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Immunohistochemical study of copper-zinc and manganese superoxide dismutases in the lungs of human fetuses and newborn infants: developmental profile and alterations in hyaline membrane disease and bronchopulmonary dysplasia

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Abstract. To determine the late gestational development of copper-zinc (CnZn) and manganese (Mn) superoxide dismutases (SOD) in human lung, immunohistochemical localization was performed for each SOD. The lung samples were taken from five aborted fetuses, four fetuses in which intrauterine death occurred, one full-term neonate, two premature infants with hyaline membrane disease and one premature infant with bronchopulmonary dysplasia (BPD). Morphometry was performed, and the percent area of positive staining was computed. The bronchial epithelium was intensely stained from the early stages of gestation (i.e. 17 weeks), while the staining intensity for both CuZnSOD and MnSOD in the peripheral airways increased gradually during lung development. The mean percent area of the staining for CuZnSOD and MnSOD from 16 to 38 weeks was increased 30-fold and 8-fold, respectively, and further increases were observed postnatally. CuZnSOD staining was markedly decreased in lungs with respiratory disorders. However, proliferating type II pneumocytes were intensely stained for MnSOD in the BPD lungs, making the staining area 3-fold larger than that in the control lungs. These results clearly depict age-related increases in staining for both CuZnSOD and MnSOD and an alteration in SOD distribution associated with neonatal respiratory disorders.

Key words: Fetal development – Superoxide dismutase – Free radicals – Hyaline membrane disease – Bronchopulmonary dysplasia

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Introduction

The antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (EC 1.11.1.9) and catalase (EC 1.11.1.6) afford primary protection against intracellular oxidative stress by reducing the concentration of active oxygen species. Superoxide, which is generatcd by one-electron reduction of molecular oxygen, is postulated to be an initiator of free-radical chain reactions in hyperoxic lung injury (Freeman and Crapo 1981). Thus, SOD, which catalyses the dismutation of superoxide anion radicals, may play a key role in protection against oxidative lung injury. Two forms of SOD exist in animal cells. One contains copper and zinc (CuZnSOD) and is found uniformly throughout the nucleus and cytoplasm. The other contains manganese (MnSOD) and occurs predominantly in the mitochondrial matrix (Slot et al. 1986).

The results of previous studies in several experimental animals suggest that maturation of tissue antioxidant capacity takes place mainly during the late gestational period as a general mechanism of preparation for birth (Frank 1991; Munim et al. 1992). We have reported that the immunoreactivity of both CuZnSOD and MnSOD was low in fetal rat lungs and kidneys at mid-gestation, and that it increased rapidly during late gestation (Hayashibe et al. 1990) and that the late gestational increase in CuZnSOD and MnSOD occurred in the bronchiolar epithelium of fetal rat lungs as well as in the alveolar epithelium, including the capillary endothelium (Asayama et al. 1991).

Thus, experimental evidence suggests that the immature intracellular antioxidant defence system affords inadequate protection against oxidative injury in premature neonates, thereby playing a primary role in the pathogenesis or bronchopulmonary dysplasia (BPD) and retinopathy of prematurity. However, little is known about the developmental profile of CuZnSOD and

MnSOD in the human lung during the perinatal period, and data are conflicting. Roberts (1979) reported that SOD activity was significantly higher in human adult lungs than in fetal lungs. Strange et al. (1988), however, reported that human lung SOD content remained at the same level throughout prenatal and postnatal development.

The present study was designed to investigate changes in human lung SOD distribution associated with perinatal maturation. Immunohistochemical localization of both CuZnSOD and MnSOD was performed in fetal and neonatal lungs at various developmental stages. The effects of hyaline membrane disease (HMD) and BPD on lung SOD distribution were also studied. We found that the staining intensity of both CuZnSOD and MnSOD in the epithelium of the peripheral airway increased gradually during lung development, as was observed in the fetal rat lung (Asayama et al. 1991). Our study also delineated the alteration in SOD distribution associated with neonatal respiratory disorders.

Materials and methods

From the cases of fetuses and newborn infants listed in the autopsy files at Yamanashi Medical College Hospital and Tokyo Metropolitan Children's Hospital, 50 formalin-fixed, paraffin-embedded lung tissue blocks were selected on the condition that they were fixed within 12 h after death and kept for no more than 7 years after preparation. The 4-μm sections stained with haematoxylin and eosin were examined by light microscopy to identify cases in which both bronchiolar and alveolar linings were intact. From these, lung samples of 13 cases were selected for SOD staining. These samples were taken from five aborted fetuses (15, 16, 17, 18 and 21 weeks of gestation, respectively), four fetuses in which intrauterine death occurred because of non-cardiac or pulmonary disorders (24, 30, 33 and 38 weeks of gestation, respectively), one 1-month-old, full-term (40 weeks of gestation) neonate that died from neuroblastoma, two premature infants that died from HMD (25 and 30 weeks of gestation, respectively) and 1 premature infant (26 weeks of gestation) that died from BPD.

The antisera used in this study were raised by immunizing New Zealand white rabbits with purified human CuZnSOD or MnSOD as has been described previously (Asayama and Burr 1985). The specificity of each antiserum was established by the development of radioimmunoassays for the respective SODs (Asayama et al. 1985).

Immunostaining of both SODs was performed according to the indirect immunoenzyme method previously described (Dobashi et al. 1989). In brief, following deparaffinization and rehydration, tissue sections were exposed to 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity and then to 10% normal goat serum for 30 min to block nonspecific binding. The sections were incubated with diluted antisera (1:10,000 for both anti-CuZnSOD and anti-MnSOD) overnight at 4° C. The steps for reacting horseradish peroxidase with diaminobenzidine were those previously described (Dobashi et al. 1991). As controls, tissue sections were incubated with nonimmune rabbit serum at a dilution of 1:10,000 instead of with the first diluted antiserum. The control sections showed negligible non-specific background staining.

Morphometry was performed with an image analyser (IBAS–2000; Zeiss, Munich, Germany). The sections were observed under a light microscope at a magnification of 200x. Histological images were transmitted to the image analyser by a colour television camera (ITC–350M; Ikegami, Tokyo, Japan). The images of the staining profiles were projected on the screen by the specific grey threshold level (Asayama et al. 1991). All of the images in five randomly selected visual fields were measured for each sample. The percent area of positive staining was computed and expressed as the mean and standard deviation.

Results

Table 1 summarizes the staining profile for CuZnSOD in the lungs of the 13 cases. CuZnSOD immunoreactivity was detected in the bronchiolar epithelium, but not in other immature structures, in the lungs of the 15-week-old fetus (Fig. 1A). In the lungs of the 16-week-old fetus, positive structures were found in more peripheral tissues. Significant staining was seen in the nucleus of the stromal

Table 1. Immunolocalization of copper-zinc superoxide dismutase

Age[a]	Bronchi	Terminal bronchioles	Respiratory bronchioles	Terminal air spaces[b]	Type II pneumocytes
15	0[c]	+	0[d]	0[d]	0[d]
16	0[c]	+	0[d]	0[d]	0[d]
17	++	++	+	0[d]	0[d]
18	++	++	+	±	±
21	++	++	+	±	±
24	++	++	+	±	±
30	++	++	+	±	±
33	++	++	+	+	+
38	++	++	++	+	+
1M	++	++	++	+	+
HMD1 (25)	++	+	+	±	±
HMD2 (30)	++	+	+	±	±
BPD (26)	++	+	+	±	±

++, Intensely stained; +, moderately stained; ±, equivocally stained, difficult to distinguish from background; −, clearly unstained; HMD, Hyaline membrane disease cases 1 and 2; BPD, bronchopulmonary dysplasia

[a] Gestational age is given in weeks and in parentheses for the disease groups

[b] Peripheral respiratory bronchioles (18–24 weeks, disease groups), saccules (30, 33 weeks), and alveoli (38 weeks, 1 month). The terminology conforms to the report by Langston et al. (1984)

[c] Tissue was not available

[d] Tissues not yet developed

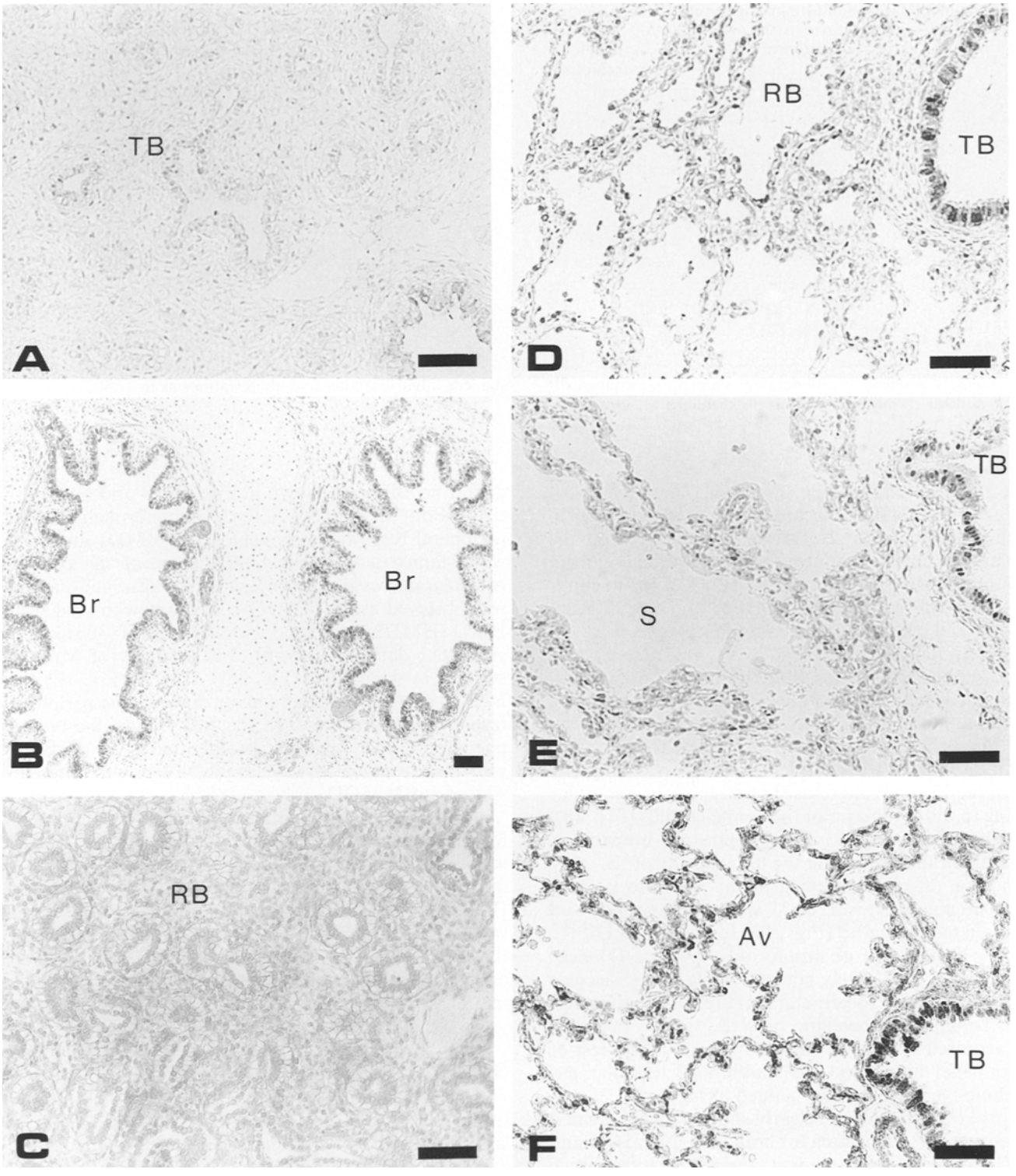

Fig. 1A–F. Immunohistochemical staining for copper-zinc superoxide dismutase (CuZnSOD) in the human lung. **A** Fifteen weeks of gestation; **B** 17 weeks of gestation; **C** 17 weeks of gestation; **D** 24 weeks of gestation; **E** 33 weeks of gestation; **F** 1 month after birth. (**A, C–F** × 200; **B** × 100). *Bars* = 50 μm. *Av*, Alveolus; *Br*, bronchus; *RB*, respiratory bronchiole; *S*, saccule; *TB*, terminal bronchiole

cells, which appeared to be the primordial alveolar pneumocytes. In the lungs of the 17-week-old fetus, the well-developed bronchial epithelium was intensely stained (Fig. 1B). Similarly, the terminal bronchioles were also intensely stained, and the intensity of the staining in the epithelial lining down to this level remained unaltered thereafter. Moderate staining occurred in the epithelial cells of the respiratory bronchioles at the nuclei that were

Table 2. Immunolocalization of manganese superoxide dismutase

Age[a]	Bronchi	Terminal bronchioles	Respiratory bronchioles	Terminal air spaces[b]	Type II pneumocytes
15	0[c]	±	0[d]	0[d]	0[d]
16	0[c]	+	0[d]	0[d]	0[d]
17	+	+	±	0[d]	0[d]
18	+	+	+	−	−
21	+	+	+	−	−
24	+	+	+	±	±
30	+	+	+	±	±
33	+	+	+	±	+
38	+	+	+	±	+
1M	+	+	+	+	++
HMD1 (25)	+	+	+	±	±
HMD2 (30)	+	+	+	±	±
BPD (26)	+	+	+	±	++

++, Intensely stained; +, moderately stained; ±, equivocally stained, difficult to distinguish from background; −, clearly unstained; other footnotes and abbreviations as in Table 1

located on the luminal side (Fig. 1C). The intensity of staining for CuZnSOn appeared to increase gradually in the epithelium of the respiratory bronchioles and the alveolar cells during the late gestational period (i.e. from 24 to 38 weeks of gestation) (Fig. 1D, E). In the lungs of the 1-month-old neonate, the type I and type II pneumocytes and the capillary endothelial cells were all moderately stained (Fig. 1F).

The staining profile for MnSOD in the lung is summarized in Table 2. In the lungs of the 15-week-old fetus, only the terminal bronchioles carried equivocal staining (Fig. 2A). Bronchiolar staining was clearly recognized in the lungs of the 16-week-old fetus. The bronchial epithelium of the 17-week-old fetus showed moderate granular staining in the cytoplasm of the epithelial cells (Fig. 2B), while the epithelial cells of the respiratory bronchioles were equivocally stained on the luminal side of the cytoplasm (Fig. 2C). The staining in the respiratory bronchioles became moderate at 21 weeks of gestation and was unaltered thereafter (Fig. 2D). In the saccules of the lungs at 33 weeks of gestation, only the type II pneumocytes were moderately stained, while other saccular structures were equivocally stained (Fig. 2E). In the lungs of the 1-month-old neonate, only the type II pneumocytes acquired an intense staining (Fig. 2F). The rest of the epithelial lining and the interlobular vascular endothelium were moderately stained at most.

Morphometric analysis clearly showed an age-related increase in the staining area for both lung CuZnSOD and MnSOD. The staining area was larger in each respective later stage than in the earlier stage. When the values at 16 weeks of gestation were compared with those at 38 weeks, the mean percent area of the staining for CuZnSOD and MnSOD was increased 30-fold and 8-fold respectively. The staining areas appeared to be further increased from 38 weeks of gestation to 1 month of postnatal life.

Photomicrographs of the lungs stained for CuZnSOD of the control (24 weeks of gestation), the 25-week-old infant that died from HMD (HMD1) and the infant that died from BPD are shown in Fig. 3A, B and C, respectively. Both the epithelia of the terminal bronchioles and peripheral respiratory bronchioles in HMD1 and BPD were stained less intensely than those of the control. A similar decrease in the intensity of CuZnSOD staining was observed in the 30-week-old infant who died from HMD (HMD2) compared with the control (30 weeks of gestation; data not shown). The intensity of MnSOD staining in the control (Fig. 3D), HMD1 (Fig. 3E) and BPD lungs (Fig. 3F) was similar down to the peripheral respiratory bronchioles. In the BPD lungs, the type II pneumocytes had markedly proliferated in the thickened wall of the terminal airway, and these cells were intensely stained for MnSOD.

Morphometric analysis revealed that the staining area for CuZnSOD was markedly decreased in lungs with respiratory disorders (Table 3). HMD did not appear to induce a consistent change in the MnSOD staining area. However, the MnSOD staining area in the BPD lungs was 3 times larger than that in the control lungs. Thus, the quantitative data obtained by morphometry were essentially consistent with those depicted in the photomicrographs.

Discussion

In the present study, the distribution of both CuZnSOD and MnSOD changed significantly in human lungs during perinatal development. Although well-developed bronchial epithelium was intensely stained from the early stages of gestation, the development of more immature peripheral tissues was associated with an increase in the intensity of staining for both SODs. The observed distribution in the lung of the full-term neonate was very similar to that in previously studied adult human lungs (Munim et al. 1990) and neonatal rat lungs (Asayama et al. 1991).

A late gestational increase in lung antioxidant enzymes has previously been reported in rats, hamsters,

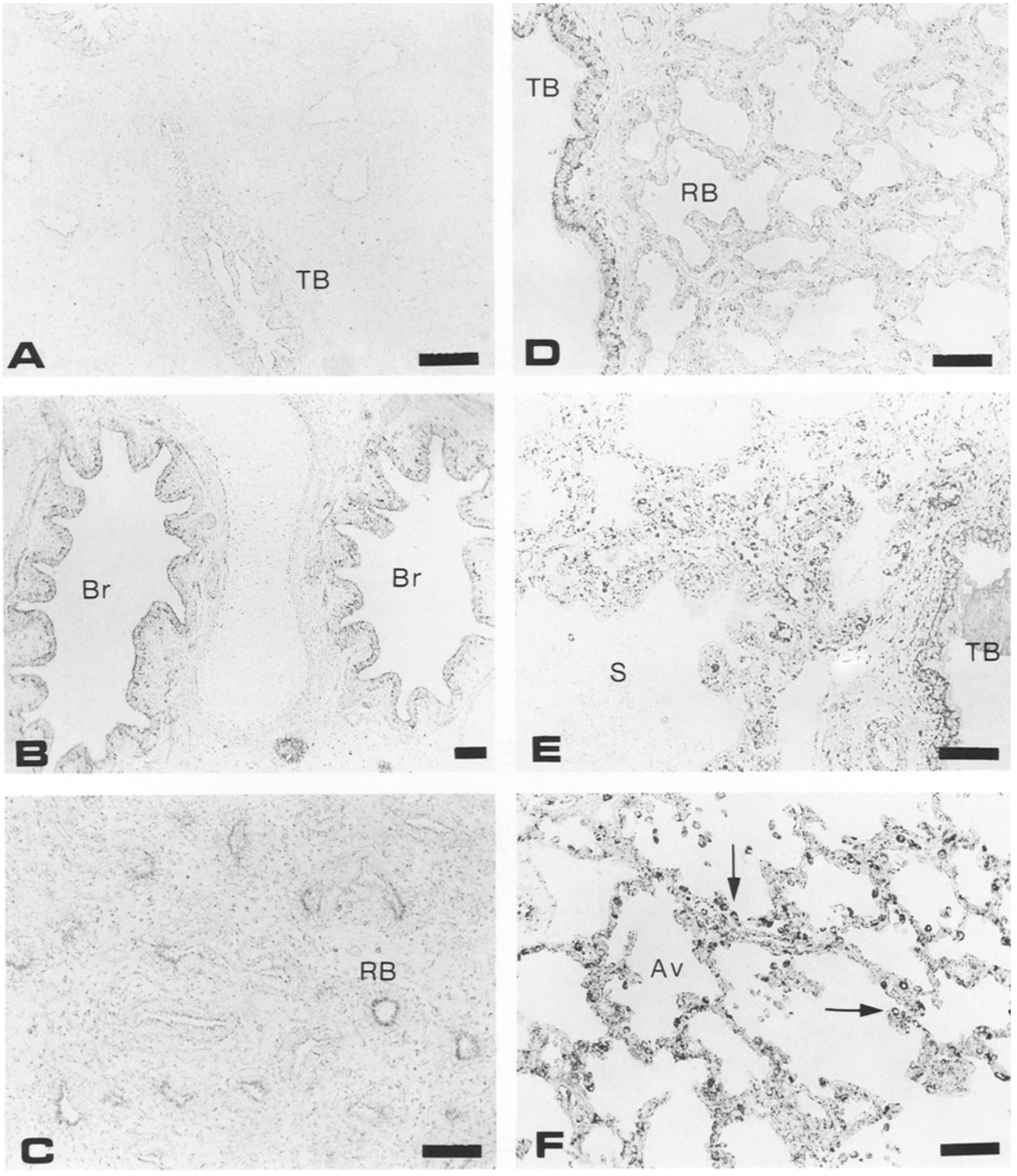

Fig. 2A–F. Immunohistochemical staining for manganese superoxide dismutase (MnSOD) in the human lung. **A** Fifteen weeks of gestation; **B** 17 weeks of gestation; **C** 17 weeks of gestation; **D** 24 weeks of gestation; **E** 33 weeks of gestation; **F** 1 month after birth. (**A**, **C–F** × 200; **B** × 100). *Bars* = 50 μm. *Arrow* indicates type II alveolar pneumocytes

guinea pigs, rabbits and sheep (Sosenko et al. 1989; Rickett et al. 1990; Frank 1991) and in man (Roberts 1973). In contrast, Strange et al. (1988, 1990) found that human lung SOD activity and immunoreactivity were unaltered throughout the gestational period. They also performed immunohistochemical staining for CuZnSOD in human lungs at various developmental stages, including cases as early as 14 weeks of gestation and adults, and reported that the distribution was unaltered (Strange et al. 1988). They noted proliferation of interstitial tissues

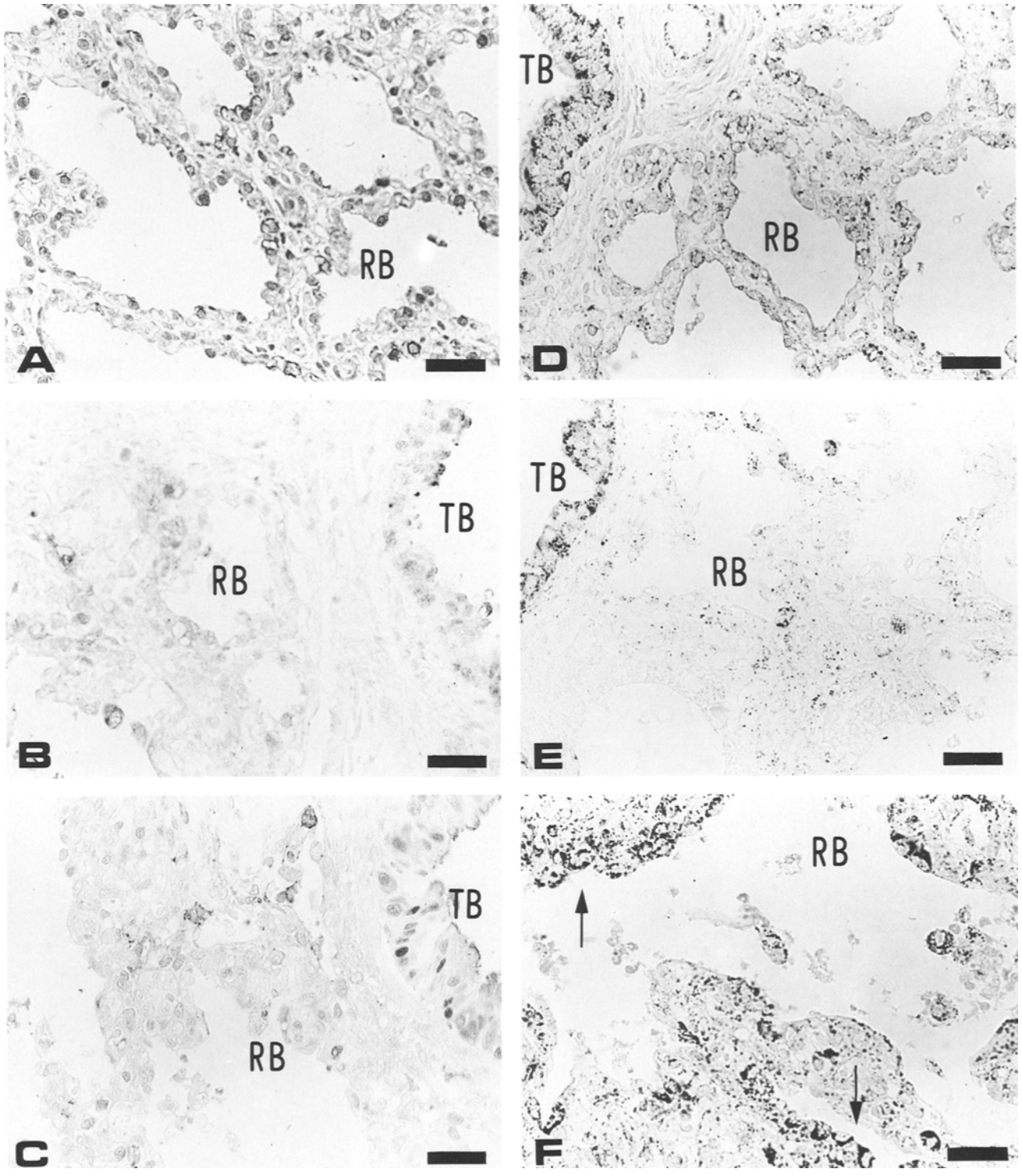

Fig. 3A–F. Immunohistochemical staining for either CuZnSOD or MnSOD in lungs with respiratory disorders. **A** Control (24 weeks of gestation), CuZnSOD staining; **B** hyaline membrane disease (HMD1; 25 weeks of gestation), CuZnSOD staining; **C** bronchopulmonary dysplasia (BPD; 26 weeks of gestation), CuZnSOD staining; **D** control, MnSOD staining; **E** HMD1, MnSOD staining; **F** BPD, MnSOD staining. **A–F** × 400. *Bars* = 25 μm

and an increased number of type II pneumocytes in the terminal air spaces of BPD lungs, but failed to observe changes in the distribution of immunoreactivity of CuZnSOD or MnSOD (Strange et al. 1990).

The discrepancy between the immunohistochemical data of Strange et al. and ours may be due to differences in methodology. They used the peroxidase-antiperoxidase method with the antiserum dilution at 1 to 20

Table 3. Percent area of staining for copper-zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) ind fetal human lungs

Age	CuZnSOD (%)	MnSOD (%)
15 weeks	0.058 ± 0.04	0.005 ± 0.002
16 weeks	0.104 ± 0.04	0.249 ± 0.10
17 weeks	0.225 ± 0.13	0.272 ± 0.13
18 weeks	0.600 ± 0.67	0.914 ± 0.16
21 weeks	0.882 ± 1.40	1.01 ± 0.28
24 weeks	0.944 ± 0.63	1.06 ± 0.20
30 weeks	1.04 ± 0.51	1.68 ± 0.25
33 weeks	1.13 ± 0.42	2.01 ± 0.32
38 weeks	3.32 ± 1.60	2.04 ± 0.68
1 month	4.19 ± 1.34	3.22 ± 0.55
HMD1 (25 weeks)	0.136 ± 0.06	1.41 ± 0.44
HMD2 (30 weeks)	0.164 ± 0.06	0.752 ± 0.24
BPD (26 weeks)	0.233 ± 0.11	3.24 ± 0.34

The image of the staining was measured, and the percent area of the visual field was computed ($n = 5$ for each sample). Data are means ± SD.

(Strange et al. 1990), and the dilution was not free from significant background staining with preimmune serum. We used an indirect method with an antiserum dilution that gave negligible background staining (i.e. 1 to 10,000). They described that all tissues, including abnormal fibrous components, were positive for both CuZnSOD and MnSOD, but did not appear to detect a higher intensity of MnSOD staining in type II pneumocytes than in the rest of the epithelial components as observed by us and other investigators (Coursin et al. 1992).

The prenatal development of staining for CuZnSOD and MnSOD in the peripheral airway observed here was similar to that seen previously in fetal rat tissues (Asayama et al. 1991; Munim et al. 1992). However, the large bronchi of the human lung appear to accumulate both SODs at a very early stage of development, and presumably at an earlier period than the corresponding developmental stage in rats. Thus, failure to detect a significant late gestational increase in SOD concentrations in the previous series of human lung homogenates (Strange et al. 1988, 1990) may be due, at least in part, to the early occurrence of SODs in the large airways. Furthermore, our immunohistochemical and morphometric data suggest that human lung SOD increases postnatally. In our previous studies in rats, the intensity of SOD staining in the lungs did not increase from day 0 to day 6 after birth (Munim et al. 1992). Human lung SOD activity is reported to be increased postnatally (Roberts 1979). These results suggest that the intracellular antioxidant enzyme defence system is established earlier in man than in rats for large airways and, conversely, earlier in rats than in man in small airways. Studies on a wide variety of cell types indicate that total SOD increases during cellular differentiation (Allen and Balin 1989). The present data appear to conform to this general trend.

In the HMD and BPD lungs studied, CuZnSOD staining was decreased both in intensity and area, while MnSOD staining was unaltered in the HMD lungs and actually increased in the BPD lungs. This selective decrease in CuZnSOD may have pathophysiological significance. The SOD content is postulated to be closely related to lung maturation. The maturation of fetal lungs induced by dexamethasone administered to the mother before delivery is known to be associated with an increase in lung antioxidant enzymes (Frank et al. 1980; Asayama et al. 1992). Furthermore, the intravenous administration of liposome-entrapped CuZnSOD is reported to afford protection against hyperoxic injury to alveolar capillary endothelial cells (Turrens et al. 1984), suggesting the primary importance of CuZnSOD as a lung antioxidant enzyme.

Thus, the evidence suggests that the low levels of CuZnSOD observed in the lungs reflects their immaturity as well as their susceptibility to hyperoxic injury. The fact that lung CuZnSOD levels remained low after respiratory therapy in our cases was in keeping with the finding that exposure to hyperoxia results in the induction of lung CuZnSOD in full-term but not in preterm neonates (Frank and Sosenko 1991). In BPD lungs, type II pneumocytes are generally found to proliferate as part of the tissue repair mechanism (Margraf et al. 1990), as was seen in this study. The proliferation of intensely stained type II pneumocytes was responsible for the marked increase in MnSOD staining area observed in the BPD lungs.

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