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Increased Basigin in Bleomycin-Induced Lung Injury

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Basigin is expressed in many tissues during development, including lung. It is also found on tumor cells and in wounds where it is thought to stimulate adjacent fibroblasts to produce matrix metalloproteinases. To investigate whether basigin might be expressed in fibro-inflammatory lung processes, we generated bleomycin-induced lung injury in mice. At 14 d after intratracheal bleomycin, we found basigin prominently in areas of fibrosis, alveolar macrophages, and bronchiolar epithelium, whereas it was only weakly present in bronchiolar epithelium in untreated mice. Western blots of radioimmunoprecipitation assay RIPA-insoluble fractions of bleomycin-treated lungs showed increased basigin compared with RIPA-insoluble fractions of lung from untreated mice. By quantitative reverse transcriptase-polymerase chain reaction, lung basigin mRNA was significantly increased 14 d after bleomycin, and by *in situ* hybridization, basigin mRNA was prominent in bronchiolar epithelium. Western blots of bronchoalveolar lavage fluid (BALF) showed various forms of basigin after bleomycin that were not present in BALF from untreated lung. These results demonstrate that bleomycin-induced lung injury is associated with increased basigin expression in bronchiolar epithelium, deposition of basigin in fibrotic sites, and increased basigin in BALF. Accordingly, basigin may play a role in diffuse alveolar injury.

Basigin (also called EMMPRIN [extracellular matrix metalloproteinase inducer], CD147, and M6 in humans; HT7, neurothelin, and 5A11 in the chicken; gp42 in the mouse; and OX-47 and CE9 in the rat) is a member of the immunoglobulin superfamily of transmembrane proteins (1). It is a highly glycosylated protein of 43–66 kD with a protein core of ∽ 27 kD. The glycosylation varies considerably among different tissues, suggesting diverse functions (2). Basigin is expressed in many tissues during development, including lung (3), but it is essentially absent in normal adult lung tissue. There is an undefined requirement for basigin during development (most basigin-deficient embryos do not survive to birth) (4). Homology between human and murine basigin is 96% and 70% for the transmembrane and intracellular domains, respectively, and 51% for the extracellular domains (5).

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Abbreviations: bronchoalveolar lavage fluid, BALF; extracellular, EC; glyceraldehyde-3-phosphatase dehydrogenase, GAPDH; glutathione-S-transferase, GST; matrix metalloproteinase, MMP; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; sodium dodecyl sulfate, SDS.

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Basigin was identified as a product of human malignant cells (6, 7) able to upregulate expression of interstitial collagenase by fibroblasts. Subsequently, it was found to induce several matrix metalloproteinases (MMPs) (8–10). Recently, murine basigin was shown to upregulate MMP expression by fibroblasts (11). Because MMPs have been implicated in diffuse interstitial lung diseases (12–15) and bleomycin-induced lung fibrosis (16–18), we hypothesized that basigin may be involved in fibro-inflammatory lung processes. To investigate this possibility, we generated bleomycin-induced lung injury in mice and assessed the lungs for basigin expression.

Materials and Methods

Experimental Protocol

Male and female 129SvEv mice, 3–4 mo of age, were housed in a pathogen-free animal facility. After an intraperitoneal injection of ketamine and xylazine for sedation and anesthesia, 0.05 U of bleomycin (Blenoxane; Nippon Kayaku, Tokyo, Japan) was instilled intratracheally. At 7, 14, and 21 d, the animals were killed, and their lungs were subjected to bronchoalveolar lavage and processing for analyses of the tissue as described below. Mice without manipulation served as controls. All procedures were approved by the Washington University Animal Studies Committee.

Antibodies to Murine Basigin

A polyclonal antibody to the entire extracellular (EC) domain of murine basigin was raised in rabbits as previously described (19). Briefly, to construct an expression vector of the EC, a cDNA fragment corresponding to the EC domain of mouse basigin, nt. 74-637 (1) was inserted into the *Bam*HI/*Xho*I sites of pGEX-5X-1 (Pharmacia, NJ), which was then used to transform bacteria. The resultant glutathione-S-transferase (GST)-basigin EC fusion protein was used to immunize rabbits. To obtain basigin-specific polyclonal antibodies, the immune rabbit serum was passed sequentially over a GST-affinity column and a basigin EC affinity column. This antibody recognized basigin by Western blotting with the GST-basigin protein but did not develop any signal in extracts of tissues from basigin-deficient mice. This antibody was used for immunohistochemistry. For studies of basigin in bronchoalveolar lavage fluid (BALF) and in RIPA-soluble and RIPA-insoluble extracts of tissues, the monoclonal antibody MRC OX-47 (Serotec, Oxford, UK) to the EC of basigin was used in Western blots.

Bronchoalveolar Lavage

At 14 d after intratracheal bleomycin, animals were killed by CO_2 narcosis, and the lungs were lavaged three times with 0.6 ml of saline through a tracheal cannula (20). The BALF was centrifuged, and the supernatant was frozen at −70°C until use.

Tissue Processing

Immediately after the animals were killed, the thorax was opened, and the lungs were perfused with saline through the right ventricle to remove blood. The lungs were inflated with 10% buffered formalin at a constant pressure of 20 cm H_2O via the trachea for 20 min, excised, immersion fixed with 10% buffered formalin for 24 h, dehydrated, embedded in paraffin, and cut into 3-μm sections. The testes were excised and processed in the same way.

Immunohistochemistry

To localize basigin in the lung, immunohistochemistry was performed on paraffin-embedded tissue using a CSA kit (DAKO Japan, Kyoto, Japan) according to the manufacturer's protocol. Briefly, after deparaffinization and rehydration in graded ethanol, sections were washed with phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide at room temperature for 15 min. The sections were incubated with the rabbit anti-basigin EC antibody in PBS at 1:1,000 for 15 min at room temperature. After being washed with PBS, the sections were incubated with goat anti-rabbit immunoglobulin G-conjugated immunoperoxidase. Peroxidase activity was visualized by incubation with diaminobenzidine tetrahydrochloride. The tissue sections were counterstained with methyl green and mounted. Sections that were not exposed to the primary antibody were used as negative controls.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from whole lungs by ToTally (Ambion, Austin, TX) immediately after animals were killed (18). Reverse transcription was carried out, and 5′ exonuclease-based fluorogenic polymerase chain reaction (PCR) was performed using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) as previously described (18). Oligonucleotide primers for basigin were derived from exon 5 of the murine basigin gene sequence (GenBank accession number D00611). The primers and probe, designed to meet the specific criteria for using Primer Express software (PE Applied Biosystems), were as follows: sense-primer: 5′-GGGAAACCATCTCACTGCGT-3′; antisense-primer: 5′-TAG ATAAAGATGATGGTAACCAACACCA-3′; probe: 5′-FAM-CCTTCCTAGGCATCGTGGCTGAGGT-TAMRA-3′.

The predicted size of the amplified product was 102 bp, and the specific amplification was confirmed by ethidium bromide staining on an agarose gel. Primers and a labeled probe for glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) (TaqMan GAPDH control reagents), for an endogenous control, were purchased from PE Applied Biosystems. A mock PCR (without cDNA) was used to exclude contamination. The relative amount of basigin mRNA in the samples was normalized by GAPDH mRNA.

In Situ Hybridization

A fragment of cDNA encoding mouse basigin (nucleotides 1–891) (1) was inserted into pBluescript KSII$^+$ (Stratagene, La Jolla, CA) at the *Sma*I site. Sense and antisense probes were prepared using restriction enzyme-digested templates and RNA polymerases (sense: *Eco*RI, and T7 RNA polymerase; antisense: *Bam*HI and T3 RNA polymerase). [^{35}S]-labeled riboprobe was prepared from a linearized basigin cDNA template. A detailed description of the methods used for *in situ* hybridization was published previously (21). Sense controls were included with each hybridization and were handled identically to the corresponding antisense samples.

Western Blotting

Mouse lungs and testes were weighed and homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 nM Tris-HCl, pH 8.0) at 0.25 mg wet wt/μl (22). The lysates were spun for 15 min at 4°C in a microcentrifuge, and the soluble fractions were removed. RIPA-insoluble pellets (ECM-enriched fractions) were weighed and washed once in RIPA buffer and boiled for 10 min in SDS-reducing sample buffer at 1 mg wet wt/μl. BALF was boiled for 3 min in SDS-reducing sample buffer. Samples were electrophoresed in a 10% SDS-polyacrylamide gel with pre-stained molecular weight markers (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) with a semi-dry transfer cell (AE-6677; ATTO, Tokyo, Japan). Nonspecific binding sites were blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk for 1 h at room temperature. Membranes were incubated with the monoclonal antibody OX-47 overnight at 4°C. After three washes with Tris-buffered saline-containing 0.1% Tween 20, the membranes were incubated in horseradish peroxidase-conjugated anti-mouse immunoglobulin at 1:10,000 dilution (DAKO). Sites of antibody binding were visualized using the ECL Plus Western blotting detection system (Amersham, Aylesbury, UK).

Deglycosylation of Basigin

To determine whether the increased basigin in BALF after bleomycin was glycosylated, unconcentrated BALF obtained 7 d after intratracheal bleomycin was denatured by boiling for 3 min in 0.5% SDS and 0.1 M β-mercaptoethanol followed by incubation with or without 250 U/ml of N-glycanase (Calbiochem, San Diego, CA) at 37°C for 24 h. The reaction mixture was then subjected to Western blot analysis as described above.

Statistical Analysis

All data are expressed as means ± SEM. Statistical differences among groups were determined using a one-factor analysis of variance. Significance was defined at $P < 0.05$.

Results

Lung Basigin Is Increased and Associated with Fibrotic Areas After Intratracheal Bleomycin

Using polyclonal anti-basigin EC antibody, we found that basigin was present only weakly in untreated lung and was confined to bronchiolar epithelial cells (Figure 1A). In contrast, at 14 d after bleomycin, this antibody revealed basigin in fibrotic areas, associated macrophages, and bronchiolar epithelium (Figure 1B). To determine whether the basigin detected by the EC antibody in fibrotic areas reflected full-length basigin, we attempted immunostaining with antibody raised to the intracellular domain of basigin, but this antibody stained tissues only weakly—even tissues such as normal testes that are known to have abundant full-length basigin (data not shown). Thus, whether the basigin detected in the fibrotic areas represents only basigin EC, fragments of the EC, or the EC together with other basigin domains is unknown. However, the results of *in situ* hybridization, shown below, indicated that basigin mRNA is restricted to airway epithelial cells and is not present in areas of fibrosis. Accordingly, the basigin seen in fibrotic areas

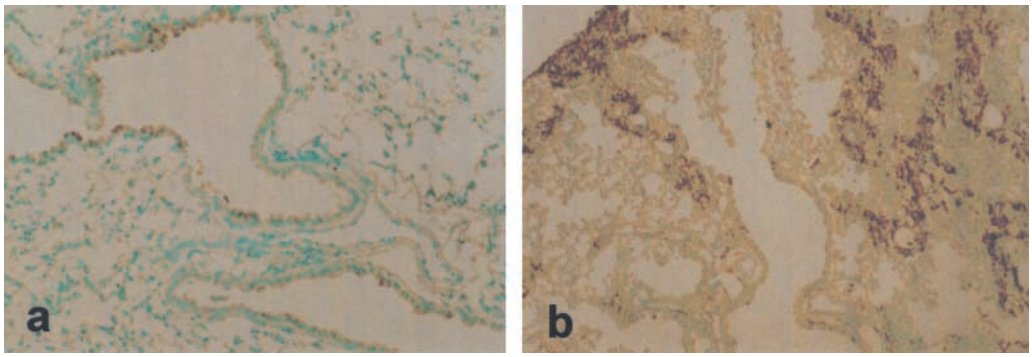

Figure 1. Immunohistochemical localization of basigin in mouse lungs using a polyclonal antibody to the extracellular domain of mouse basigin. (*A*) Basigin is detected weakly only in airway epithelium in the normal lung. (*B*) Basigin is prominent in fibrotic areas and associated macrophages and in airway epithelium in bleomycin-treated lung at 14 d. Original magnification: ×100.

after bleomycin seems to be basigin that has become associated with extracellular matrix after release from its cellular source.

To confirm the apparent association of basigin with lung extracellular matrix in the bleomycin-treated lung, Western blots were performed on RIPA-insoluble fractions of untreated lung and lung 14 d after bleomycin. Positive controls were done using testes. Basigin in testes was almost exclusively present in the RIPA-soluble fraction and appeared at ∽ 27 kD and 50 kD; there was little detectable basigin in the RIPA-insoluble fraction (Figure 2, *lanes 1* and *2*). In untreated lung, a basigin band was present at ∽ 27 kD in the RIPA-insoluble fraction of normal lung (Figure 2, *lane 3*). In comparison, there was a much stronger signal at ∽ 27 kD in the RIPA-insoluble fraction of bleomycin-treated lungs, and there was a prominent band at ∽ 58 kD that was not present in the RIPA-insoluble fraction of untreated lungs (Figure 2, *lane 4*). These results support an association of increased basigin with the fibro-inflammatory lesions in the bleomycin-treated lung and suggest that at least some of the basigin associated with the matrix is full length.

Lung Basigin mRNA Is Increased After Intratracheal Bleomycin

By quantitative reverse transcriptase-PCR, lung basigin mRNA was present only at low levels in untreated mice (Figure 3). However, at 14 d after intratracheal bleomycin instillation, the lung basigin mRNA was significantly increased compared with controls and with lung basigin mRNA 7 d after intratracheal bleomycin instillation ($P < 0.05$). The basigin mRNA level was back to baseline at 21 d.

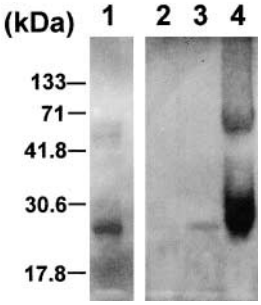

Figure 2. Western blots of lung tissue homogenates after extraction with RIPA-buffer using a monoclonal basigin antibody. *Lane 1:* RIPA-soluble fraction of testis. *Lane 2:* RIPA-insoluble fraction of testis. *Lane 3:* RIPA-insoluble fraction of normal lung. *Lane 4:* RIPA-insoluble fraction of bleomycin-treated lung at 14 d. Untreated lung is similar to normal testes in that there is minimal basigin associated with the RIPA-insoluble fraction, whereas in bleomycin-treated lung, basigin is strongly present in the RIPA-insoluble fraction.

Lung Basigin mRNA Is Expressed by Epithelial Cells

To investigate the site of expression of increased basigin mRNA after intratracheal bleomycin, *in situ* hybridization was performed. Testis, used as a positive control, showed strong signals (Figures 4A and 4B), which is consistent with previous data (4). There was minimal signal detectable in the normal lung (Figures 4C and 4D). In contrast, basigin mRNA was prominent in airway and alveolar epithelium in bleomycin-treated lungs at 7 and 14 d (Figures 4E, 4F, 4G, and 4H). Signals for basigin mRNA were not observed in fibrotic areas at 14 d (data not shown), indicating that fibroblasts and inflammatory cells at the fibro-inflammatory sites are not significant sources of basigin, despite the prominent deposition of basigin protein at those sites.

Glycosylated Basigin Is Increased in BALF After Bleomycin

Using the monoclonal antibody OX-47, basigin was detectable in BALF from untreated mice and mice lavaged 14 d

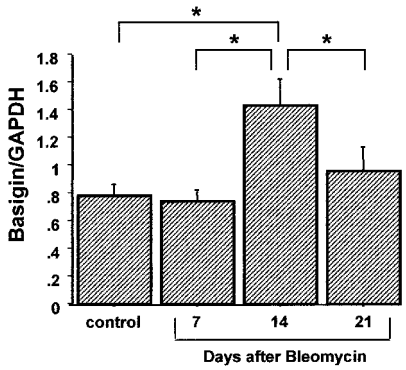

Figure 3. Quantitative reverse transcriptase-PCR analysis of lung basigin mRNA after bleomycin. The mean values (± SEM) are expressed as the ratio of basigin mRNA to GAPDH mRNA at each time point ($n = 5$ mice in each group). Basigin mRNA in the lungs was significantly upregulated at 14 d after bleomycin treatment and returned to baseline at 21 d. $^*P < 0.05$. One representative experiment from a series of three is shown.

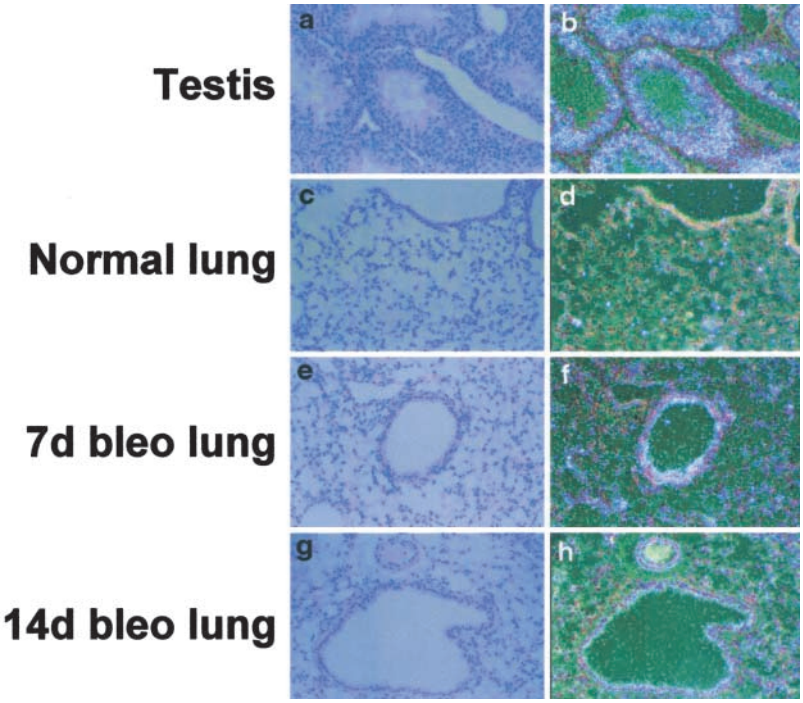

Figure 4. Localization of basigin mRNA in murine adult testis and lung. Shown are paired brightfield (*A*, *C*, *E*, and *G*) and darkfield (*B*, *D*, *F*, and *H*) views of testis or lung sections hybridized *in situ* for basigin mRNA (original magnification: ×100). Normal testis (*A*, *B*) shows strong signals. No signal is detected in the normal lung (*C*, *D*). At 7 d (*E*, *F*) and 14 d (*G*, *H*) after bleomycin instillation, basigin mRNA is evident in airway and alveolar epithelium. Controls using a sense probe demonstrated minimal hybridization in all samples (not shown).

after intratracheal bleomycin. However, the pattern and amount of signal was different between the two groups. In BALF from untreated animals, there was a single band at ∽ 55 kD (Figure 5A, *lanes 1–3*). In BALF from bleomycin-treated animals (Figure 5A, *lanes 4–6*) the ∽ 55-kD band was present but was much more prominent than in BALF from untreated animals. In addition, there was a heavy band at ∽ 27 kD and faint bands intermediate between the upper and lower bands. Thus, post-bleomycin BALF has increased basigin compared with BALF from untreated lungs, and basigin probably consists of unglycosylated and glycosylated forms. To confirm the presence of glycosylated forms of basigin in BALF after intratracheal bleomycin, BALF was exposed to N-glycanase to remove asparagine-linked carbohydrates. By Western blot, it was evident that N-glycanase produced a decreased mass of high-molecular-weight basigin and an increase in unglycosylated basigin at ∽ 27 kD (Figure 5B).

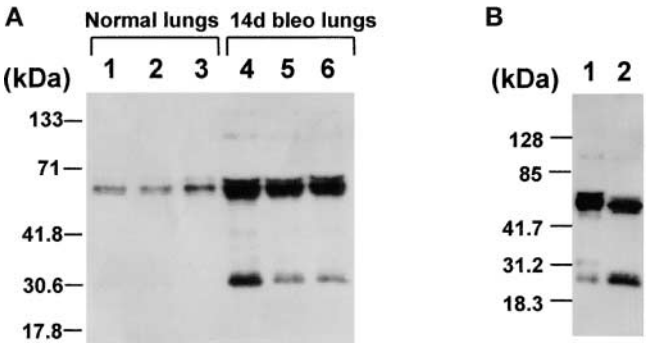

Figure 5. Western blots of BALF using a monoclonal antibody to the extracellular domain of basigin. (*A*) *Lanes 1–3:* Fluids from normal lung. *Lanes 4–6:* Fluids from bleomycin treated lungs at 14 d. All lanes show basigin at ∽ 55 kD, but after bleomycin there is increased basigin at 55 kD and prominent basigin at a molecular weight corresponding to basigin core protein. (*B*) BALF from bleomycin treated lungs at 7 d. *See* Materials and Methods for details. *Lane 1:* Without N-glycanase treatment. *Lane 2:* With N-glycanase treatment. N-glycanase has resulted in a downward shift of the prominent *upper band*, a marked increase in the size of the lowest *band* corresponding to basigin core protein, and the disappearance of *bands* between the *upper* and *lower bands.*

Discussion

Basigin is expressed at low levels in normal adult tissues, but its expression is increased in diverse pathologic processes, including malignant tumors of the skin and breast (23), failing myocardium (24, 25), rheumatoid arthritis synovial membrane (26), hepatitis C virus-associated cirrhotic liver (27), and ventilator-injured lung (28). As is evident from the tissues in which basigin expression is increased, basigin can be expressed by malignant cells, structural cells, and inflammatory cells. In the present study, basigin was prominent in airway epithelium, fibro-inflammatory foci, and alveolar macrophages after intratracheal bleomycin.

The time course of increased basigin expression in pathologic conditions has not been defined in most circumstances, but the increase can occur rapidly. In ventilator-induced lung injury in rats, basigin mRNA increased in alveolar macrophages and airway epithelial cells within ∽ 1 h of ventilation with high volumes, and the mRNA responses were matched by increases in basigin protein (28). We observed increased basigin mRNA by *in situ* hybridization at

7 d after bleomycin treatment. Studies at earlier time points may have detected increases.

The molecular basis for the upregulation of basigin expression in bleomycin-induced lung injury and in other settings is not known. Binding of Sp1 and Sp3 to an element in the promoter of human basigin seems to be critical for the regulation of basigin gene expression in macrophages (29). The levels of Sp1 and Sp3, and their activation and binding, are affected by a variety of stimuli; thus, different factors may be involved in different pathologic situations.

The distribution of basigin in the lung after intratracheal bleomycin was notable. By *in situ* hybridization, basigin mRNA at 7 and 14 d was largely confined to airway epithelial cells, whereas by immunohistochemistry it was prominent at fibro-inflammatory sites where there was minimal basigin mRNA. The apparent association of basigin with extracellular matrix was confirmed by Western blotting of insoluble extracellular matrix. Also, basigin was increased in BALF after bleomycin, with various forms not seen in BALF from untreated lungs. In ventilator-induced lung injury, the levels of 31 kD basigin, presumably nonglycosylated basigin, and other forms increased. Taken together, these results indicate that in the injured lung a large fraction of the increased basigin becomes dissociated from airway epithelial cells and macrophages, which are the principal cellular sources.

The basis for an association between basigin and fibro-inflammatory foci is not clear. It is possible that fibroblasts or inflammatory cells embedded in the fibrotic areas may bind and internalize basigin. Alternatively, basigin may have an affinity for fibrotic extracellular matrix in bleomycin-injured lungs, although basigin did not localize to sites of fibrosis in studies of hepatic fibrosis (27). Although the validity of bleomycin-induced lung injury as a model of human idiopathic pulmonary fibrosis is questioned (30), we have observed strong immunostaining for basigin in hyperplastic epithelium in idiopathic pulmonary fibrosis, so that, with regard to basigin expression, there is some similarity (T. Betsuyaku, Y. Fukuda, and R. M. Senior, unpublished observations).

The mechanisms that account for liberating basigin from the cells that produce it so that it is found in BALF and at sites of fibrosis are not known. Previous studies have concluded that basigin can become adherent to cells that do not make the protein, as in the case of peri-tumor fibroblasts (23). The present results are consistent with this notion; however, the fibro-inflammatory sites that show basigin are not necessarily adjacent to the epithelial cells that are the source of the basigin, suggesting that under some circumstances basigin undergoes significant translocation.

Basigin stimulates production of many MMPs by fibroblasts *in vitro*, including interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), MMP-2 (8, 9), MMP-9 (31), and MT1-MMP (10), and this property has been the focus of much interest since its discovery in 1982 (6). Basigin also induces the expression of MMPs in mesenchymal cells *in vivo*, and one consequence is to promote tumor cell invasiveness. It was shown recently that clones of breast cancer cells transfected with basigin cDNA were more tumorigenic and invasive than control plasmid-transfected cancer cells and expressed increased gelatinase A (MMP-2) and gelatinase B (MMP-9) (32). Binding of collagenase, and possibly other MMPs, to the basigin on tumor cells may be another property of basigin that promotes its effectiveness in enhancing tumor cell invasiveness (32). The terminal airway cells and cells comprising alveolar bronchiolization that we found to express gelatinase B (MMP-9) after intratracheal bleomycin previously were the cells showing basigin expression in the present study (18).

The mechanisms of basigin induction of MMPs have been only partially defined. Basigin co-immunoprecipitates and co-localizes with $\alpha3\beta1$ and $\alpha6\beta1$ integrins and can be cross-linked to these at the cell surface, suggesting an interaction with integrins (33). Downstream effects seem to involve the MAP kinase p38 (34). We have found that recombinant extracellular domain of murine basigin has the capacity to induce MMP-1 expression in human lung fibroblasts so that this domain of basigin alone may affect MMP expression by other cells (unpublished observation).

Because basigin and gelatinase B (MMP-9) expression at the mRNA level increase and peak at approximately the same time after bleomycin (i.e., 14 d), it is not obvious that basigin is a major factor in the upregulation of gelatinase B. Coincident expression of basigin and gelatinases also occurred in ventilator-induced lung injury. The increases in lung basigin after bleomycin and ventilator-induced injury included the low-molecular-weight form (28), which is presumably nonglycoslylated and not active in MMP induction (9). Accordingly, the impact of increased basigin expression on MMP production in these models may be less than expected based on basigin mRNA.

The role of MMPs in pulmonary fibrosis is not well understood, although increased MMPs have been demonstrated in human and experimental lung fibrosis. In human pulmonary fibrosis, increased MMP-9 expression involving macrophages and epithelial cells is associated with progressive fibrosis (14, 15). We (18) and others (16) have reported increased MMPs in bleomycin-induced lung injury in mice. However, we did not observe more severe fibrosis in MMP-9–deficient mice given bleomycin (18), so that MMP-9 does not seem to have an obvious role in limiting the fibrotic reaction, whereas MMP-7 (matrilysin) deficiency is associated with protection from fibrosis (35). Accordingly, the results with MMP-deficient mice suggest that there is not a simple link between tissue expression of MMPs and the magnitude of fibrosis post-bleomycin.

Although the capacity to promote MMP expression has been a focal point of many studies of basigin, basigin may have functions apart from the regulation of MMPs. Basigin deficiency, produced by gene targeting, is associated with a high level of embryonic lethality before E 8.5 (4). Analysis of basigin-knockout mice that have survived has revealed profound disturbances in reproductive function associated with the blockage of spermatogenesis at the first phase of meiosis (4), defective implantation, and endometrial abnormalities (36). In addition, there are multiple disturbances in the central nervous system (19) and an altered mixed lymphocyte reaction (37). These surviving animals have poor growth, and most die before 2 months of age with diffuse infiltration of the lungs with lymphocytes and granu-

locytes (4). Basigin (CD147) facilitates HIV-1 infection by interacting with virus-associated cyclophilin A, thereby promoting entry of the virus into cells (38).

In summary, we found that basigin is increased in the lungs in bleomycin-induced lung injury. The increase is reflected in BALF and at sites of fibro-inflammatory lesions; the cells expressing basigin are principally airway epithelial cells. The capacity of basigin to induce MMP expression in other situations raises the possibility that basigin affects MMP expression in the lung injury induced by bleomycin, but other effects of basigin cannot be excluded. These data suggest that basigin may participate in epithelial–mesenchymal interactions, leading to changes in tissue architecture during injury/repair of the lungs.

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