Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung

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Endo, Motoyoshi, Seiichi Oyadomari, Yasuhiro Terasaki, Motohiro Takeya, Moritaka Suga, Masataka Mori, and Tomomi Gotoh. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. Am J Physiol Lung Cell Mol Physiol 285:L313–L321, 2003. First published April 4, 2003; 10.1152/ajplung.00434.2002.—Arginase, which hydrolyzes arginine to urea and ornithine, is a precursor for the synthesis of polyamines and proline, which is abundant in collagen. The supply of proline can be a crucial factor in the process of lung fibrosis. We investigated the induction of arginine metabolic enzymes in bleomycin-induced mouse lung fibrosis. Histological studies and quantification of lung hydroxyproline showed that lung fibrosis develops in up to 14 days after bleomycin treatment. Under these conditions, collagen I mRNA was induced gradually in up to 15 days, and the content of hydroxyproline reached a maximum at 10 days. Arginase I mRNA was undetectable before bleomycin treatment but was induced 5–10 days after this treatment. Arginase I protein was induced at 7 days and remained little changed for up to 10 days and decreased at 14 days. On the other hand, arginase II mRNA that was detectable before treatment was increased gradually for up to 10 days and decreased at 14 days. Arginase II protein began to increase at day 5, increased for up to 10 days, and was decreased at day 14. mRNAs for cationic amino acid transporter-2 and ornithine decarboxylase were induced in a manner similar to that seen with collagen I mRNA. Immunohistochemical analysis showed that arginase I is induced in macrophages, whereas arginase II is induced in various cell types, including macrophages and myofibroblasts, and roughly colocalizes with the collagen-specific chaperone heat shock protein 47. Our findings suggest that arginine metabolic enzymes play an important role in the development of lung fibrosis, at least in mice.

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arginase in some inflammatory models (27, 45). Ornithine, and involvement of several cytokines were also noted in the lungs of LPS-treated mice, whereas arginase I is important role in cell proliferation and growth (22). The activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of the ornithine-polyamine pathway, parallels intensity of the repair processes in injured tissues (13).

Arginase exists in two isoforms, the hepatic type (arginase I) and the extrahepatic type (arginase II) (92). Arginase I and II genes were mapped to chromosomes 6q23 and 14q24, respectively. We reported that CCAAT/enhancer binding protein family transcription factors bind to the arginase I gene promoter and enhancer and play crucial roles in arginase I expression (14, 24, 25). It was also reported that Th1 cytokines suppress arginase I expression, whereas Th2 cytokines enhance it (30). Regulation of arginase II expression by cytokines was reported (26, 50). We found that both arginase I and II can prevent NO-dependent apoptosis in murine macrophage-derived RAW 264.7 cells by depleting intracellular arginine and thus decreasing NO production (15). We also showed that inducible nitric oxide synthase and arginase II are induced early in the lungs of LPS-treated mice, whereas arginase I is induced late in the lung (40). The induction of arginase and involvement of several cytokines were also noted in some inflammatory models (27, 45). Ornithine, which is formed by arginase I and/or II in endotoxemia, may be utilized for synthesis of polyamines and proline (and thus collagen), which are required for cell growth and tissue repair. Ignarro et al. (19) showed that ornithine, which is formed by arginase, is utilized for polyamine synthesis in rat aortic smooth muscle cells. They also reported that arginase activity is induced in the process of collagen synthesis (19). Durante et al. (10) reported that the physiological cyclic stretch directs arginine transport and metabolism to collagen synthesis in vascular smooth muscle, and Shearer et al. (41) noted induction of arginase activity in wounded healing. Therefore, it was deemed necessary to determine whether arginase isoforms and other arginine metabolic enzymes are involved in the process of lung fibrosis.

We now report that both arginase I and II are induced in the process of bleomycin-induced fibrosis of mouse lung. We also found that arginase I is induced in macrophages, whereas arginase II is induced in various cells, including macrophages and myofibroblasts. mRNAs for CAT-2 and ODC were also induced.

MATERIALS AND METHODS

Mice and treatment. Specific pathogen-free male C57BL/6 mice weighing 19–22 g (6 wk of age) were given 10 mg/kg of bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) intratracheally in 40 μl of saline and the controls in the same volume of saline. All these mice were killed at the indicated times following anesthetization with ether. All procedures involving animals were approved by the Animal Care and Use Committee of Kunamoto University.

RNA blot analysis. Total RNAs from mouse tissues were prepared by the guanidium thiocyanate-phenol-chloroform extraction procedure (8). After electrophoresis in formaldehyde-containing agarose gels, the RNAs were transferred to nylon membranes. For the hybridization, we used the following probes: digoxigenin-labeled antisense RNAs for rat arginase I (45), rat arginase II (16), rat CAT-2 (40), rat ODC (40), rat AS (56), and rat collagen type IV. The template plasmid for collagen I was prepared as follows. A partial cDNA for rat collagen I corresponding to nucleotides 3,415–3,882 (GenBank accession number S67530) was isolated by using RT-PCR and rat liver RNA, then inserted into the HindII site of pGEM-3Zf(+) (Promega, Madison, WI). Chemiluminescence signals derived from the hybridized probes were detected with a Las-1000 Plus bioimage analyzer (Fuji Photo Film, Tokyo, Japan) and the digoxigenin luminescence detection kits (Roche Molecular Biochemicals, Indianapolis, IN).

Immunoblot analysis. For immunoblot analysis of the cytosolic proteins arginase I and heat shock cognate protein (Hsc) 70, the mouse lungs were homogenized in 25 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 1% Triton X-100, and the homogenates were centrifuged at 50,000 g for 20 min at 4°C. The supernatants served as tissue extracts. For analysis of arginase II and Tom20 (55), both of which are mitochondrial proteins, the lungs were homogenized in 0.2 M KCl containing 0.25 M sucrose, and the homogenates were centrifuged at 650 g for 5 min at 4°C. The supernatants were centrifuged at 11,000 g for 20 min at 4°C. The pellet was dissolved in 25 mM Tris-HCl (pH 7.4) containing 300 mM NaCl and 1% Triton X-100 and was used as mitochondrial extracts that were subjected to SDS-PAGE. The proteins were electrotransferred to nitrocellulose membranes. Immunodetection was done with ECL kits (Amersham, Buckinghamshire, UK) according to the protocol provided by the manufacturer.

Immunohistochemical staining. Lungs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), and the excised tissues were embedded in optimum cutting temperature compound (Miles, Elkhart, IN) and frozen in dry ice, and then the sections (5-μm thick) were cut and air-dried. The digested sections were pretreated with 5 mM periodic acid for 10 min at room temperature to inhibit endogenous peroxidase activity. The specimens were incubated for 1 h with 1,000-fold-diluted rabbit antiserum against rat arginase I (20) or rat arginase II (38) or mouse monoclonal antibody F4/80 (28), which recognizes a 160-kDa glycoprotein on the cell surface of most mouse macrophage populations, then were washed three times with PBS for 5 min. For the single immunostaining of arginase I or II, the sections were incubated for 1 h with 500-fold-diluted sheep anti-rabbit IgG[F(ab')2] conjugated with peroxidase (Amer-
sham) as a second antibody, and peroxidase activity was visualized by incubation with a 3,3'-diaminobenzidine solution. For double staining of arginase I or II and macrophages, sections were incubated for 1 h with Alexa Fluor 488-labeled anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 546-labeled anti-rabbit IgG as second antibodies. For the double staining of arginase II and heat shock protein (HSP) 47 (6), specimens were incubated for 1 h with 200-fold-diluted antisera against rat arginase II and mouse HSP47. The sections were then incubated for 1 h with Cy2-labeled anti-mouse IgG (Amersham Pharmacia Biotech, UK) and Cy3-labeled anti-rabbit IgG as second antibodies. For the double staining of arginase II and \( ^{\alpha} \)-smooth muscle actin, specimens were incubated for 1 h with 200-fold-diluted antisera against rat arginase II and 500-fold-diluted mouse \( ^{\alpha} \)-smooth muscle actin (Sigma Chemical, St. Louis, MO). The sections were then incubated for 1 h with Cy2-labeled anti-mouse IgG and Cy3-labeled anti-rabbit IgG as second antibodies.

Hematoxylin-eosin and AZAN staining. Lungs were fixed by perfusing with 4% paraformaldehyde in PBS (pH 7.4), embedded in paraffin, and sectioned 5-\( ^{\mu} \)m thick. The procedures of hematoxylin-eosin (HE) and AZAN staining were as described (52).

Hydroxyproline quantification. To assess collagen contents in the lung, we measured hydroxyproline content, as described (54). In brief, a minced lung was homogenized in 6 N HCl and hydrolyzed for 5 h at 130°C. The pH was adjusted to 6.5–7.0 with NaOH, and the sample volume was adjusted to 30 ml with H\(_2\)O. The sample solution (1.0 ml) was mixed with 1.0 ml of chloramine T solution (0.05 M), and then the mixture was incubated at room temperature for 20 min. When 1.0 ml of 20% dimethyl benzaldehyde solution was added, the mixture was incubated at 60°C for 20 min. The absorbance at 560 nm was measured.

RESULTS

Development of lung fibrosis in bleomycin-treated mice. We induced lung fibrosis in male C57BL/6 mice by giving bleomycin intratracheally. In preliminary experiments, we titrated the dose of bleomycin, assessed the degree of fibrosis by histochemical staining, and analyzed induction of mRNAs for arginase I and II by Northern blot analysis (see Induction of arginase I in macrophages and of arginase II in various cells in bleomycin-treated mouse lungs). Maximal fibrosis and maximal induction of arginase I and II mRNAs were obtained with 10 mg/kg of bleomycin hydrochloride, although about one-third of mice died before 14 days. Thus bleomycin hydrochloride at 10 mg/kg was used in the following experiments. The development of lung fibrosis is shown in Figure 2.

Fig. 2. Development of lung fibrosis in bleomycin-treated mice. Lungs of control (A, C) and a bleomycin-treated mouse (14 days) (B, D) were stained with hematoxylin-eosin (HE) (A, B) or AZAN (C, D). Original magnification: \( \times 200 \).

Fig. 3. Measurement of hydroxyproline of the bleomycin-treated mouse lung. Bleomycin was given to mice intratracheally. After the indicated number of days, hydroxyproline of the lungs was measured as described in MATERIALS AND METHODS. Results are shown as means \pm SD (\( n = 3 \)).
fibrosis was evaluated 14 days after this treatment (Fig. 2). HE staining revealed diffuse alveolar destruction with collapse and obliteration of alveolar spaces, and collagen deposition was evident with AZAN staining. Collagen contains hydroxyproline as a unique component. To evaluate collagen contents in the lung, we measured hydroxyproline (Fig. 3), which was \( \sim 50 \mu \text{g} \) in control lungs. The hydroxyproline markedly increased 5 days after treatment and reached a maximum (200 \( \mu \text{g} \)) at 10 days with little decrease at 14 days. Thus lung fibrosis is indeed induced by bleomycin with development of lung fibrosis being completed in \( \sim 10 \) days.

Induction of arginine metabolic enzymes in the bleomycin-treated mouse lung. We analyzed the induction of collagen I and arginine metabolic enzymes in bleomycin-treated mouse lung by using RNA blot analysis (Fig. 4). Collagen I mRNA was present at a low level in untreated mice, began to increase at 3 days, and increased gradually for up to 15 days, a finding in good agreement with data in Fig. 3. Arginase I mRNA was not detected in the control lung, was induced 5 days after bleomycin treatment, reached a maximum at 7 days, and decreased to undetectable levels at day 14. Arginase II mRNA, which was present at a low level in untreated mice, began to increase on the third day and...
reached a maximum level at day 10. Arginase I and II mRNAs were induced somewhat earlier than collagen I mRNA. ODC mRNA, which was present at a low level in untreated mice, began to increase on day 5, and reached a maximum level on day 10. OAT mRNA evident in untreated lungs remained little changed by bleomycin. ODC mRNA was also induced by bleomycin treatment, whereas OAT mRNA was highly expressed before treatment and did not change much after treatment. These results suggest that arginase I and II are induced in the process of lung fibrosis and supply ornithine. Then, ornithine produced by arginase is assumed to be used for proline synthesis by OAT and for polyamine synthesis by ODC. CAT-2 mRNA, which was weakly detectable in untreated mice, began to increase on day 3 and increased gradually for up to 15 days. Therefore, arginine may be derived from outside the cells by CAT-2. In contrast, mRNA for AS, an arginine biosynthetic enzyme, was present before treatment and remained little changed after treatment.

Figure 5 shows the time course of induction of arginase I and II proteins in the bleomycin-treated mouse lung. Arginase I protein was not detected in untreated controls, began to increase on day 7, increased markedly by day 10, and was much decreased by day 14. This time of induction agrees well with that for arginase I mRNA. The two polypeptides of arginase I apparently arose by alternative translation initiation from the two methionine residues located 30 base pairs apart (23). Arginase II protein was hardly detectable when lung extracts were subjected to immunoblot analysis. Because arginase II is located in the mitochondria, we used the mitochondrial fractions for analysis. Arginase II protein was hardly detectable in untreated controls, began to increase on day 5, had increased markedly by day 10, and decreased little by day 14. This time of induction agrees with that for mRNA.

Induction of arginase I in macrophages and of arginase II in various cells in bleomycin-treated mouse lungs. To identify cells expressing arginase I and II, we performed immunohistochemical analysis of the lung (Figs. 6 and 7). As mentioned above, the dose of bleomycin used in these experiments is sublethal. Therefore, the extent of induction of arginine metabolic enzymes may be underestimated. However, we believe that our conclusion is not affected. Arginase I immunoreactivity was undetectable in the control lung (Fig.
whereas strong immunoreactivity was seen in the bleomycin-treated mouse lung. Types I and II lung epithelial cells were negative for arginase I, and positive cells were morphologically considered to be macrophages. Double immunostaining with anti-arginase I and macrophage-specific antibodies showed that arginase I is induced in a group of macrophages (Fig. 6B). Arginase II immunoreactivity was weakly evident in some cells in control lungs yet was strong in many cells in bleomycin-treated lung, apparently including type I
and II epithelial cells (Fig. 7A). Double immunostaining with anti-arginase II and macrophage-specific antibodies showed that arginase II is induced in macrophages but is also induced in many other cells (Fig. 7B). α-Smooth muscle actin is a marker of myofibroblasts that plays an important role for the development of the fibrotic lesion (Fig. 7C). α-Smooth muscle actin staining was not detected in control mouse lung, and arginase II immunoreactivity was evident in a small number of cells. In bleomycin-treated lung, many cells positive for α-smooth muscle actin staining were observed. Some of them were positive for arginase II, whereas others were negative. These results also indicate that arginase II is induced in many cells other than macrophages and fibroblasts.

HSP47 is a collagen-specific molecular chaperone in the endoplasmic reticulum that is involved in synthesis, folding, and assembly of various collagens, and its expression parallels that of collagen (36). Therefore, we performed double immunostaining of arginase II and HSP47, to see whether arginase II-expressing cells produce collagen (Fig. 7D).

Immunostaining with anti-HSP47 revealed that the HSP47 is expressed in various cells in lungs and that it is clearly induced with bleomycin-treatment. Distribution of bleomycin-induced arginase II was similar with that for HSP47. Our observations suggest that arginase II is induced in collagen producing cells and that this enzyme provides the ornithine needed for proline synthesis.

**DISCUSSION**

Arginine is a precursor for the synthesis of proline and polyamines, and because proline is an important component of collagen, the supply of proline can be a crucial factor in the process of lung fibrosis. Other investigators have reported the involvement of arginine metabolic enzymes in the production of proline or polyamines (11, 19). The successful healing of wounds requires the local synthesis of significant amounts of collagen. Albina et al. (2) showed that ornithine may contribute to the synthesis of protein-incorporated proline in wounds by increasing the extracellular pool of free proline. Arginase I and II knockout mice were recently generated. In arginase I knockout mice, the plasma ornithine level is fourfold greater than that for wild type, and the plasma proline level is twice as great as that for wild type, and the plasma proline level is not changed (42). These results show that arginase regulates plasma arginine and proline levels. Wei et al. (53) reported that arginase I plays a potentially important role in controlling proliferation of rat aortic smooth muscle cells by providing ornithine for the production of polyamines. Our present data show that mRNA for ODC, a rate-limiting enzyme in polyamine synthesis, was induced by bleomycin treatment. Therefore, we propose that ODC is induced to provide polyamines for the proliferation of cells such as fibroblasts during processes of tissue repair.

Under normal conditions, arginase I is expressed almost exclusively in the cytosol of hepatocytes, where it serves as the final enzyme of the urea cycle. Expression of arginase I in the liver is regulated by dietary protein (33) and by hormones such as glucagon and glucocorticoids (46). More recently, arginase I was found to be induced also by LPS, interleukin (IL)-4, cAMP, and hypoxia in macrophages (26, 27, 34, 51) and by LPS in macrophages of various tissues of rats and mice (40, 45). IL-4 is also known to induce collagen synthesis in fibroblasts in vitro (31). On the other hand, lung IL-4 expression was detected in a murine model of bleomycin-induced pulmonary fibrosis (12). These results suggest a potential role for IL-4 in pulmonary fibrosis. Perhaps because of the potential to stimulate and amplify inflammatory responses, IL-4 stimulates collagen synthesis in lung cells and thus promotes the progression of fibrosis in the end stage of lung disease. If this proposal is tenable, bleomycin may induce arginase I through the induction of IL-4.

Transforming growth factor (TGF)-β is known to enhance the expression of the lung matrix that causes fibrosis. Boutard et al. (4) reported that TGF-β stimulates arginase activity in macrophages. Durante et al. (11) found that TGF-β stimulates arginine transport and metabolism in vascular smooth muscle cells. Induction of TGF-β in the bleomycin-treated lung has been repeatedly reported. Therefore, TGF-β is a good candidate of arginase-inducing cytokines.

We observed that arginase I is induced in alveolar macrophages by bleomycin treatment. Although there have been reports (18, 41) concerning the expression of arginase in alveolar macrophages, this is apparently the first report showing a relationship between arginine metabolic enzymes and bleomycin-treated lung fibrosis. In the process of fibrosis, the demand for proline increases as it is required for the synthesis of collagen fiber. Arginase I in alveolar macrophages may contribute to the supply of proline for collagen synthet.
sisis in collagen-synthesizing cells. It is also possible that arginase I supplies ornithine for synthesis of polyamines, which were found to be required for the functional activation of macrophages (29).

Arginase II is primarily expressed in the kidney, small intestine, and lactating mammary glands and at low levels in other tissues, under normal conditions (38). Arginase II, like arginase I, is induced in LPS-activated mouse peritoneal macrophages (34, 40). In contrast to arginase I, arginase II expression is not increased by IL-4 (26, 27). The expressions of arginase I and II are regulated differentially. We found that arginase II is induced in the mouse lung after bleomycin-treatment. Induced arginase II is present in various cells including alveolar macrophages and epithelial cells. Expression of HSP47 in macrophages has been reported (1). Expression of HSP47 always parallels that of collagen in developing tissues and various cell lines and in collagen-related pathological conditions such as fibrosis (35). The appearance of myofibroblasts (characterized by α-smooth muscle actin expression) is characteristic in lung fibrotic lesions. We showed that major portions of myofibroblasts are positive for arginase II. The distribution of HSP47 (39) is similar to that of arginase II. These results suggest strongly that induced arginase II plays an important role in collagen synthesis by providing proline in the bleomycin-treated mouse lung.

IPF is a rapidly progressive illness of unknown cause, and no effective drug therapy is currently available. Our findings suggest that arginase may prove to be a new target for the prevention of the development of lung fibrosis.

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DISCLOSURES
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1. Abe K, Ozono Y, Miyazaki M, Koji T, Shioshita K, Furusu A, Tsukasaki S, Matsuya F, Hosokawa N, Harada T, Taguchi T, Nagata K, and Kohno S. Interstitial expression of heat shock protein 47 and alpha-smooth muscle actin expression is characteristic in lung fibrotic lesions. We showed that major portions of myofibroblasts are positive for arginase II. The distribution of HSP47 is similar to that of arginase II. These results suggest strongly that induced arginase II plays an important role in collagen synthesis by providing proline in the bleomycin-treated mouse lung.


