressed as a percentage of the thymidine incorporated by fibroblasts not given CM. In their study, bleomycin-macrophage CM (at 0.3 μg/ml bleomycin) contained only 15% more MDGF than parallel control macrophage CM. In light of our findings concerning the effects of No Cell CM on fibroblast thymidine incorporation, it is conceivable that the slight increase in MDGF in bleomycin-macrophage CM reported previously[8] would be accentuated if the relative inhibitory activity of the bleomycin and control culture medium were considered in calculating this activity. Aside from this difference in expression of data, there are also differences in methods that could account for the divergent conclusions. These relate to the use of 0.2% plasma-derived serum and confluent fibroblasts in their MDGF assay, while 0.4% FCS and nonconfluent fibroblasts whose cell cycles were synchronized by preincubation in serum-free medium, were used in this study. Furthermore, fibroblasts were exposed to CM for 48 hours in this study *vs.* 18 hours in the previous study. How such differences could account for the divergent findings is unclear at this time; however, Kouzan et al[36] have recently demonstrated that the presence or absence of serum during adherence or stimulation of macrophages has a significant effect on the resulting products in the medium. This serum effect may also account for our finding that there is insignificant Il-1 activity in bleomycin-stimulated macrophage CM, whereas a recent report by Jordana et al[31] found that bleomycin can stimulate macrophage Il-1

secretion in the presence of serum. Despite the differences in conclusions between the study just cited[8] and those from our data, there is a common finding with respect to the combined effects of LPS and bleomycin on macrophages *in vitro* (Table 1). In both, macrophages treated with LPS and bleomycin showed greatly reduced MDGF production at high doses of LPS (10 and 30 μg/ml). LPS-treated macrophages and macrophages from bleomycin-treated animals have been shown to have enhanced PGE_2 synthesis compared with control cells.[19,37] The addition of bleomycin to LPS-stimulated cells may enhance PGE_2 levels enough to inhibit fibroblast proliferation. Previous work[10] has demonstrated that the addition of PGE_2 to LPS-stimulated macrophages inhibits MDGF secretion.

The MDGF activity in CM of bleomycin-stimulated macrophages has not yet been characterized. The term MDGF has been used here in the general sense, as it has been by others,[6,8,15–17,20,21,23] to describe product(s) of macrophages that enhance fibroblast proliferation. Several groups[9–11,19,35,37] have demonstrated that macrophages secrete multiple forms of fibroblast stimulatory factors, some of which are known monokines such as PDGF and TNF, whereas others are as yet only partially characterized and collectively referred to as MDGF. In the present study, measurements of Il-1 and TNF activity in bleomycin-stimulated macrophage CM have failed to detect significant amounts of either monokine, implying that neither substance is responsible for the MDGF activity in this CM.

The work presented here reinforces the important role of the macrophage in pulmonary fibrosis. Alveolar macrophages come into contact with bleomycin and increase their MDGF secretion. Bleomycin has been shown previously to promote fibroblast collagen[26,28] and glycosaminoglycan synthesis. A very low concentration of bleomycin (0.01 ng/ml) also has been shown to directly increase fibroblast proliferation, an effect that is augmented by PDGF,[38] another macrophage product.[13] The combined action of this drug on macrophages and fibroblasts could be a potent driving force to the fibrotic process in the lungs.

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