Matrix metalloproteinase induction in fibrosis and fibrotic nodule formation due to silica inhalation


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Matrix metalloproteinase induction in fibrosis and fibrotic nodule formation due to silica inhalation. Am J Physiol Lung Cell Mol Physiol 288: L709–L717, 2005. First published December 17, 2004; doi:10.1152/ajplung.00034.2004.—Matrix metalloproteinases (MMPs) are the principle enzymes that initiate degradation of collagen. We examined the role of MMPs during alveolar wall fibrosis and fibrotic nodule formation from silica exposure. Rats were exposed to filtered air or 15 mg/m³ silica by inhalation for 5 days/wk, 6 h/day. Lungs were preserved by intratracheal instillation of fixative at 20, 40, 60, 79, and 116 days of exposure. Additional groups were fixed after 20, 40, and 60 days of exposure followed by 36 days of recovery. The number of nodules, defined by a collagenous core and a bounding cell layer detached from the alveolar wall, was determined by morphometry. Lungs showed increased alveolar wall collagen and fibrotic nodules at 79 and 116 days of exposure with increased collagenase and gelatinase activity. The number of nodules per lung in exposed groups increased from 619 ± 447 at 40 days to 13,221 ± 1,096 at 116 days (means ± SE, n = 5). No nodules were seen in control lungs. Silica-exposed rats with a 36-day recovery in filtered air showed enhanced MMP activity over exposure to silica for the same duration with no recovery. MMP-2 and MMP-9 were significantly elevated in alveolar macrophages after 40-day exposure. TIMP-1 expression was not significantly altered. In summary, MMP activity was upregulated at 40 days of silica exposure and progressively increased during ensuing fibrotic responses. Early expression of stromelysin was found in fibrosing alveolar walls and fibrotic nodules.

DESPITE IMPLEMENTATION of rules and regulations for safer limits to silica exposure in the workplace, some cases of acute and chronic silicosis continue to occur. Workers in occupations such as rock drilling, silica flour milling, and sandblasting (1, 5) remain susceptible to acute silicosis, defined as silicoproteinosis that develops within a few months up to 5 yr of inhalation of relatively high doses of silica (3, 30). Chronic silicosis, the form of the disease characterized by fibrotic nodules, can take 15 yr or more to develop and is also still prevalent in the modern worker (3, 22).

The more insidious form of the disease, chronic silicosis, results from recurring exposure to relatively low levels of silica (3, 22) and is frequently found in workers in occupations such as road construction, where clouds of silica particles are produced (19). These workers eventually develop restrictive lung disease with chronic cough and difficulty in breathing. Upon medical examination, silicotic nodules are found in the workers’ lungs, confirming the diagnosis of chronic silicosis (3, 22). These lesions, which are often seen in chest radiographs of workers’ lungs before the appearance of symptoms, are the hallmark of chronic silicosis.

Relatively low-dose, chronic occupational exposures to silica can lead to the development of silicotic nodules. These nodules, diagnostic for chronic silicosis, are described as having a cell-free concentrically arranged whorl of hyalinized collagen fibers with a cellular boundary (3, 24). The majority of silicotic nodules appear in the periphery of the lungs, mainly in the respiratory bronchioles and alveoli (4). Systematic studies show that silica nodules symmetrically enlarge with time (9), but the formation and development of these lesions in the early stages of chronic silicosis have not been characterized.

To follow the progression of nodules, researchers have studied how cytokines might affect their development. Investigators (6, 20) have studied the effects of interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-α) expression during murine silicosis. Deletion of the TNF receptor has been shown to significantly reduce the fibrotic response due to silica (21). These cytokines are upregulated in the lungs of the silica-exposed mice and appeared to be involved in the disease process, including granuloma formation. There is strong support for a role for TNF-α and IL-1β in granulomatous inflammation, but fibrotic nodules are not granulomas (15).

Another aspect of nodule development is the connective tissue remodeling that occurs at the core of these lesions. Remodeling, a normal event that continually occurs in healthy lungs, is a process whereby extracellular matrix (ECM) is recycled. In this process, the connective tissue in the lungs is broken down, and new ECM is synthesized to replace it. Various cells of the lung, particularly fibroblasts, produce the new matrix components for reconstruction. During this normal process, there is a balance between degradation and synthesis of matrix components (33). However, an aberrant remodeling process occurs from damage to the ECM due to silica inhalation, and the balance seen in the normal lung no longer exists. There is now increased degradation of the damaged matrix material and an oversynthesis of collagen for the repair of the injured alveolar region of the lung. This aberrant remodeling is thought to cause a continuous accumulation of collagen in...
fibrotic nodules and a continuation of fibrotic responses in the lung.

In fibrotic responses of the lung, the enzymatic degradative process is a critical step in the tissue remodeling process. Matrix metalloproteinases (MMPs) play a significant role in remodeling, as these enzymes can degrade every major component of the ECM. MMPs are zinc-dependent, highly regulated enzymes produced by virtually every type of cell in the alveolar region of the lungs (34). There are four major groups of these enzymes: 1) collagenases, 2) gelatinases, 3) stromelysins, and 4) matrilysins. Each group of MMPs plays a specific role in the degradation of components of the ECM. However, these are also powerful enzymes that have the enzymatic capacity to destroy a large portion of the lung ECM in a short period of time. For this reason, MMPs must be well regulated. As one type of regulation, these enzymes are secreted from the cell in a nonactive, proenzyme form. The pro-MMPs must then be cleaved by another MMP or lung enzyme to initiate enzymatic activity.

During fibrotic responses of the lung, there is an imbalance in remodeling mechanisms. Injured matrix from silica exposure must undergo remodeling to repair alveolar wall damage. MMPs, initiators of this process, are upregulated during early stages of silica exposure. Indeed, Perez-Ramos et al. (23) showed an upregulation of MMPs in silica-exposed rats. They showed an increase in MMP-2 and MMP-9, the endogenous MMPs of the lungs. However, the involvement of MMPs in the formation and development of nodules and alveolar wall fibrosis has not been elucidated. In this current study, MMPs and the continual remodeling process during silicosis were studied as pivotal fibrotic responses of the lung.

MATERIALS AND METHODS

Experimental model. Pathogen-free male Fischer 344 rats (strain CDF, 75–100 g initial weight; Charles River, Raleigh, NC) were housed in an animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. All rats were exposed and killed according to a standardized experimental protocol that complied with the Guidelines for the Care and Use of Laboratory Animals and was approved by the National Institute for Occupational Safety and Health Animal Care and Use Committee. After a 2-wk acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass chambers. Chamber exchange rate was 10–12 air exchanges per hour. After the acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass of chambers. Chamber exchange rate was 10–12 air exchanges per hour. After the acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass of chambers. Chamber exchange rate was 10–12 air exchanges per hour. After the acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass of chambers. Chamber exchange rate was 10–12 air exchanges per hour. After the acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass of chambers. Chamber exchange rate was 10–12 air exchanges per hour. After the acclimation period, rats were exposed to 15 mg/m3 silica (Min-U-Sil 5; U.S. Silica, Berkeley Springs, WV; average particle mass.
sample by adding 1 μl of the lung homogenate to 199 μl of 100 mM Tris (pH 7.6). For the activated sample, 1 μg of trypsin (in 1 mM HCl) was added to 50 μl of the lung homogenate and 145 μl of Tris (pH 7.6). For the activated sample, 1 μl of the lung homogenate and 145 μl of Tris. The solution was then incubated at 22°C for an additional 30 min. For measurement, the entire contents of the sample (activated or latent) was added to a 1-ml fluorescent cuvette in a water-jacketed bath, and 850 μl of incubation medium were added (100 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 5 mM CaCl2, 0.1 mM ZnCl2). One microgram of the fluorescent substrate was then added (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2; Sigma, St. Louis, MO), and the rate of change in fluorescence (328 nm excitation, 393 nm emission) was determined over 10 min. A standard curve of fluorescence vs. nanomoles of substrate cleaved was constructed using a cleaved fluorescent peptide (Mca-Pro-Leu-Gly-NHOH and p-NH2-Bx-Gly-Pro-D-Leu-Ala-NHOH, Sigma). Protein concentrations of the samples were measured by Lowry assay, and activity was expressed as nanomoles of fluorescent substrate cleaved per microgram of protein per minute.

Specificity of the assay for MMP activity was verified by a number of mechanisms inhibiting of MMP activity (N-CBZ-Pro-Leu-Gly-NHOH and p-NH2-Bx-Gly-Pro-D-Leu-Ala-NHOH, Sigma) were shown to have dose responses with 50% inhibition in the 4- to 30-μM range. The chelating agent EDTA and the zinc chelator 1,10-phenanthroline completely eliminated MMP activity. The rate of fluorescence was linearly proportional to the volume of added lung tissue and/or protein. Inhibitors of other classes of proteases had no significant effect on the activity of lung tissue homogenates. These included aprotinin, leupeptin, pepstatin, or soybean trypsin inhibitor (Sigma). Incubation with phenylmethanesulfonyl fluoride or N-ethylmaleimide (Sigma) produced <10% loss of activity.

**Immunohistochemistry.** Paraffin sections were deparaffinized and rehydrated with xylene-alcohol series to distilled water. For antigen retrieval, slides were then placed in citrate buffer solution (0.01 M, pH 6.0) and microwaved at a high setting for 105 s to bring the solution to 100°C. The solution was maintained at that temperature for an additional 6 min. Sections were then equilibrated to room temperature and rinsed in distilled water. Endogenous peroxidase was blocked by placing the sections in 3% hydrogen peroxide-methanol (1:1 vol/vol) for 30 min at room temperature for 20 min. After being rinsed in Tris buffer (0.1 M, pH 7.4), the sections were loaded into a Sequenza staining tray (Shandon-Lipshaw, Pittsburgh, PA), rinsed, and blocked with 1% BSA in PBS for 30 min. A primary antibody solution was then added, and the section was incubated for 16 h at 4°C. After incubation, the sections were washed with buffer and incubated with the secondary antibody. In this study, the secondary antibody was a biotinylated anti-rabbit and anti-mouse immunoglobulin adsorbed to abolish cross-reactivity with serum proteins of the rat (Dako, Carpinteria, CA). The sections were then washed in buffer, incubated with streptavidin peroxidase for 30 min, and washed with buffer. Then the antibody complex was detected by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Zymed Laboratories, San Francisco, CA) for 7 min. After detection, sections were washed with distilled water, counterstained with Mayer’s hematoxylin for 1 min, rinsed, dehydrated, and mounted. Primary antibodies for MMP were obtained from NeoMarkers (Fremont, CA) and consisted of antibodies to detect MMP-2 or gelatinase A (Ab-1 mouse monoclonal IgG, 1:100), MMP-7 (Ab-1 mouse monoclonal IgG, 1:1,000), MMP-9 or gelatinase B (Ab-8 mouse monoclonal IgG, 1:500), MMP-10 or Stromelysin-2 (Ab-2 mouse monoclonal IgG, 1:500), and tissue inhibitor of metalloproteinase-1 or TIMP-1 (Ab-1 mouse monoclonal IgG, 1:100).

**Confocal microscopy.** Fixed lung tissue was sectioned in blocks ~5 mm thick, dehydrated in a graded series of ethanol washes, fluorescently labeled by incubation for 24 h with a 0.1 mg/ml concentration of Lucifer yellow-dilithium salt according to the manufacturer’s protocol (Molecular Probes, Eugene, OR), and then embedded in Spurr’s epoxy for analysis by confocal microscopy. Images were recorded using a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA) fitted with an argon-ion laser (488 nm) at ×60 magnification. Emission spectra >510 nm were diverted to a photodetector and used to image lung tissue and cells. Scans of the lung tissue were recorded at a photomultiplier tube setting of 400, pinhole aperture setting of 50 μm, and a laser voltage setting of 20 mW. Three-dimensional projections were generated from 75 serial optical sections of lung tissue 0.7 μm thick each. The area of the x- and y-plane of view measured 3.0 × 10^5 μm. Three-dimensional reconstructions were generated, using Voxel View Ultra software (Vital Images, Fairfield, IA).

**Morphometric analysis.** The number of fibrotic nodules per lung was determined by morphometric methods in a manner similar to that previously described for counting alveoli in the lungs (17). To accomplish this, we cut spaced serial sections for a total distance of 250 μm (25 μm apart, 5 μm thick) from paraffin sections of the lung. Photographs were taken with a SONY Progressive 3CCD Digital DXC-9000 camera (Tokyo, Japan) on an Olympus AX70 microscope (Tokyo, Japan) and printed on 8 × 10-in photographic paper with a Codonics NP-1660 printer (Middleburg Heights, OH) at a final magnification of ×200. The dissector method of counting was then used to determine the number of nodules per unit volume in each series of sections. Basically this consisted of identification of nodules present in the first and last prints of the series. Each print covered a 1 × 0.8-mm area of the lung section with a guard region of ~0.2 mm. Nodules present in the last print but not present in the first print were counted unless their boundary crossed the forbidden line for unbiased counting described by Gundersen (11). Prints of the sections between the first and last print were examined to be certain that no small nodules were completely contained within the selection volume. For each series, the number of nodules per unit volume was then computed from the nodule count divided by the selection volume. To determine the total number of nodules per lung, we then multiplied the number of nodules per unit volume by the alveolar volume of the lung. Two series were measured in each animal, and six animals per group were studied.

**Statistical analysis.** Analysis of variance and Duncan’s multiple-comparison test (8) were used to evaluate the significance between measurements. All tests were two-sided tests, and P < 0.05 was considered to be significant. Data are given as mean ± SE.

**RESULTS**

Figure 1 displays a continuum of Sirius red-stained micrographs of lung sections. Sirius red stains collagen bright red. Figure 1A is lung tissue from a filtered-air control slide showing normal lung parenchymal structure. Figure 1, B, C, and D, shows micrographs from rats exposed to crystalline silica for 40, 79, and 116 days respectively. In Fig. 1B, foci of inflammatory cells are noted after 40 days of exposure; air spaces have an increased number of inflammatory cells, but the appearance of the alveolar walls remains normal. However, by 79 days of silica exposure (Fig. 1C), collagen has accumulated in the alveolar walls; a fibrotic nodule is shown forming within the alveoli as it “buds” from the alveolar wall; and there is a build-up of phospholipid in the air spaces. Figure 1D displays an accumulation of collagen in a fully formed fibrotic nodule after 116 days of exposure. The excessive Sirius red staining in these nodules and the alveolar walls demonstrates that the fibrotic process is occurring in these silica-exposed lungs. As with the 79-day exposure group, an excessive amount of phospholipid is seen in the air spaces at 116 days of silica exposure.

Figure 2 depicts the increase in the number of fibrotic nodules versus the duration of silica inhalation. Morphometric methods show that fibrotic nodules begin to form by 40 days of

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silica inhalation. The number of nodules progressively increases with increasing duration of silica exposure. Another group of animals was also exposed to silica for a period of 20, 40, or 60 days and then were allowed to “recover” from the exposure by breathing filtered air for 36 days. The number of silica nodules found in rat lungs after 36 days of filtered-air recovery was comparable to that expected if silica exposure had continued for these 36 days. No nodules were seen after 20 days of exposure in either the recovery or the nonrecovery group. This result indicates that once the fibrotic process reaches a certain threshold, nodule formation progresses even after silica exposure has terminated.

The connective tissue network of a rat lung exposed to silica for 116 days was imaged in Fig. 3 using laser scanning confocal microscopy. We generated a three-dimensional reconstruction of an alveolus containing a fibrotic nodule (Fig. 3, left). Collagen can be seen within the alveolar walls and in the core of the nodule. Within the alveolus, the nodule has expanded to fill the alveolar space. In the single section image (Fig. 3, right) the collagen appears as a bright white thread-like material encased in an aggregation of cuboidal epithelial cells, which covers the periphery of nodules and surrounds the accumulating collagen in the core. Collagen fibers projecting from the alveolar wall to the nodule are shown in the right micrograph. This micrograph illustrates the general observation that nodules form by collagenous, avascular buds from an alveolar wall. It is also of interest to note the concentration of alveolar macrophages at the periphery of the nodule in the air space.

Zymographs of lung homogenates from filtered-air control rats, silica-exposed rats with no recovery period, and silica-exposed rats with a 36-day recovery period are shown in Fig. 4. The 62-kDa and 92-kDa demarcations denote the molecular masses of the active form of gelatinase A and gelatinase B, respectively. These are also marked on the left-hand margin of the figure as MMP-2 and MMP-9. Along with these active forms, the proenzyme, or latent, form of these MMPs are indicated. These two MMPs are commonly found in the lungs as illustrated by the filtered-air control sample. As shown in Fig. 4, the active form of the MMPs demonstrates the most gelatinase activity in filtered-air control rats. For MMP-2, the latent form of the MMP is of comparable gelatinase activity to the activated MMP. Typically the latent form of MMP-9 is barely detectable in zymographs of filtered-air control rats. The MMP activity at 20 days of silica exposure was approximately comparable to the filtered-air controls. By 40 days there was a noticeable increase in gelatinase activity that was further increased with 60 days of exposure in the nonrecovery, silica-exposed rats.

The other groups examined were the rats exposed to silica for 20, 40, and 60 days followed by 36 days of filtered-air exposure.
Gelatinase activity in most groups increased when compared with filtered-air controls. The intensity of gelatinase activity in the 20-day exposure with recovery was comparable to the intensity of the bands at the 60-day exposure group with no recovery. The 40- and 60-day silica exposure with recovery groups showed an increase in activity compared with the corresponding 40- and 60-day silica exposed groups without recovery. The rats with silica exposure followed by a 36-day recovery period showed a larger increase in gelatinolytic activity in the pro- or latent form of both MMP-2 and MMP-9 compared with corresponding nonrecovery group.

Figure 5 shows the results from the MMP assay performed on lungs from the rats exposed to silica and the rats exposed to silica followed by 36 days of filtered-air exposure. The results are similar to those for gelatinase activity demonstrated in the zymograph of Fig. 4. Minimal MMP activity was seen after 20 and 40 days of exposure, but a significant increase is seen in the 60-day exposed group compared with controls. In addition, the 20-, 40-, and 60-day exposure groups with the 36 days of recovery (equivalent to 56, 76, and 96 days from the start of silica exposure, respectively) displayed a significant increase in MMP activity compared with the nonrecovery counterparts. The 60-day exposure group had MMP activity comparable to the 20-day exposure/recovery group. The activity at 76 and 96 days from the start of silica exposure with filtered-air recovery was higher but not statistically different from the MMP activity of the 60-day silica-only exposure group.

Localization of gelatinase A (MMP-2) in the fibrotic lung is shown in Fig. 6. In Fig. 6A, anti-MMP-2 was used to localize the enzyme in a 79-day silica-exposed lung. MMP-2 was highly expressed, shown by the brown precipitate of DAB, in a large number of alveolar macrophages in these silicotic lesions.
Fig. 6. Immunohistochemistry of gelatinase A (MMP-2) showing the lack of MMP-2 expression in nodules. Gelatinase A was intensely expressed in alveolar macrophages (arrow) at 79 days of exposure in A. B and C: adjacent sections displaying a fibrotic nodule. In B a Sirius red stain defines the collagen core of the fibrotic nodule. The immunolocalization of gelatinase A in an adjacent section is shown in C. Gelatinase A was diffusely expressed in alveolar macrophages (arrows) but not localized in the nodule. Comparable results were seen with gelatinase B (MMP-9).

Fig. 7, A–C, depicts the significant expression of stromelysin-2 (MMP-10) and its immunolocalization in alveolar macrophages at 40 days of silica exposure with increased expression at 79 and 116 days. Adjacent sections, such as illustrated
in Fig. 7, C and D, were utilized to identify immunopositive cells within the fibrotic nodules. Figure 7C shows the immunolocalization of MMP-10, which is displayed as brown precipitate in interstitial cells in fibrotic nodules and within alveolar walls of silica-exposed lungs. The adjacent section in Fig. 7D illustrates a fibrotic nodule using Sirius red staining in a rat lung exposed to silica for 116 days. With its collagenous core budding from the alveolar wall and an encircling cell layer, these nodules appear to be forming in the air space. Figure 7C is representative of the high level of immunostaining that was present in the fibrotic nodule in the case of stromelysin-2 (MMP-10). This finding is in contrast to that described for MMP-2 and MMP-9. Whereas MMP-2 and MMP-9 were expressed at high levels in alveolar macrophages and at lower levels in the alveolar walls, MMP-2 and MMP-9 were not found in fibrotic nodules. Stains for TIMP-1 and MMP-7 were also negative in fibrotic nodules (data not shown).

DISCUSSION

A critical requirement for fibrotic responses in the lung is the degradation of the interstitium by MMPs. These enzymes are known to degrade the connective tissue in fibrotic nodules and alveolar walls. When crystalline silica inhalation caused damage to ECM components, there was significant upregulation of MMP expression in the lung. Secretion of this zinc-dependent enzyme can occur in most cell types of the alveolar region; however, in our study, MMPs were predominantly expressed in the alveolar macrophages. We also observed that the number of macrophages in the air space increased with prolonged silica inhalation. As with continued silica exposure, the increased macrophage population in the air spaces displayed an upregulation of MMP expression. Indeed, earlier studies of ours (32) and others (23) detected the secretion of gelatinase A (MMP-2) and gelatinase B (MMP-9) by alveolar macrophages. As described in RESULTS, we did not find high levels of MMP-2 or MMP-9 in cells located in the alveolar walls such as epithelial type I, type II cells, interstitial, and endothelial cells. Expression of MMP-2, MMP-9, or any of the other MMPs in fibrotic nodules or cells in the alveolar wall during fibrotic lung response to silica has not been demonstrated.

One report (23) showed this same pattern of MMP-2 and -9 upregulation in alveolar macrophages in response to silica exposure as reported in the present study. These researchers also showed an increase in MMP-labeled granulomas at 15 days postintratracheal instillation (IT) exposure. Nevertheless, in their study, this early MMP expression was followed by a subsequent decrease in activity at the 60-day time point. In our study, the initial increase of MMP activity occurred at the same time as the onset of initial pathology (40 days). However, contrary to the findings of Perez-Ramos et al. (23), MMP activity continued to increase with increased exposure time.

A possible explanation for the discrepancy between these studies is the type of exposure method used. Our study utilized the inhalation of silica particles as opposed to the Perez-Ramos et al. study (23) where IT was the mode of exposure. The instillation method has some positives such as ease of exposure and accuracy of the amount instilled. Also, the amount instilled is the full lung burden for intratracheally instilled rats. In the Perez-Ramos et al. study, the rats were instilled with 50 mg of silica. However, the single bolus exposure by this technique poses some key physiological questions compared with inhalation. Dose rates are quite different between the two methods. Instillation introduces the whole dose in seconds. The inherent possibility of excessive dose by this material poses the risk of overwhelming the lung defense and producing effects that are not relevant to those that may occur at lower doses and dose rates (7). By comparison, lung burden by inhalation can occur over a time period of minutes, hours, days, weeks, and even months. The lung burden for the present study was examined by our colleagues (25) who found that from 10 to 116 days of exposure, there was a steady increase in lung silica burden to a maximum of ~7 mg at 116 days. Also, inhalation results in a relatively homogeneous distribution of silica throughout the lungs as opposed to gravitational distribution by IT (2).

Our inhalation study demonstrated an increase in MMPs in alveolar macrophages, but these cells were not directly involved in the interstitial remodeling of the ECM for nodule formation. However, budding from the ECM of the alveolar wall may begin the process of nodular formation. The process involves the separation of connective tissue and cuboidal-type cells from the alveolar wall forming a collagenous, cellular expansion into the alveolus. This budding eventually leads to a completely separated, avascular nodule with a collagen core and periphery of cells. These nodules appear as lesions in the air space of fibrotic alveoli. We attempted to discern if there was a progression of the budding morphology of nodules as they detached from the alveolar wall. However, at all time points, there appeared to be a mix of nodules at different stages.

By confocal microscopy, we examined the “anatomy” of these nodules. The fibrotic nodule contained a collagen core but did not have the extensive “whorl” pattern seen in the human silicotic nodule form. Surrounding this collagen is a number of cuboidal-like cells which appear to outline the lesion. Nodules were contained in an alveolus with a tether of collagen attaching the nodule to the alveolar wall bud point (Fig. 3). Again, this morphology supports the concept of a budding process that forms nodules from the alveolar wall. Others have suggested the genesis of nodules occurred in large clumps of cells (13, 26). By contrast, our light and confocal microscopy studies illustrated that a budding of a fibrotic portion of the alveolar wall can lead to nodular formation. Although unique in structure, the fibrotic nodules in the alveoli appear similar to scar tissue in the wound-healing process.

One well-studied theory of wound healing involves the removal of damaged collagen and other matrix components by the MMPs, thus allowing matrix synthesis and turnover (29). Intestinal damage caused by silica inhalation leads to mechanisms similar to wound healing and scar-tissue formation. The MMP involved in this process, and the focus of many wound studies, was stromelysin. In situ studies by Saarialho-Kere et al. (27) found stromelysin inside ulcers of the skin but never in adjacent or distal areas of scar tissue. Also, gelatinases A and B (MMP-2 and MMP-9) were not actively synthesized in these wound tissues. Another wound study, involving intestinal ulceration (31), found stromelysin again in the scar-tissue cells but absent from normal intestinal cells. Similar results were reported with corneal injury (10). In all of these studies, as with the present investigation, stromelysin was detected in the interior of scar tissue.
Because of these various wound studies, we focused our research on the possibility of stromelysin being the MMP present during the formation of nodules from silica inhalation. The nodules, which form in the alveolar region of the lung, appear quite similar to wound scar-tissue formations. Research cited earlier by Perez-Ramos et al. (23) studied some of the common MMPs, including MMP-2 and MMP-9, but did not examine fibrotic nodules for stromelysin (MMP-10). Our inhalation study showed no MMP-2 or MMP-9 activity within the nodular formations; however, stromelysin, the wound-healing MMP, was localized in the core of nodules and alveolar walls (Fig. 7). This agrees with the results seen in the wound-healing research referred to earlier. In our study, stromelysin is localized in nodular and interstitial cells as early as 40 days of silica exposure. These localized cells may break down damaged connective tissue and play a key role in fibrotic responses and remodeling in fibrotic nodule formation and alveolar wall thickening.

Using the rat model, we were able to follow the progression of fibrotic responses from the inhalation of crystalline silica for up to 116 days. The fibrosis in silica-exposed lungs occurred in the respiratory unit. Silica-induced disease progressed from alveoli containing foci of inflammatory cells during early exposure to fully formed fibrotic nodules. In our study, we found silica nodules as early as 40 days of silica exposure. After this time point, continued exposure generated a striking increase in the number of fibrotic nodules.

Another group of rats was exposed to crystalline silica followed by 36 days of filtered-air exposure. The lungs of these animals showed similar increases in fibrotic responses similar to those with continued exposure. That is, the number of nodules for the filtered-air recovery and nonrecovery group coincided solely with the number of days elapsed from the initiation of silica inhalation rather than the number of days of inhalation exposure. Once nodule formation occurred, at ~40 days of exposure, nodules continued to form at the same rate regardless of continuation of silica exposure. From these data, it appears that once a threshold for initiation of the fibrotic response is achieved, nodules will continue to grow and proliferate without further exposure.

Not only was there an increase in size and number of nodules in these silicotic lungs, there was also an increase in collagen content (Fig. 1). By 116 days of exposure, the fibrotic response increased the connective tissue synthesis and resulted in significant remodeling of the alveoli. There was also an upregulation of MMPs, the enzymes capable of degrading the ECM components. The degradation of matrix components and the oversynthesis of collagen indicate that remodeling of the lungs was occurring. As silica inhalation continued to the 116 day exposure, aberrant remodeling of the alveolar region appeared to be occurring, leading to fibrotic responses such as nodule formation and alveolar wall thickening. The results demonstrate that animals given acute, low-level exposure to silica followed by filtered-air recovery develop elevated MMP expression and fibrotic nodules.

Our results are consistent with the following scheme for the development and growth of fibrotic nodules. The interstitium of the nodule and alveolar wall in a silicotic rat continually undergoes remodeling. Indeed, Porter et al. (25) reported an increase in hydroxyproline with increased exposure from rats in the same inhalation study as ours. The nodule, with a large amount of connective tissue and a considerable collection of cells, continues to grow outwardly from a fibrotic alveolar wall. Eventually, the walls of the alveolus tend to collapse in on the fibrotic nodule, rendering that particular alveolus dormant. Finally, the incessant growth of this nodule leads to expansion outside its particular alveolus, allowing it to merge with other progressing nodules and ultimately cause the formation of large nodules similar to human fibrotic nodules.

The appearance of nodules around 40 days of silica inhalation suggests that nodule formation is a seminal event in silicosis. Silica exposure induces nodule genesis, but the continued formation and enlargement of these nodules appear to involve remodeling mechanisms similar to those described in wound healing. Once formation of fibrotic nodules has begun to occur, further exposure is not necessary for existing nodules to enlarge and new nodules to form. Stromelysin, an MMP found in scar tissue remodeling, was induced by silica exposure. Stromelysin was observed at the core of fibrotic nodules and appears to play an important role in the development of nodules.

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