Fibronectin Fragments Containing the RGDS Cell-binding Domain Mediate Monocyte Migration into the Rabbit Lung

A Potential Mechanism for C5 Fragment-induced Monocyte Lung Accumulation

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Abstract

Many inflammatory processes are characterized by an early phase of neutrophil migration and a later phase of monocyte migration into the inflammatory site. Mechanisms that govern the transition between phases are the subject of these investigations. Acute lung inflammation induced by C5 fragments in the rabbit leads to an initial neutrophil influx and plasma leakage into the alveolar space, followed by monocyte influx that we have previously shown to be dependent on prior emigration of neutrophils. Neutrophil enzymes are known to cleave intact fibronectin into fragments that are monocyte chemotaxins in vitro. Accordingly, generation of appropriate fibronectin fragments in situ by proteolytic enzymes from infiltrating neutrophils might represent a potential mechanism for attraction of monocytes into the lung. The studies reported herein demonstrate that a 120-kD fragment of fibronectin containing the RGDS fibroblast cell-binding domain induced monocyte migration into the rabbit lung in vivo. Intact fibronectin was inactive. A significant proportion of the monocyte migration was neutrophil independent. Intact fibronectin was present in bronchoalveolar lavage fluid from C5 fragment-treated animals rendered neutropenic, but absent in lavage from normal C5 fragment-treated animals. Fibronectin fragments were present in bronchoalveolar lavage fluid from both C5 fragment-treated and control rabbits. In addition, the amount of fibronectin was significantly increased in lavage of C5 fragment-treated normal but not neutropenic animals. Monoclonal antibodies directed against an epitope of fibronectin containing the RGDS cell-binding domain significantly inhibited the C5 fragment-induced monocyte migration, but not neutrophil migration. These studies suggest that chemotactic fibronectin fragments may in part be responsible for the recruitment of monocytes into areas of acute lung inflammation. (*J. Clin. Invest.* 1990; 86:1065-1075.) **Key words:** fibronectin • monocytes • neutrophils • proteases • chemotaxis

Introduction

Inflammatory processes are often characterized by an orderly influx of leukocytes into areas of tissue injury: an initial phase

of neutrophil accumulation followed by a later phase of mononuclear cell (including monocyte) emigration (1-6). The mechanisms that govern the transition from one phase to the other are largely unknown. In the lung, fragments of the fifth component of complement (C5f) induce a complete inflammatory process characterized by an early neutrophil influx into the alveolar space (3, 5, 6) followed by a monocyte influx that is dependent on the prior emigration of neutrophils (2). We have previously suggested that during C5 fragment-induced acute lung inflammation neutrophils generate signal(s) which promote the subsequent accumulation of monocytes in the lung, and that these signals are potentially distinct from signals which promote neutrophil accumulation (2). The enzymatic degradation products of the extracellular matrix proteins collagen (7), elastin (8), and fibronectin (9), which are present in the lung, have been shown to stimulate monocyte, but not neutrophil, migration in vitro. The in vivo generation of these biologically active monocyte chemoattractants at areas of inflammation, potentially by neutrophil enzymes such as elastase (8, 10) and collagenase (7), make these molecules candidates for stimuli that may promote a later neutrophil-dependent monocyte influx into inflammatory sites.

Fibronectins are a family of adhesive glycoproteins spliced from one gene (11) and are produced by hepatocytes (12), endothelial cells (13), and fibroblasts (14, 15). Fibronectins accumulate at sites of inflammation in the skin (16, 17), kidney (18), synovium (19), and lung (20-22). They are proteins with several functional domains that bind different cell types as well as different extracellular molecules such as heparin and collagen (14, 23). Neutrophil enzymes, elastase and cathepsin G, are capable of digesting fibronectin (10, 24) into fragments, some of which have chemotactic activity for monocytes but not for other leukocytes in vitro (25). Thus, a 120-kD thermolysin-generated fragment of fibronectin, similar in size and sequence to a neutrophil elastase-generated fibronectin fragment (10), which contains the RGDS fibroblast cell-binding domain has chemokinetic and chemotactic activity for monocytes but not for neutrophils or lymphocytes in vitro (9). In contrast, intact plasma fibronectin has no chemotactic activity for monocytes or other leukocytes.

We have previously shown that C5 fragment-induced monocyte migration into the alveolar space of rabbits is a neutrophil-dependent event (2). C5 fragments instilled into the lung do not induce monocyte migration into the alveolar space of neutrophil-depleted rabbits. Monocyte migration is reestablished if neutrophils are repleted before induction of the inflammatory response by C5 fragments in these neutrophil-depleted animals (2). Neutrophils, which localize at sites of C5 fragment-induced lung inflammation, might degrade fibronectin into chemotactically active fragments that subsequently

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recruit monocytes into the inflammatory sites. The purpose of these studies was therefore fourfold: (a) to determine whether fibronectin fragments could induce monocyte migration into the rabbit lung in vivo; (b) if so, to determine whether this monocyte migration was neutrophil-independent; (c) to determine the content of intact and/or fragmented fibronectin in the alveolar space of rabbits under unstimulated conditions and after the induction of an acute inflammatory process by C5 fragments; and, then, (d) to attempt to block the influx of monocytes into an inflammatory site induced by C5 fragments with antifibronectin antibodies.

Methods

Materials. Intact fibronectin was isolated by a modification of the method of Furie and Rifkin (26). The 30- and 120-kD fibronectin fragments were isolated after thermolysin digestion of intact fibronectin (27). The eluate used as a control in these experiments was the void fraction from the final column used in preparation of the 120- and 30-kD fibronectin fragments. Biologically active C5f were purified from serum as previously described (28, 29). The terminal arginine residue was removed by reaction with carboxypeptidase B covalently linked to Sepharose-6B beads.

Monoclonal mouse anti-human fibronectin antibodies were kindly provided by J. A. McDonald (Washington University, St. Louis, MO) and prepared as previously described (30). Ab 295 is directed against an epitope of fibronectin containing the fibroblast cell-binding domain. Ab 296 is directed against an epitope of fibronectin near the carboxy terminal of the fibronectin molecule (heparin binding site) and does not bind to the cell-binding domain or epitopes of the 120-kD fragment used herein.

Polyclonal goat anti-rabbit fibronectin antibody (Organon Teknica-Cappel, Malvern, PA), rabbit fibronectin (Biomedical Technologies Inc., Stroughton, MA), rabbit anti-goat IgG antibody (Organon Teknica-Cappel), prestained high molecular mass standards (Bio-Rad Laboratories, Richmond, CA), and ^{125}I -protein A (New England Nuclear, Boston, MA) were used in ELISA and immunoblot assays.

In vivo experimental design. New Zealand white rabbits of both sexes were anesthetized with xylazine and ketamine, and under bronchoscopic guidance, the appropriate stimuli $\pm$ antibodies were selectively instilled into the right cranial lung lobe (RCL)¹ bronchus. Monocytes labeled with ^{111}In -tropolonate were infused intravenously 15 min after the instillation of stimuli. Rabbits were killed 48 h after the infusion of labeled monocytes (except in migration time course experiments and stimulus + antibody experiments). Experimental and control lung lobes were lavaged to determine the total fibronectin and total protein content, the migration of radiolabeled monocytes and unlabeled neutrophils into the alveolar space, and the content of unlabeled mononuclear cells in the alveolar space.

Isolation and indium labeling of monocytes. Rabbit peripheral blood monocytes (> 91% pure) free of detectable red blood cell and platelet contamination were isolated by a combination of discontinuous plasma Percoll density gradients and counterflow centrifugation cell elutriation to separate monocytes from lymphocytes in the absence of an adherence step (2). The elutriation system that consisted of a Beckman J2-21 centrifuge (J2-21; Beckman Instruments, Inc., Fullerton, CA), a strobe RPM JE-6B Elutriator Rotor (Beckman Instruments, Inc.), and a Masterflex controller pump (Cole-Parmer Instrument Co., Chicago, IL) was sterilized as previously described (2). The purified monocytes were labeled with 50–75 μCi of ^{111}In -indium chloride solution, 2 mCi/ml; New England Nuclear Research Products) tropolonate (Fluka AG, Buchs, Switzerland) in a 0.5 cc vol of

platelet poor plasma according to the method of Danpure (31, 32). Autoradiography confirmed that greater than 99% of the cells were discretely labeled in a uniform fashion before their reinfusion into experimental animals (2). The label was shown to remain cell associated throughout the experiment as cells in the circulation, lung parenchyma, and bronchoalveolar lavage were discretely labeled by autoradiographic analysis (2) and < 1% of the label was present in plasma or lavage supernates at any time. The functional integrity of radiolabeled monocytes prepared in this fashion has been extensively tested in vivo (2).

Bronchoscopic instillation of experimental materials. New Zealand white rabbits of either sex (2.5–3.5 kg) were anesthetized with 2–3 mg/kg xylazine (Moby Corp., Shawnee, KS) and 30–35 mg/kg ketamine (Vetinary Products, Bristol Laboratories, Syracuse, NY). A polyethylene catheter (P.E. 50; Fisher Scientific Co., Pittsburgh, PA) was advanced through the suction port of an Olympus BF-7 pediatric bronchoscope and guided under direct vision into the dorsal branch of the right cranial lung lobe bronchus (RCL). Animals received a 1-ml vol of PBS alone into either the RCL or 1-ml vol of PBS with one of the following: 80 μg intact plasma fibronectin (0.33 nmol), 40 μg 120-kD fibronectin fragment (0.33 nmol), 10 μg of 30-kD fragment of fibronectin (0.33 nmol), a volume of eluate equal to the volume of the 120-kD fibronectin fragment used, or 20 μg C5 fragments. In selected experiments, 80 μg of Ab 295 or 296 were added to the C5 fragments before instillation. The infusate remained localized to the RCL area of the right lung (2). The left cranial lung lobe (LCL) of the left lung therefore served as an internal control and accounted for intrarabbit variability in these experiments.

Bronchoalveolar lavage fluid analysis. Rabbits were sacrificed by pentobarbital overdose. The right and left cranial lung lobes were selectively lavaged with 10 ml of heparinized PBS. In time course experiments, PMSF (5 mM) and EDTA (5 mM) were included in the lavage to prevent fragmentation of fibronectin during the procedure. The lavage recovery was > 75% of the volume infused. The total nucleated cell count was determined by use of a Neubauer hemacytometer. A differential cell count of 200 cells was carried out on Wright-stained cytopsin preparations. Absolute neutrophil counts were used as an index of neutrophil migration, < 1% of resident alveolar cells in controls were neutrophils. Because of the presence of resident alveolar macrophages in control and experimental animals, cell-associated radioactivity in the lavage was used as an index of monocyte migration. Therefore, the remaining lavage sample was centrifuged. The supernatant was analyzed separately from the pellet in a gamma well counter (Beckman Instruments, Inc.) and consistently contained < 0.1% of the total lavage radioactivity. An aliquot of the ^{111}In -monocytes before infusion was routinely saved and counted simultaneously with the lavage cell pellets so as to avoid the variable of radioactive decay of ^{111}In . Counts present in the cell pellet were expressed as a fraction of total radioactivity initially infused. A gamma counter (7000; Beckman Instruments, Inc.), set to count both 173 KeV and 247 KeV peaks of ^{111}In was used to count radioactivity in all samples. Throughout the experimental protocol the label remained cell associated.

Neutrophil depletion. Circulating and marginating neutrophils were depleted by intravenous infusion of mechlorethamine hydrochloride MSD (Merck and Co., West Point, PA) into rabbits 4 d before experimentation as previously described (2). Blood samples taken on the day of experimentation (day 4) showed that the total white blood cell counts were 1,500–2,000/mm³ with < 1% neutrophils. This protocol effectively depletes neutrophils from the lung as well as the peripheral circulation (2). PBS $\pm$ 40 μg of 120 kD fibronectin fragments were instilled into the RCL lobe bronchus of neutrophil-depleted rabbits according to the above protocol.

Histologic and morphometric analysis. Rabbit lungs were excised and fixed via the intratracheal instillation of glutaraldehyde-paraformaldehyde fixative in 0.2 M cacodylate buffer (400 mOsm) at 25 cm of water pressure. After 24 h of fixation, three sections from the RCL and three sections from the LCL were dissected and prepared for 2- μm

1. Abbreviations used in this paper: LCL, left cranial lung lobe; RCL, right cranial lung lobe.

paraffin block face sectioning, mounted on glass slides, and stained with hematoxylin and eosin. Light photomicrographs were taken at 675 $\times$.

Nine microscopic fields on each slide were selected according to a predetermined pattern using the stage micrometer. Thus, a total of 27 fields were examined per condition studied. The number of mononuclear cells and neutrophils in the interstitium, as well as in the alveoli and nonparenchyma (i.e., airways), were counted at the given coordinates.

Quantification of fibronectin and total protein. Fibronectin content in bronchoalveolar lavage fluid was determined using an inhibition ELISA assay modified from that of Rennard et al. (33). Briefly, known concentrations of rabbit fibronectin or lavage samples diluted in PBS + 0.05% Tween-20 were incubated overnight at 4°C with a 1:8,000 dilution of a polyclonal goat anti-rabbit fibronectin antibody. Controls without antibody or without antigen were also prepared. Rabbit fibronectin (2 μ g/ml) was used to coat 96-well MAXISORP immunoplates (Nunc, Roskilde, Denmark) overnight at 4°C. The next morning, plates were washed and incubated 1 h with 10% BSA to block nonspecific binding sites. After further washing the plates, controls, antigen-antibody fibronectin standards, or lavage samples were incubated in respective wells for 2 h. After washing, 1:1,000 HRP-goat anti-rabbit IgG was incubated 1 h, and wells were developed, and read at a wavelength of 490 nm. Standards were plotted as a percent of OD max, and fibronectin concentration in lavage samples was determined by use of linear regression calculations. Total protein content of lavage samples was determined using the Pierce BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL).

Qualitative immunoblot analysis of intact and fragmented fibronectin. The content of intact and fragmented fibronectin in lavage of untreated and C5 fragment-treated rabbit lungs was assessed qualitatively by Western immunoblot analysis. C5 fragments (20 μ g) were instilled into the RCL of rabbits. The RCL and control LCL were lavaged with saline containing 5 mM PMSF and 5 mM EDTA 4 h later. The RCL and LCL of additional control rabbits, not treated with C5 fragments, were also lavaged under the same conditions. The fibronectin content of these lavage samples was determined quantitatively as outlined above by an indirect ELISA assay. Lavage samples and control intact rabbit fibronectin were loaded onto 7.5% NaDodSO₄-

PAGE gels and run in Laemmli buffer (34) under reducing conditions. Samples were transferred to nitrocellulose membranes (35), incubated with a goat anti-rabbit fibronectin IgG antibody, a rabbit anti-goat IgG antibody, and 1 μ Ci of ¹²⁵I protein A (36, 37) sequentially, and then developed by autoradiography overnight on Kodak XAR-5 film.

In experiments to determine whether instilled intact fibronectin was being fragmented over time, ¹²⁵I-fibronectin (ICN Radiochemicals, Irvine, CA) or unlabeled intact fibronectin were incubated in bronchoalveolar lavage. Aliquots were removed at times 0, 0.5, 2, 4, 24, and 48 h and loaded onto 7.5% gels as outlined above under reducing and nonreducing conditions. Analysis by autoradiography of ¹²⁵I-fibronectin and quantitative densitometry of Coomassie blue-stained gels loaded with unlabeled intact fibronectin showed that there was no fragmentation of intact fibronectin over the 48-h time course.

Statistics. Data were expressed as mean $\pm$ standard error and analyzed by analysis of variance using the Fisher PLSD and Scheffe F-test through the software Statview 512+ (Brainpower, Calabasas, CA) operating on a Macintosh PC. Statistical significance was considered to be present if $P < 0.05$.

Results

Monocyte migration: bronchoalveolar lavage fluid analysis. Fragments of fibronectin containing the fibroblast cell binding domain (120 kD) were selectively instilled into the RCL bronchus of rabbits. Indium-labeled monocytes were infused intravenously 15 min later, and their migration into the RCL and control untreated LCL alveolar space was assessed by determination of cell-associated radioactivity present in lavage fluid 48 h after monocyte infusion. The 120-kD fragment induced a significant monocyte migration as compared to intact fibronectin, a 30-kD fibronectin fragment (containing a heparin-binding domain), or the vehicle PBS $\pm$ isolation column eluate (Fig. 1A). The migration induced by the 30-kD fragment was minimal and not significantly different from the migration induced by eluate from fibronectin fragment isolation columns. A similar pattern of unlabeled mononuclear cell accu-

Figure 1. Fibronectin fragment-induced monocyte migration into the alveolar space. Intact fibronectin (0.33 nmol), a 30-kD fibronectin fragment (0.33 nmol), a 120-kD fibronectin fragment (0.33 nmol), eluate from fibronectin isolation columns, or saline were instilled into the RCL bronchus of rabbits. ¹¹¹Indium-labeled monocytes were infused intravenously 15 min later. The RCL was lavaged 48 h after monocyte infusion and cell-associated radioactivity (A) was measured in a gamma well counter and expressed as a percentage of radioactivity initially infused. Total mononuclear cell content in lavage was also measured (B). There was a significant monocyte migration and mononuclear cell accumulation in the alveolar space in response to the 120-kD fragment but not to the intact fibronectin or 30-kD fragment compared to controls. The data are expressed as mean $\pm$ SE, $n = 7$ for saline, and $n = 5$ for all other conditions.

mulation in the RCL was observed 48 h after the instillation of 120-kD fragments or controls (Fig. 1 B). At 48 h, under any of these experimental conditions, there was no detectable neutrophil accumulation in the RCL alveolar space as assessed by Wright-stained cytopspins of lavage fluid. There was no monocyte nor neutrophil migration detected in the control LCL under any experimental condition (data not shown). As we have previously reported with C5 fragment-induced lung inflammation (2), the majority of radiolabeled monocytes were retained in the lung, liver, or spleen with a small percentage emigrating into the alveolar space.

The time course of 120-kD fibronectin fragment-induced monocyte and neutrophil migration was determined. There was a significant monocyte migration into the RCL 6 h after monocyte infusion. The monocyte accumulation continued to increase 24–48 h after fragment instillation (Fig. 2). Significant neutrophil influx was observed by 6 h, and had disappeared by 48 h after the instillation of the 120-kD fibronectin fragment. Neutrophil migration appeared to be a nonspecific event since it also occurred, although to a lesser extent, with this same time course after instillation of 30-kD fibronectin fragments (Fig. 2) or other control substances (saline or eluate, data not shown).

Histologic sections were taken from the 120-kD fibronectin fragment-treated RCL and control untreated LCL (Fig. 3 A), and detailed morphometry was done to determine the content of neutrophils and mononuclear cells in lung tissue. By 6 h after fragment instillation, there was a significant neutrophil accumulation in both the alveoli and alveolar septa of the RCL (Fig. 3 B), 36% neutrophils and 64% mononuclear cells. By 24 h after the instillation of fragments, there was an increased alveolar and septal infiltration of leukocytes, predominantly mononuclear cells with a continued neutrophilic component in the RCL (Fig. 3 C), 15% neutrophils and 85%

Figure 2. Time course of 120-kD-induced monocyte and neutrophil migration into the alveolar space. Radiolabeled monocytes were infused intravenously 15 min after instillation of 120 kD fibronectin fragments into the RCL bronchus. Animals were lavaged at times indicated. ¹¹¹Indium-labeled monocytes (open rectangles) or unlabeled neutrophils in lavage were used as an index of leukocyte migration. There was a significant monocyte migration detected 6–48 h after 120-kD instillation. There was a significant 120-kD-induced neutrophil migration into the RCL (black circles) by 6 h that had disappeared by 48 h. Neutrophils also migrated into the RCL in response to 30-kD fibronectin fragments 6 h after instillation (open circles) and were absent by 48 h. Data expressed as mean ± SE, *n* = 6 for 6- and 48-h points, *n* = 4 for other times.

mononuclear cells. By 48 h, there was less leukocyte accumulation in the alveolar space, but a continued mononuclear cell accumulation in the thickened RCL alveolar septa was apparent, 2% neutrophils and 98% mononuclear cells (Fig. 3 D). There was no evidence of significant neutrophil or mononuclear cell accumulation in alveoli or alveolar septa of the control untreated LCL at the 6-, 24-, or 48-h time points (Fig. 3 A). Of those cells present in control lobes, > 99% were mononuclear.

Neutrophil independence of 120 kD fibronectin fragment-induced monocyte migration. To determine whether the monocyte emigration that was induced by 120-kD fibronectin fragments was neutrophil dependent, experiments to determine monocyte migration were repeated in neutropenic animals. There was a significant monocyte migration and total mononuclear cell accumulation in the RCL alveolar space 48 h after the instillation of the 120-kD fragment as compared to PBS controls (Fig. 4). As expected, neutrophils did not accumulate in the alveolar space. The magnitude of monocyte migration and mononuclear cell accumulation were decreased as compared to that observed in animals with normal neutrophil counts (Fig. 1).

Fibronectin content of lavage from the alveolar space after C5 fragment instillation. The fibronectin content in bronchoalveolar lavage fluid from C5 fragment-treated rabbits was determined by inhibition ELISA assay in lavage from both the RCL that was treated with C5 fragments and from the LCL that received no treatment. There was a significant increase in the fibronectin content of lavage from C5 fragment treated RCL by 2 h as compared to controls (Fig. 5 A). There was also a substantial, although not statistically significant, increase at the 1- and 4-h time points. There was no increase in fibronectin content of lavage at any time point from saline-treated RCL as compared to lavage from untreated LCL (data not shown). When fibronectin content was expressed with respect to total protein leak into the alveolar space, there was an appreciable rise at 1 and 2 h, but these levels did not reach statistical significance compared to control values (Fig. 5 B). In contrast, there was no increase in the fibronectin content of lavage over time from animals rendered neutropenic before the instillation of C5 fragments into the RCL (Fig. 5, A and B).

The ELISA assay showed a relative increase in the fibronectin content of lavage in C5 fragment-treated animals, but could not distinguish between intact and fragmented fibronectin. Qualitative analysis of lavage was therefore done by Western immunoblot analysis to determine the character of the increased fibronectin. Intact fibronectin was detected in lavage from both the RCL and LCL of normal untreated animals (Fig. 6, lanes 2 and 3). Fragments of fibronectin were detected in lavage from the RCL and control LCL of both C5 fragment-treated rabbits and untreated rabbits at all times examined (Fig. 6, all lanes). No intact fibronectin was seen in lavage from the C5 fragment-treated RCL of normal animals (Fig. 6, lane 4). In contrast, the majority of fibronectin detected in the lavage of C5 fragment-treated lung lobes in neutropenic animals was intact (Fig. 6, lane 6). Fragments of fibronectin of ~ 100–120 kD in size were among those fragments detected in all samples.

Inhibition of C5 fragment-induced monocyte migration by an antifibronectin antibody. To determine whether C5 fragment-induced monocyte migration into the rabbit lung could

be inhibited by antifibronectin antibodies, a monoclonal antibody directed against an epitope of fibronectin containing the fibroblast (RGDS) cell-binding domain (295 Ab) and a monoclonal antibody directed against an epitope distant from the cell-binding domain near the carboxy terminal of fibronectin (296 Ab) were instilled alone or in the presence of C5 fragments into the RCL. Animals were sacrificed 4 h after C5 fragment instillation, a time at which there was maximal monocyte migration into the RCL (data previously published; reference 2), and a time when antibody would be more likely to remain present in the alveolus. There was a 70% inhibition of C5 fragment-induced monocyte migration with coinstitution of the 295 Ab as compared to C5 fragments alone. In contrast, there was not a significant inhibition of monocyte migration into the RCL by the 296 Ab (Fig. 7 A). The antibodies themselves did not induce significant monocyte migration, and neither antibody altered C5 fragment-induced neutrophil migration into the alveolar space (Fig. 7 B).

Discussion

Fragments of fibronectin that contain the RGDS fibroblast cell-binding domain have been shown *in vitro* to have chemokinetic and chemotactic activity for monocytes (9). To determine whether these fragments could induce monocyte migration into the lung *in vivo*, a thermolysin-generated 120-kD fibronectin fragment that contains the fibroblast cell-binding domain (27) was locally instilled into the RCL bronchus of rabbits. The migration time course of intravenously infused ¹¹¹indium-labeled monocytes and total mononuclear cell content in lavage fluid revealed a significant monocyte accumulation in the RCL alveolar space by 6 h with a continued accumulation up to 48 h (Figs. 1–3). There was no significant monocyte migration in response to equivalent molar amounts of intact fibronectin or a 30-kD fibronectin fragment that contained a heparin-binding domain (Fig. 1). These data established that fragments of fibronectin that contained the RGDS cell-binding domain, but not intact fibronectin, lead to monocyte migration *in vivo*, and that the majority of neutrophil migration observed was not specifically in response to the 120-kD fibronectin fragment.

We have previously shown that C5 fragment-induced monocyte migration into the alveolar space of rabbits requires the prior emigration of neutrophils into the inflammatory site (2). Neutrophils are known to release a plethora of enzymes at sites of inflammation. *In vivo* studies have shown that neutrophil elastase, instilled into lungs of dogs or rats, localizes in alveolar septa (38) and leads to the degradation of alveolar septal components (39). *In vitro*, these enzymes can digest fibronectin into fragments that have chemotactic activity specifically for monocytes (10, 24). Accordingly, it is possible that neutrophils localized at sites of C5 fragment-induced lung in-

Figure 4. Migration of monocytes induced by 120-kD fibronectin fragments in neutropenic animals. The experimental protocol outlined in Fig. 1 was used. The 120-kD fragment of fibronectin induced a significant monocyte migration and total mononuclear cell accumulation in the RCL of neutropenic animals compared to PBS controls 48 h after instillation. The data are expressed as mean ± SE, $n = 4$ for ¹¹¹Indium-monocyte migration, and $n = 7$ for total mononuclear cell accumulation.

flammation could degrade fibronectin into chemotactically active fragments. These fragments could then potentially recruit monocytes into the inflammatory sites and in part account for the neutrophil dependence of C5 fragment-induced monocyte migration.

It was therefore initially important to determine the content of intact fibronectin and fibronectin fragments in the alveolar space of normal and C5 fragment-treated lungs. There was an increase in alveolar lavage fluid fibronectin over control values by 2 h after C5 fragment instillation (Fig. 5 A). These data confirmed our previous findings that C5 fragments induced a generalized protein leak into the alveolar space (40), and, in addition, showed that fibronectin was among those proteins. When this fibronectin content was normalized to total protein leak into the alveolar space, there was an appreciable, though not statistically significant, increase of fibronectin as compared to controls (Fig. 5 B), indicating that the major source of fibronectin in the alveolar space was most likely plasma. It was apparent by qualitative immunoblot analysis that fragments of fibronectin were present in lavage from both control and C5 fragment-treated animals (Fig. 6). Intact fibronectin, however, was present in lavage from untreated lung lobes but absent in lavage from C5 fragment-treated lung lobes (Fig. 6). Quantitation of fibronectin content in lavage by ELISA, however, showed that there was a significantly greater concentration of fibronectin in lavage obtained from C5 fragment-treated RCL as compared to controls (Fig. 5). Accordingly, it appeared that fragments of fibronectin were being retained in the alveolar space or that the formation of

Figure 3. Photomicrographs (675×) of a typical microscopic field from the RCL or LCL. (A) No leukocyte accumulation was noted in the LCL 6, 24, or 48 h after the instillation of 120-kD fibronectin fragments (histology shown at 24 h). (B) A significant neutrophil accumulation in the RCL airspaces and alveolar septa 6 h after the instillation of 120-kD fibronectin fragments (36% neutrophils, 64% mononuclear cells; determined by morphometric analysis). (C) A mononuclear cell accumulation with some neutrophils within the RCL alveolar spaces and thickened alveolar septa 24 h after the instillation of 120-kD fibronectin fragments (15% neutrophils, 85% mononuclear cells). (D) A mononuclear cell predominance in the thickened RCL alveolar septa and less leukocyte accumulation in the alveolar air spaces 48 h after the instillation of 120-kD fibronectin fragments (2% neutrophils, 98% mononuclear cells).

Figure 5. Fibronectin content in bronchoalveolar lavage from normal (open squares) and neutropenic (black circles) animals treated with C5 fragments. C5 fragments (20 μ g) were instilled into the RCL bronchus, and both the C5-treated RCL and untreated control LCL were lavaged individually at the different time points. (A) There was a significant increase in the fibronectin content of the RCL lavage as

compared to the LCL lavage at the 2-h time point in normal but not neutropenic animals. (B) When the fibronectin content was normalized to the total protein content of lavage fluid, there was an appreciable though not significant increase in fibronectin at the 1- and 2-hour time points in normal animals only. The data are expressed as mean $\pm$ SE, $n = 4$ for RCL points. LCL points in A and B represent the combination of values obtained at all time points ($n = 14$).

fragments was accelerated by some mechanism in the setting of C5 fragment-induced lung inflammation. It was notable that 100- and 120-kD fibronectin fragments were present in lavage in sizes similar to those fibronectin fragments that we have been described *in vitro* to be chemotactic for monocytes (9). This increase above control values of fibronectin in lavage was shown to be neutrophil dependent in that there was not an increase of fibronectin in lavage obtained from neutropenic animals treated with C5 fragments (Fig. 5, A and B). It was also observed qualitatively by immunoblot analysis that intact fibronectin was present in lavage from C5 fragment-treated lung lobes in neutropenic animals but, as mentioned above, absent in similar lavage from normal C5 fragment-treated animals. The increase of fibronectin observed in lavage (~ 400 ng/ml) is appreciably lower than the amount used in our experimental system (40 μ g/ml), although we have observed a small monocyte migration in response to 400 ng/ml of the

120-kD fragment (data not shown). These data are not meant to establish a concentration of fibronectin necessary to induce monocyte migration. Lavage analysis serves only to reflect the presence of mediators in the lung and, in most instances, does not quantitate the actual tissue concentration of these mediators. Fibronectin fragment content in lung tissue may be greater and persist for longer periods of time than that measured in lavage. This could explain the continued monocyte emigration into the lung over 48 h (Figs. 2 and 3).

These data do not exclude that cellular fibronectin is generated and fragmented in this system. Peters et al. have shown that oxidant-induced vascular injury leads to release of intact tissue-derived fibronectin in both the intravascular (21, 22) and alveolar compartments in either an isolated perfused rabbit lung or an intact *in vivo* rabbit system (22). Protease-induced lung injury in this isolated perfused lung system was further shown to lead to the accumulation of fragments of

Figure 6. Qualitative assessment of fibronectin in lavage fluid from untreated control and C5 fragment-treated rabbit lungs. C5 fragments (20 μ g) were instilled into the RCL of normal and neutropenic animals 4 h before lavage of both the treated RCL and the untreated control LCL. In addition, both the RCL and LCL were lavaged in normal control animals not treated with C5 fragments. The lavage used was saline plus 5 mM PMSF and 5 mM EDTA. These samples and control intact rabbit fibronectin were loaded onto 7.5% NaDodSO₄-PAGE gels and run in Laemmli buffer (34) under reducing conditions. Samples were transferred to nitrocellulose membranes, probed with a goat anti-rabbit fibronectin IgG antibody, and then with a rabbit anti-goat IgG antibody, incubated with 1 μ Ci of ¹²⁵I protein A, and developed by autoradiography overnight on Kodak XAR-5 film. Lane 1 is intact rabbit fibronectin; lanes 2 and 3 are RCL and LCL lavage from a normal control rabbit, respectively; lanes 4 and 5 are

C5 fragment-treated right and untreated control LCL lavage of a normal animal, respectively. Lanes 6 and 7 are C5 fragment-treated right and untreated LCL lobe lavage from a neutropenic animal. Intact fibronectin was present in all lanes except lane 4. Fibronectin fragments of ~ 100 - and 120-kD size predominated in these samples. The arrow indicates these 100–120-kD bands seen in lavage. Hatch marks represent molecular mass standards. Samples are representative of 1 animal for each condition listed. Each condition was examined in four separate animals.

Figure 7. Inhibition of C5 fragment-induced monocyte migration. Radiolabeled monocytes were infused intravenously 15 min after the instillation of 20 μ g C5 fragment plus 80 μ g of either a monoclonal antibody directed against an epitope of fibronectin containing the fibroblast RGDS cell-binding domain (295 Ab) or against an epitope of fibronectin not containing this domain (296 Ab). Animals were lavaged 4 h

after instillation, and leukocyte migration was assessed as in Fig. 2. There was a significant inhibition of C5 fragment-induced monocyte migration (A), but not neutrophil migration (B), by the 295 Ab and not by the 296 Ab. The antibodies themselves did not induce significant monocyte migration (white portions of bars). The data are expressed as mean $\pm$ SE, $n = 3$ for 296 Ab + C5 fragments, and $n = 5$ for all other conditions.

cellular (fibroblast) fibronectin in alveolar lavage fluid (22). Fibronectins have also been shown to accumulate in human cutaneous delayed-type hypersensitivity reaction sites (16), areas of renal (18), and synovial (19) inflammation, as well as being associated with alveolar macrophages during acute C5 fragment-induced lung inflammation (1) and in areas of chronic lung inflammation (20).

We speculated that monocyte migration in this system could in part be due to the fragmentation of fibronectin by neutrophils and that these chemotactically active fragments might be capable of inducing *in vivo* migration in the absence of neutrophils. There was a significant monocyte migration into the alveolar space of neutrophil-depleted animals in response to instillation of the 120-kD fragment (Fig. 4) showing that the 120-kD fragment of fibronectin was capable of inducing monocyte migration independent of preexisting neutrophil accumulation in the lung. The magnitude of monocyte migration and total mononuclear cell accumulation was less than that observed in animals with normal neutrophil counts. Wachfogel et al. have shown that fibronectin fragments containing the RGDS fibroblast cell-binding domain, but not intact fibronectin, induce neutrophil degranulation and elastase release (41). Because instillation of the 120-kD fibronectin fragment led to a nonspecific neutrophil accumulation in the lung and alveolar space of normal rabbits, a further *in vivo* generation of fibronectin fragments chemotactic for monocyte migration may have ensued and potentially amplified the monocyte migration response in those animals not neutropenic and with neutrophil migration into the lung. Alternatively, neutrophils may initiate further monocyte migration by other mechanisms as well. Despite a low grade influx of neutrophils in response to the eluate and 30-kD fibronectin fragments, monocyte migration was not significantly stimulated. Possible explanations for this were that the number of neutrophils present in lavage were not adequate to induce monocyte migration by the above proposed mechanisms, or that the neutrophils were not stimulated to release enzymes or other factors which could generate sufficient monocyte chemoattractant activity to initiate monocyte migration into the alveolar space under these conditions.

To further implicate fibronectin's role in monocyte migration, monoclonal antibodies directed against specific epitopes

of fibronectin were coinstilled with C5 fragments into the alveolar space of rabbits. An antibody directed against an epitope of fibronectin containing the RGDS fibroblast cell-binding domain significantly inhibited the monocyte migration. In contrast, an antibody directed against an epitope of fibronectin near the carboxy terminal of the molecule, downstream from and excluding the cell binding domain, was inactive (Fig. 7A). Neither antibody inhibited neutrophil migration (Fig. 7B), showing that the antibody was not inhibiting leukocyte migration globally in this model, but rather more specifically abrogating monocyte migration.

These data suggest that fibronectin fragments containing the RGDS fibroblast cell-binding domain and/or epitopes near the cell binding domain participate in monocyte migration in these experiments. These findings are in agreement with our previous *in vitro* studies of chemotaxis (9), where the 120-kD fibronectin fragment exhibited chemotactic activity for monocytes and intact fibronectin was inactive. We speculate that monocytes respond to a cryptic site of the fibronectin molecule which is uncovered after cleavage into the 120-kD fragment. Others have also suggested that the fibronectin molecule contains cryptic sites that interact with peripheral blood monocytes. Czop et al. (42) have shown that monocyte phagocytosis of particulate activators of the human alternative complement pathway is enhanced by fibronectin fragments but not by intact fibronectin.

These data do not directly prove that neutrophil proteases are responsible for the cleavage of intact fibronectin into fragments that are chemotactically active for monocytes. However, these data in conjunction with the above cited data (2, 9, 10, 22) strongly support this hypothesis. Certainly the proteolytic cleavage of fibronectin by neutrophil enzymes or by proteases from other sources, i.e., eosinophils, monocytes, macrophages, bacteria, or other cells are not the only mechanisms by which monocytes are stimulated to migrate into tissues. Other mechanisms not involving proteases could induce monocyte migration into inflammatory lesions, i.e., the stimulation of macrophages, lymphocytes, and neutrophils to release other monocyte-specific chemoattractants, the generation of nonspecific leukocyte chemoattractants, and potentially the generation of collagen and elastin-derived monocyte chemoattractants.

In summary, we report that (a) a 120-kD fragment of fibro-

nectin stimulates monocyte migration into the alveolar space of the rabbit lung; (b) this monocyte influx is in part neutrophil independent; (c) the concentration of fibronectin in the alveolar space of rabbits with C5 fragment-induced pulmonary inflammation is increased and largely fragmented in association with an increased plasma protein leak in normal but not neutropenic animals; and (d) a significant proportion of C5 fragment-induced monocyte influx into the alveolar space is abrogated by the administration of intraalveolar monoclonal antifibronectin antibodies directed against an epitope of fibronectin that includes the RGDS fibroblast cell-binding domain. We suggest that one mechanism by which monocytes are stimulated to migrate into pulmonary inflammatory lesions may be by initial neutrophil localization and the subsequent cleavage of proteins such as fibronectin into molecules that are chemotactic for monocytes.

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