0031-3998/93/3406-0729$03.00/0
PEDIATRIC RESEARCH

Vol. 34, No. 6, 1993
Printed in U.S.A.

Tissue Inhibitor of Metalloproteinase-1 mRNA is Specifically Induced in Lung Tissue after Birth

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ABSTRACT. Interactions between extracellular matrix, proteins, metalloproteinases, and their inhibitors play a major role in determining the structure of the lung during *in utero* development and after birth. To better understand the molecular mechanisms underlying lung development and morphogenesis, expression of the tissue inhibitor of metalloproteinases (TIMP-1) gene was examined *1*) through the course of late fetal development, *2*) when normal fetal development was interrupted by premature birth and extrauterine survival, and *3*) during exposure of prematurely delivered neonates to hyperoxia. Total RNA isolated from lung tissue of fetal baboons (*Papio* sp) at 140, 150, and 180 d of gestation (term gestation = 180 d); baboons prematurely delivered at 140 d of gestation, 1, 2, 6, and 10 d old; and premature baboons ventilated for 6 and 10 d with 100% oxygen was examined by Northern blot analysis. The results demonstrated that TIMP-1 mRNA, which is expressed at low levels during fetal development, undergoes a marked increase in abundance shortly after both premature and term birth. This parturition-induced pattern of gene expression appears to be tissue specific to the lung and, contrary to results reported for adult and neonatal animals, is not affected by ventilation of the premature lungs with 100% inspired oxygen. Although the physiologic consequences of TIMP-1 mRNA induction by birth are not yet known, these data suggest a possible role for TIMP-1 in postnatal adaptation of lung tissue. (*Pediatr Res* 34: 729–734, 1993)

Abbreviations

ECM, extracellular matrix
FiO_2 1.0, 100% oxygen
PRN, clinically appropriate
TIMP, tissue inhibitor of metalloproteinases
SSC, standard saline citrate

Mammalian development and morphogenesis is accomplished by spatiotemporally specified patterns of cellular proliferation and differentiation. These processes occur within an architectural framework known as the connective tissue or ECM and are guided by the action of diffusible factors such as cytokines. Collagen fibers are the major constituent of the ECM, and their breakdown is a key step in processes requiring scheduled (developmental) or unscheduled (injury) tissue remodeling. Collagen metabolism is regulated in part by a balance between the activities of two sets of proteins: metalloproteinases such as collagenase, which degrade collagen, and TIMP, which counteracts their activity. TIMP-1 is a glycoprotein member of the metalloproteinase inhibitors that binds to activated interstitial collagenase, activated stromelysin, and type IV collagenase (1–3). Cells that produce interstitial collagenase can also synthesize and secrete TIMP-1. In these cells, the net collagenolytic activity can be modulated by the activated enzyme and TIMP-1 levels (4). The human TIMP-1 gene has been isolated, sequenced, and mapped (5, 6).

Morphogenesis of the lung, particularly during early development and perhaps postnatally, is sensitive to the pattern and composition of the ECM (7, 8). Branching morphogenesis of the lung, which is dependent on mesenchymal-epithelial interactions, does not occur in the absence of a functional ECM at the interface of the two interacting tissue layers (7, 8). Similarly, postnatal lung development probably occurs on a functional ECM whose structural modification may be necessary for adaptation of the lung from a liquid breathing phase to an air breathing phase at birth. One important role for the ECM is in regulating the growth and differentiation of various cell types. Thus, some components of the ECM, such as certain proteolytic fragments of laminin, have growth factor activity on cultured cells (9). However, a novel mechanism of growth control in tissues may entail the ability of the ECM to regulate the sequestration and timely release of peptide growth factors (*e.g.* basic fibroblast growth factor) (10). This form of regulation can be mediated by the interplay between the ECM degrading enzymes (*e.g.* metalloproteinases) and their inhibitors such as TIMP-1. Thus, during tissue morphogenesis and remodeling, levels and/or activity of proteinases and their inhibitors may be modulated to regulate the release of biologically active cytokines stored in the ECM depot.

We have been interested in the regulatory mechanisms that control *in utero* and postnatal development of the lung and how they may be altered in pathophysiologic conditions. In this paper, we report studies that examined the expression of TIMP-1, a key regulatory protein involved in ECM turnover, in both fetal and premature baboon lungs. TIMP-1 transcripts have been identified during early embryogenesis in the mouse (11). However, the regulation of this gene during fetal development has not been elucidated nor is it known whether it is influenced by birth. Previous studies have demonstrated increased expression of TIMP-1 mRNA in the lungs of rabbits exposed to hyperoxia (12, 13). It is unclear whether this increase occurs in the lungs of premature infants, who as a consequence of birth itself undergo a 6-fold increase in inspired oxygen tension, and also frequently require supplemental oxygen to sustain life (14).

The present studies were performed in a unique model of fetal development, premature birth, and survival (15). This model

Received May 12, 1993; accepted July 29, 1993.
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Supported by Grants HL48298 from the National Institutes of Health and RG-109-N from the American Lung Association.

allows the characterization of genetic programs expressed during normal development and offers information on how these programs may be altered when *in utero* development is interrupted by premature birth. Our findings demonstrated that TIMP-1 expression in the lung occurs at low levels during fetal development and is increased in a tissue-specific manner in the lungs of term and premature animals upon parturition. Furthermore, in support of our previous studies on regulation of surfactant protein gene expression (16), these data demonstrated that premature infants differ from neonatal or adult animals in many aspects of gene regulation. Functional consequences of these observations are discussed. Preliminary observations of these studies were previously published in abstract form (17).

MATERIALS AND METHODS

Detailed descriptions of this model have been reported previously (14, 15). Briefly, when delivered at 140 d (of 180-d term gestation), the premature baboon develops hyaline membrane disease that is biochemically, clinically, and morphologically identical with that in human premature infants. Animals supported with positive pressure ventilation and PRN oxygen require high FiO_2 and ventilator pressures for the first few days, have onset of recovery late in the second day of life, and do not develop chronic lung disease. However, treatment with positive pressure ventilation and FiO_2 1.0 for 10 d or longer results in a predictable homologue of bronchopulmonary dysplasia with clinical, radiographic, and morphologic findings typical of the human disease.

Gestational ages were determined by matings that were timed by observation of perineal sex skin changes and confirmed by ultrasound examination at intervals during the pregnancy. Fetal animals were delivered by hysterotomy at 140, 150, or 180 d of gestation and immediately killed, without breathing air, by an overdose of barbiturates. Premature animals were delivered by hysterotomy at 140 d of gestation, intubated, and resuscitated, and placed on a time-cycled ventilator. Their intensive care management was similar to that used for human premature infants and has been reported previously (14). Premature baboons were maintained on ventilatory support for 24 h (140 + 1 d), 48 h (140 + 2 d), 144 h (140 + 6 d), or 240 h (140 + 10 d) in either PRN or 100% oxygen (FiO_2 1.0) for the duration of the experimental period. In addition, two spontaneously delivered term infant baboons were electively killed by an overdose of anesthesia at 48 h of age (180 + 2 d). At autopsy, a segment of the lung was removed, frozen in liquid nitrogen, and stored at −80°C.

Extraction and analysis of RNA. RNA was extracted from frozen lung samples using a modified method of Chirgwin *et al.* (18). Lung tissues from three animals were examined separately in each case. Approximately 0.5 g of frozen tissue was ground to a powder in a crucible containing liquid nitrogen. The pulverized tissue was transferred to a tube to which 25 mL of diethylpyrocarbonate-treated homogenization buffer (3 M LiCl, 10 mM NaAc, pH 5.0, 6 M urea, 0.2 mM vanadyl ribonucleoside complexes) were added. This mixture was homogenized with a motor-driven homogenizer, and the homogenate was stored at −20°C overnight to precipitate the RNA. The precipitate was then pelleted by centrifugation, resuspended in guanidinium thiocyanate homogenization buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, and 0.5 wt/vol Sarkosyl, Sigma, St. Louis, MO), layered over a 5.7 M CsCl cushion, and centrifuged at 32 000 rpm in a Beckman L8-70 ultracentrifuge using the SW41 Ti rotor at 20°C for 18–20 h. Pelleted RNA was subsequently dissolved in 1% wt/vol SDS, 0.5 mM EDTA, extracted with phenol-chloroform, and precipitated with ethanol. The final RNA pellet was collected by centrifugation and redissolved in diethylpyrocarbonate-treated water for further analysis.

For Northern blot analysis, RNA concentrations were determined by spectrophotometry at 260 nm and equal amounts (approximately 10 μg) of RNA were denatured in a formaldehyde/formamide solution (19), heated at 65°C for 5 min, and quenched on melting ice. RNA was resolved by gel electrophoresis on 1.2% agarose/formaldehyde gels at 50 V for 2–3 h. Northern blot analysis was performed as described by Thomas (20). The RNA was transferred to Hybond-N (Amersham, Buckinghamshire, UK) membranes overnight, washed the next day in 2 × SSC for 2 min, and baked at 80°C for 2 h before hybridization.

For quantitative slot-blot analysis, two identical but separate blots of total denatured RNA were prepared as follows. For each sample, 2 and 10 μg of total RNA were blotted identically on Nytran filters (Schleicher and Schuell, Keene, NH). After UV cross-linking of the RNA to Nytran (according to manufacturer's specifications), the filters were prehybridized and subsequently hybridized with either TIMP-1 or the β-actin probes. Steady state levels of β-actin were used as a control against which the abundance of TIMP-1 mRNA was quantified among different samples. The integrity of each RNA sample was first confirmed by Northern blot analysis before use on the slot blots. For quantification, various exposures of the autoradiograms were scanned with a densitometer (Ultroscan, XL, Pharmacia LKB, Uppsala, Sweden). Table 1 presents the calculated means ± SD of the densitometric ratios of autoradiographic signals for TIMP-1/β-actin mRNA for each experiment.

Preparation and use of DNA probes. A plasmid containing human TIMP-1 genomic DNA (ATCC 59667) was purchased from the American Type Culture Collection (Rockville, MD). A genomic clone containing the sequences for β-actin was used to determine the expression of a constitutively expressed gene and to normalize the amounts of RNA on Northern and slot blots. The inserts were subcloned into pGEM 4Z (Promega, Madison, WI) and, after growth and purification of the plasmids, were excised using the appropriate restriction enzymes and subsequently purified by agarose gel electrophoresis and electroelution. The purified inserts were radioactively labeled with [α-^{32}P]deoxycytidine 5′-triphosphate using a random primer labeling technique (21), and the products were purified by two rounds of Sephadex G-50 spin column chromatography. Blots were first prehybridized by incubation in hybridization buffer (19) for 4 h at 42°C. Hybridization was then performed at 42°C by incubating the blots in hybridization buffer (5 × SSC, 50% formamide, 5 × Denhardt's) containing 10% dextran sulfate and the desired ^{32}P-labeled probe overnight. The following morning, the blots were washed stringently for 2 h in 0.1 × SSC and 0.1% SDS at 50°C with several changes. Blots were then autoradiographed with X-OMAT AR film (Kodak) at −70°C using Kodak X-Omatic regular intensifying screens. For multiple probing of the same blot, subsequent hybridizations were performed by first removing the previous probe by soaking the blot in near-boiling water for 30 min and then hybridizing as described above.

Table 1. *Measurement of TIMP-1 mRNA during hyperoxia**

Age (d)	O_2 treatment	TIMP-1/β-actin
140 + 6	PRN	0.609 ± 0.138
	FiO_2 1.0	0.580 ± 0.120
140 + 10	PRN	0.593 ± 0.156
	FiO_2 1.0	0.623 ± 0.091

* Values represent the calculated means ± SD of the densitometric ratios of autoradiographic signals for TIMP-1/β-actin mRNA for each experiment (n = 3). Animals were prematurely delivered at 140 d of gestational age and treated with hyperoxia (FiO_2 1.0) or normoxia (PRN) for 6 and 10 d, respectively.

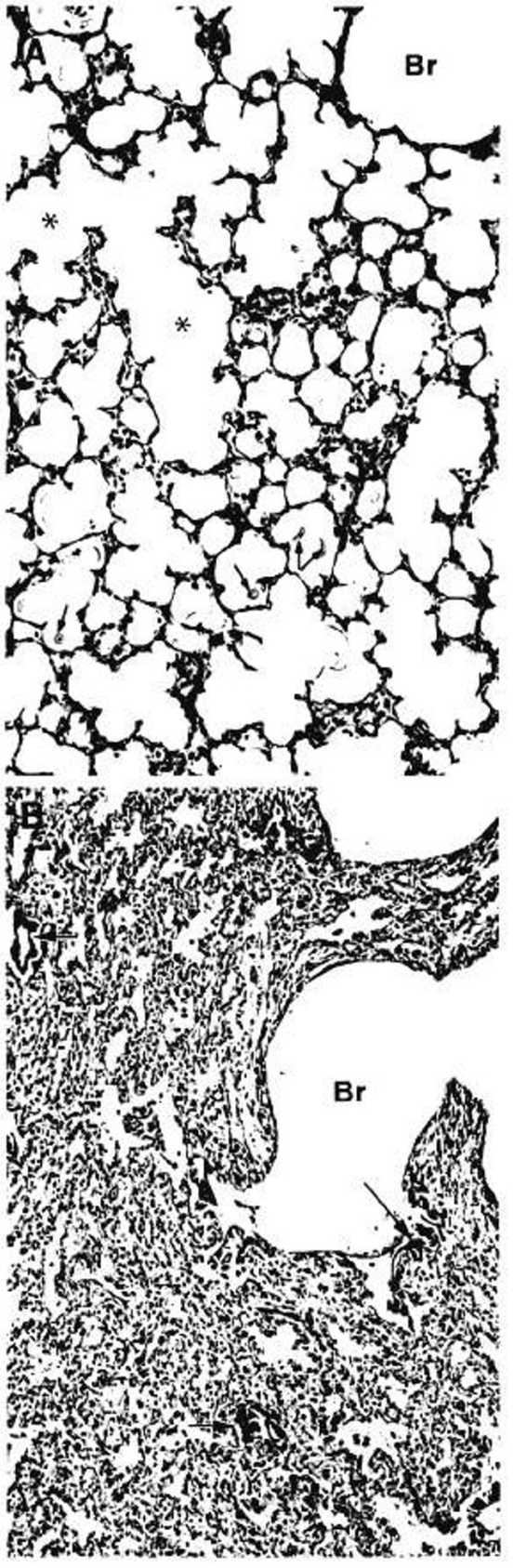

Fig. 1. Histologic findings in the lungs of fetal and premature baboons. *A*, 140-d gestation control. The bronchiole (*Br*) and several alveolar ducts (*) are present. The saccules (primitive alveoli) show secondary crest and early alveolar configurations. A few squamous cells are present in the air spaces (*arrows*). *B*, 140 + 2 d premature baboon.The bronchiole (*Br*) is dilated and partially lined with hyaline membranes (*long arrow*). The distal air spaces are collapsed and some show hyaline membranes (*short arrows*). Both specimens were fixed in phosphate-buffered 4% paraformaldehyde-0.1% glutaraldehyde and stained with hematoxylin and eosin (×160).

RESULTS

Treatment of premature baboons. The clinical course of the 1-, 2-, 6- and 10-d-old animals was similar to that previously described from our laboratory (14, 15). All the premature baboons developed hyaline membrane disease and required substantial ventilator support. Comparison of histologic findings in the lungs of 140-d baboon fetuses and 140 + 2 d premature baboon neonates showed significant lung injury by 2 d of postnatal life (Fig. 1). In spite of high ventilator pressures (mean pressure 13–14 cm H_2O), the PRN animals required an FiO_2 between 1.0 and 0.8 for the first 42 h; then they could be progressively weaned to levels below 0.3 by 96 h of age. In the hyperoxic animals, FiO_2 and Po_2 remained elevated throughout the treatment period, although, in contrast to the PRN animals, progressive disease led to a significant fall in arterial/alveolar oxygen after d 4.

Expression of TIMP-1 in fetal and postnatal lung tissue. Expression of TIMP-1 through the course of late *in utero* development and during premature delivery and adaptation to the extrauterine environment was examined by Northern blot analysis of RNA. Total RNA samples, extracted from fetal lung tissues of animals at 140, 150, and 180 d (term) gestational age as well as from prematurely delivered fetuses of 140 d gestational age who were treated with PRN oxygen for 24 h (140 + 1 d), 48 h (140 + 2 d), and 6 and 10 d (140 + 6 d and 140 + 10 d), were resolved by gel electrophoresis, transferred to Hybond-N membranes, and probed with radioactively labeled TIMP-1 genomic DNA insert. Figure 2 shows the results of autoradiography of a representative analysis. TIMP-1 DNA probe was hybridized to an mRNA with an electrophoretic mobility corresponding to approximately 0.9 kb, consistent with the size of the human TIMP-1 mRNA previously reported (22). Although expression of TIMP-1 mRNA in fetal samples was very low, it was nevertheless detectable after long exposure of the blots or overloading of the RNA quantity in specific lanes (see for example Fig. 2, *lane A*). In contrast, however, a significant increase in TIMP-1 mRNA abundance occurred within 24 h after birth (Fig. 2, *lane D*). Thereafter, TIMP-1 mRNA levels appeared to stabilize and remained stable through d 10, demonstrating that expression of TIMP-1 mRNA in the lung was low during late fetal development and underwent a marked increase within 24 h after the birth of premature fetuses.

TIMP-1 gene expression and prematurity. We next asked whether the increase in TIMP-1 mRNA abundance observed in the previous experiments occurred only as a consequence of premature birth or whether it also occurred during term delivery. To address this question, we compared TIMP-1 mRNA levels among total RNA samples isolated from lung tissues of 180-d fetuses, lung samples of 140 + 2 d prematurely delivered infants, and 180 + 2 d term delivered infants. Northern blot analysis of these samples revealed that TIMP-1 mRNA was detectable in only the two postnatal samples (Fig. 3). Therefore, expression of TIMP-1 at birth was related to delivery and extrauterine adaptation and was independent of the level of fetal maturation.

Tissue specificity of TIMP-1 mRNA induction after birth. To determine whether parturition-induced accumulation of TIMP-1 mRNA occurs in other tissues or specifically in the lung, two other tissues in the prematurely delivered fetuses were examined for the expression of this gene. RNA was isolated from liver and kidneys of prematurely delivered 140-d gestational age animals who were maintained in the extrauterine environment for 0 (control) and 48 h. Northern blot analysis of these samples revealed that TIMP-1 mRNA was specifically induced only in the postnatal lung tissue (Fig. 4, *lane B*). Therefore, although not

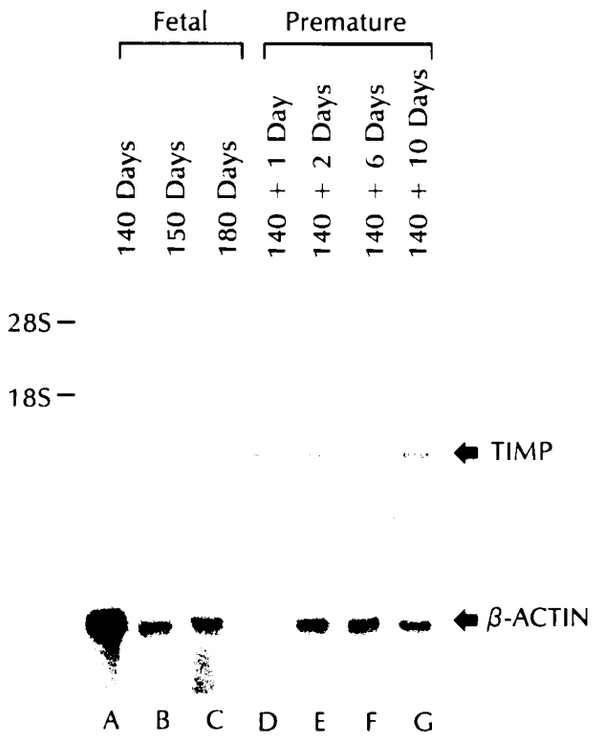

Fig. 2. A representative Northern blot of TIMP-1 mRNA abundance in fetal and postnatal lung tissue. With the exception of lane A, approximately equal quantities (10 μg) of total RNA isolated from lung of fetal and prematurely delivered baboons were resolved by electrophoresis on 1.2% formaldehyde agarose gels and blotted onto Hybond-N, as described in Materials and Methods. The amount of RNA in lane A was intentionally overloaded (5×) to demonstrate TIMP-1 mRNA in fetal samples. The filter was hybridized with a TIMP-1 ^{32}P-labeled DNA probe prepared by the random primer labeling method and autoradiographed. *Lower panel*, the same blot probed with a ^{32}P-labeled β-actin genomic clone.

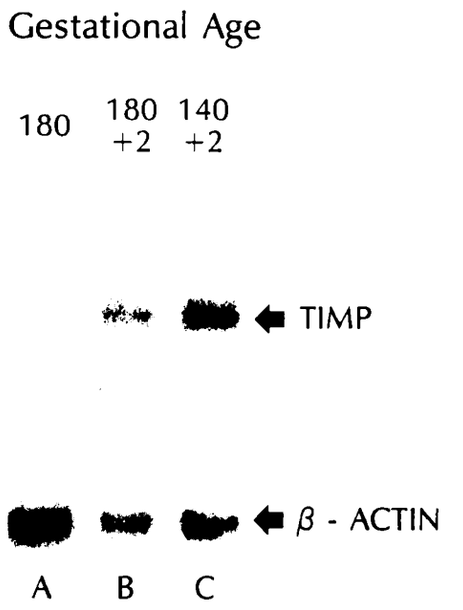

Fig. 3. Postnatal increase in TIMP-1 mRNA. The abundance of TIMP-1 mRNA in 10 μg of total RNA from the lung tissue of 180-d fetuses, 140 + 2 d premature neonates, and 180 + 2 d term neonates was examined by Northern blot analysis; *upper panel*, probed with TIMP-1 ^{32}P-labeled DNA; *lower panel*, the same filter probed with a ^{32}P-labeled β-actin genomic clone.

an exhaustive tissue survey, these experiments clearly demonstrated that accumulation of TIMP-1 mRNA within 48 h after birth was tissue specific.

Effect of hyperoxia on postnatal levels of TIMP-1 mRNA in prematurely delivered infants. Exposure to hyperoxic levels of inspired oxygen for 64–96 h increases TIMP-1 and surfactant-associated protein A mRNA levels in the lungs (12). The increase in TIMP-1 mRNA has been speculated to be an early marker of pulmonary fibrosis. In contrast to these studies, we have recently shown that the increase in surfactant-associated protein A mRNA level in response to hyperoxia does not occur in prematurely delivered baboons (16). To determine the effect of hyperoxic exposure and/or lung injury on regulation of TIMP-1 gene expression in the premature infants, the abundance of TIMP-1 mRNA was determined by quantitative slot-blot analysis. These analyses demonstrated that the abundance of mRNA for TIMP-1 does not change significantly between premature baboons ventilated with 100% oxygen for 6 and 10 d and those ventilated with PRN oxygen (Table 1), even though, as described previously (14, 15), animals exposed to 10 d of hyperoxia have significant lung injury. Therefore, postnatal levels of TIMP-1 mRNA in prematurely delivered baboons were not increased by hyperoxic lung injury.

DISCUSSION

In the present study, we have examined the effect of *in utero* development, premature birth and survival, and postnatal exposure to hyperoxia on TIMP-1 mRNA abundance in the im-

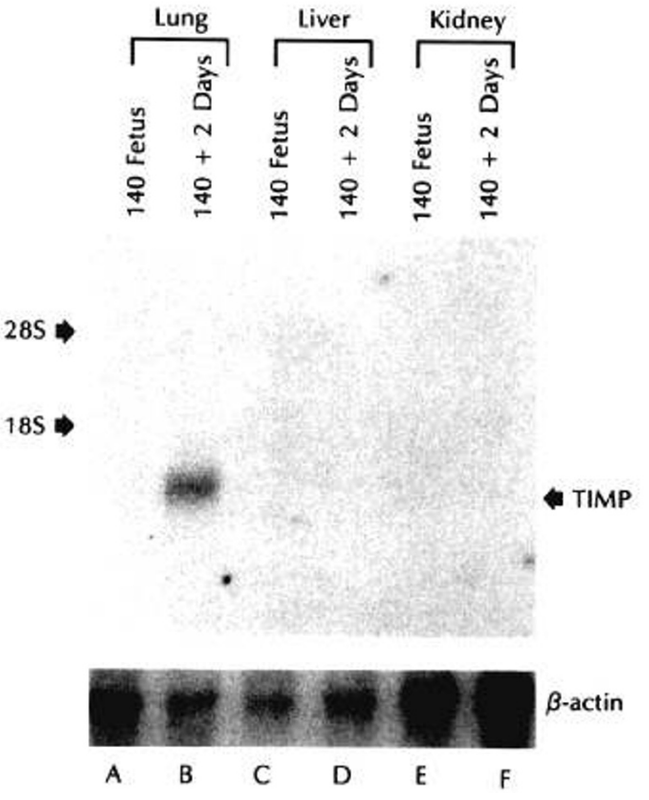

Fig. 4. Tissue-specific expression of TIMP-1 mRNA. Total RNA from liver, kidney, and lung tissues of 140-d fetal and 140 + 2 d postnatal baboons was analyzed on a Northern blot. The blot was probed with ^{32}P-labeled TIMP-1 DNA (*upper panel*) and a β-actin genomic probe (*lower panel*) and autoradiographed.

mature baboon lung. Our findings demonstrated that *1*) mRNA for TIMP-1 was expressed at low levels during late fetal development, *2*) delivery and oxygen breathing in term or premature fetuses was associated with a marked increase in TIMP-1 mRNA abundance, *3*) this parturition-induced gene expression was regulated in a tissue-specific manner, and *4*) postnatal levels of TIMP-1 mRNA were not increased by lung injury from ventilation of the premature baboon lung with 100% oxygen.

TIMP-1 is an inhibitor of a variety of metalloproteinases including collagenase, gelatinase, stromelysin, and proteoglycanase (1, 23–26). TIMP-1 is produced and secreted by human fibroblasts, smooth muscle cells, osteoblasts, and human mononuclear phagocytes (1, 27). Based on its prevalence and ability to bind degradative enzymes with high affinity, TIMP-1 is believed to be a key regulator of ECM turnover (1, 23–26). Brenner *et al.* (11) have recently identified TIMP-1 transcripts in mouse embryos, suggesting a role for TIMP-1 in ECM remodeling during early embryogenesis. TIMP-1 mRNA levels during later stages of development have not been previously examined.

Our results revealed the presence of very low levels of TIMP-1 mRNA in fetal baboon lung at 140, 150, and 180 d of gestation (*lanes A-C*, Fig. 2). The abundance of TIMP-1 mRNA did not change significantly during this developmental window. However, both term and premature delivery (at 140 d of gestation) followed by air breathing and extrauterine adaptation induced a marked increase in TIMP-1 mRNA levels (Fig. 2). It is noteworthy that the increase during the 48 h of postnatal development occurred with tissue specificity in that at least liver and kidney samples from the same animals did not exhibit increased TIMP-1 mRNA levels after birth. For the majority of genes examined to date, increased mRNA abundance results in increased protein synthesis. Where it has been studied, increased TIMP-1 mRNA is accompanied by a concomitant increase in TIMP-1 protein (28). However, we do not know whether the increase in TIMP-1 mRNA described here leads to increased TIMP-1 protein levels. We are currently developing specific anti-TIMP-1 MAb to address this issue. In future experiments, it will also be necessary to determine the specific cells involved in parturition-induced TIMP-1 gene expression. Fibroblasts and macrophages (1, 27), as well as chondrocytes of the cartilage surrounding airways and pulmonary endothelial cells (29), have been known to express TIMP-1 mRNA. In the present study, the relative paucity of fibroblasts and macrophages in the premature baboon lung at 24 h (30), coupled with the rapid increase in abundance of TIMP-1 mRNA, makes it unlikely that the observed increase is the result of cell proliferation in these two cell populations. We have previously shown that premature baboons treated with positive pressure ventilation and prolonged hyperoxia develop bronchopulmonary dysplasia with increased fibrosis (15). This observation, coupled with the findings in adult and term neonatal rabbits (12, 13) raised the possibility that exposure to hyperoxia might also increase TIMP-1 mRNA levels in the premature baboon lung. However, we found no differences in postnatal abundance of TIMP-1 mRNA between hyperoxic and normoxic animals on d 6 or 10. The fact that our results differ from those of previous studies (12, 13) might be explained by differences in animal species, maturity, and physiology. The inflammatory response to hyperoxia in the 140-d-gestation premature baboon lung is much less severe than that observed in older animals (14, 15, 30). Because mononuclear phagocytes produce TIMP-1 and fibroblast production of TIMP-1 is influenced by factors produced by inflammatory cells (28, 31), differences in the magnitude of the inflammatory response may account for maturation-related differences in TIMP-1 gene expression occurring secondarily to hyperoxia. Exposure of term neonatal baboons to hyperoxia was not examined in the present study.

The specific effectors of the observed increase in TIMP-1 mRNA are unknown. In cultured fibroblasts, retinoic acid (28), as well as growth factors in the presence of transforming growth factor-β (31), is known to increase both TIMP-1 mRNA and protein. Preliminary studies using the polymerase chain reaction have demonstrated that both platelet-derived growth factor and transforming growth factor-β mRNA are expressed in the developmental window described in the present studies (32). Changes in the expression of these and other cytokines may mediate alterations in gene expression (*e.g.* TIMP-1) associated with development and parturition.

Because an increase in the level of TIMP-1 mRNA was found after both premature and term birth, we compared some of the pulmonary and nonpulmonary factors known to be associated with each of these situations. Both term and premature births result in a significant increase in alveolar oxygen tension. Similarly, significant elevation in circulating catecholamines occurs in both cases (33, 34). Unlike the term infant, however, the 140-d premature baboon has substantial oxygen requirement, develops hyaline membrane disease, and has significant lung injury by 24 h (8, 10). Because a birth-related hyperoxic challenge is common to both premature and term infants, one hypothesis derived from these data suggests that changes in lung cellular oxygen tension mediate the birth-related induction of TIMP-1 gene expression. Such a mechanism, if present, would have to be independent of oxidant injury (at least in the premature baboon), inasmuch as both term and premature birth result in a significant induction of TIMP-1 mRNA. In this light, lack of TIMP-1 responsiveness to prolonged hyperoxic exposure in premature infants (140 + 6 d and 140 + 10 d) is not presently understood. However, among many plausible explanations, it is possible that either the TIMP-1 gene is maximally expressed in this developmental window or there are developmental differences in response mechanisms to hyperoxia between immediate postnatal and more developed infants. Furthermore, although we found no net change in the steady state levels of TIMP-1 mRNA between normoxic and hyperoxic premature baboons, we cannot rule out the possibility of other changes such as shifts in the cellular site of TIMP-1 mRNA expression. In future experiments, *in situ* localization of TIMP-1 mRNA in lung tissue may provide the necessary information to ascertain this possibility.

Acknowledgment. The authors thank the staff of the Technical Publications Department of Southwest Foundation for Biomedical Research for preparation of the manuscript.

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