ORIGINAL ARTICLE

Expression Patterns of Heat Shock Proteins in Lungs of Neonates With Congenital Diaphragmatic Hernia

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Background: Congenital diaphragmatic hernia (CDH) is associated in many cases with pulmonary hypertension. Currently, extracorporeal membrane oxygenation (ECMO) is one of the possible modalities of treatment of pulmonary hypertension and prevention of parenchymal lung injury in neonates with CDH.

Hypothesis: Molecular stress is present in the lungs of neonates with CDH. To test this hypothesis, we investigate the expression pattern of stress genes (heat shock proteins [HSPs] 27 and 70) in lungs of patients with CDH who have pulmonary hypertension, and evaluate the influence of ECMO on the expression levels of these genes to understand the underlying molecular mechanisms.

Design: Paraffin-embedded lung autopsy specimens from patients with CDH and lung hypoplasia who either did or did not receive ECMO treatment and age-matched controls were immunostained by means of monoclonal anti–human antibodies against HSP 70 and HSP 27, with the streptavidin-biotin complex method.

Setting: Level III academic children's hospital.

Main Outcome Measures: Expression levels of both HSP 27 and HSP 70 were semiquantitatively evaluated in bronchial epithelium, as well as in medial smooth muscle cells (SMCs) and endothelium of large and small pulmonary arteries, by means of a score ranging from 0 to 4. Statistical analysis of the data was performed with the nonparametric Mann-Whitney test, with significant probability value at $P \leq .05$.

Results: For HSP 70, the most pronounced immunoreactivity was observed in the bronchial epithelium, followed by the medial SMCs of small arteries (of external diameter <200 µm). The overall expression was significantly higher in patients with CDH than controls in bronchi as well as in pulmonary arteries. For HSP 27, intense expression was found in medial SMCs, followed by the bronchial epithelium in controls, with significantly increased expression in medial SMCs of large and small arteries in patients with CDH. Treatment with ECMO was associated with significantly reduced expression levels of HSP 70 in medial SMCs of both large and small arteries, whereas HSP 27 expression levels were decreased only in small arteries. In addition, the expression levels of both HSPs were significantly lower in endothelium of small arteries.

Conclusions: Increased expression of HSPs in CDH points to a condition of pulmonary stress. This pulmonary stress appears to be partially ameliorated by ECMO treatment. This may point to one of the mechanisms by which ECMO alleviates pulmonary hypertension associated with CDH.

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THE HIGH mortality and morbidity in infants with congenital diaphragmatic hernia (CDH) are largely determined by the severity of lung hypoplasia and therapy-resistant pulmonary hypertension (PH).[1] Pulmonary vascular abnormalities in CDH consist of a decreased number of pulmonary arteries per unit lung volume and peripheral muscularization of small arteries with medial and adventitial thickening.[2,3] To decrease the PH, a number of management protocols have been developed, including extracorporeal membrane oxygenation (ECMO) with delayed surgery after the patient's condition has stabilized.[4,5] The use of ECMO has been attempted to diminish the abnormal pulmonary vascular tone by guaranteed oxygen supply and to reverse the pulmonary structural abnormalities, with documented variable improvement of the survival rate in the high-risk infants with CDH.[5,6] It is unclear whether or not ECMO imposes pulmonary stress responses in infants with CDH.

A number of genes are expressed immediately when cells are subjected to stress and stretch and in the case of acute and chronic lung injury.[7,8] Among these are the

PATIENTS AND METHODS

TISSUE SPECIMENS

After the study design was approved by the departmental research committee, we obtained archival autopsy lung tissue specimens of 24 neonates who died of CDH and lung hypoplasia, as confirmed by a lung–body weight ratio index of 0.012 or less.[16] All patients with CDH belonged to the high-risk group. These specimens represent the available material from patients with CDH who died after treatment at Sophia Children's Hospital, Erasmus University, Rotterdam, the Netherlands, and whose parents' consent for autopsy was obtained, during the period from 1981 through 1997. During the same period, more than 120 neonates with CDH were treated in our hospital, with an overall survival rate of 64.7%. All cases were subjected to standard management protocols, including delayed surgery beginning in 1986 and ECMO management since its introduction in our center in 1992. All patients underwent routine cardiac ultrasonography, which indicated PH and right-to-left shunting, together with the clinical observation of preductal and postductal transcutaneous oxygen saturation differences of greater than 10%.[17]

Cases were divided into 2 groups. Group 1 consisted of 20 neonates with CDH who did not receive ECMO treatment (mean gestational age, 38.6 ± 0.4 weeks); all died in the first 2 days of life, including 4 who died in the first postnatal hour. The primary cause of death in group 1 was extreme lung hypoplasia in 11 cases, persistent fetal circulation in 8 cases, and interstitial pulmonary hemorrhage in 1 case. Group 2 consisted of 4 neonates with CDH (mean gestational age, 39 ± 2 weeks) who were treated with ECMO for a period of 3 to 21 days, with a mean bypass time of 270 hours. Venoarterial ECMO, when instituted, was performed in the neonates who did not have any of the following institutional exclusion criteria: gestational age less than 34 weeks, birth weight less than 2000 g, artificial ventilation for more than 7 days, alveolar arterial oxygen difference less than 580 mm Hg, and maximal Pa_{O_2} less than 72 mm Hg. Patients who received ECMO were all successfully decannulated and received conventional ventilatory support until death, in 3 patients from persistent fetal circulation and in the fourth patient from multiple organ failure. In the patients with CDH, hernia was left-sided in 16 cases and right-sided in 8 cases. None of the patients received surfactant treatment.

Five age-matched neonates (mean gestational age, 37.8 ± 1.2 weeks) who were subjected to ventilation periods up to 16 hours and died of birth asphyxia within the first 24 hours served as controls, constituting group 3. All of these controls showed no lung abnormalities on histological examination and had no clinical features of PH.

Patients from non-ECMO groups were subjected to ventilation therapy until the time of death. The **Table** gives the patients' demographic characteristics and data on ventilator settings, including inspiratory oxygen fraction, peak inspiratory pressure, positive end-expiratory pressure, and frequency from all groups. No case of stillbirth was included in any of the studied groups.

Autopsy was performed in the first 24 hours after death in all cases. Since we did not find significant differences between ipsilateral and contralateral lungs on screening by histological examination, we processed tissues from either side of the lung randomly for immunohistochemistry in this study.

Continued on next page

heat shock proteins (HSPs), a group of highly conserved proteins that can be induced by exposure to heat, as well as a variety of pathophysiological conditions including hypoxia and oxidative and metabolic stresses.[9,10] Heat shock proteins represent one of the lung defense mechanisms against injury, in addition to the antioxidant enzyme system.[7] The HSP 70 family of proteins binds to adenosine triphosphate and plays a role in the transport of proteins into the endoplasmic reticulum and mitochondria.[10-12] The small HSP 27 is expressed in developing organs and under conditions of cellular stress. It is localized in the cellular cytoplasm and migrates to the nucleus upon stress. The HSP 27 acts as a molecular chaperone and plays an important role in signal transduction and drug resistance.[12-14] The HSPs have been reported to participate in embryonic and fetal development.[15]

To our knowledge, no study of HSP expression in CDH has been presented. The molecular basis of the mechanism by which ECMO treatment, originally referred to as "lung rest," is of benefit in CDH remains unknown. We hypothesize that vigorous ventilatory support in CDH may result in a state of severe pulmonary stress.

The present study was carried out to investigate the pulmonary expression of the stress genes HSP 27 and HSP 70 in human CDH. In addition, we wished to study whether the institution of ECMO alters their expression levels in human lungs with CDH, aiming to understand the molecular mechanisms of hypoplastic lungs after injury.

RESULTS

IMMUNOLOCALIZATION OF HSP 70

In control cases, HSP 70 was localized in the bronchial epithelium and medial arterial SMCs. In the CDH group, the most pronounced immunoreactivity was observed in the bronchial epithelium, with maximal expression score of 2.98 ± 0.14, followed by medial SMCs of small arteries (2.13 ± 0.15), medial SMCs of large arteries (1.15 ± 0.15), and endothelium of small (0.55 ± 0.16) and large (0.2 ± 0.11) arteries. The overall pulmonary expression of HSP 70 in CDH was higher than in controls (**Figure 1**, A and B).

Statistical analysis of HSP 70 expression scores between control and non–ECMO-treated CDH groups showed significantly higher levels of expression ($P \leq .05$) in the bronchial epithelium, medial SMCs of large arteries, medial SMCs of small arteries, and endothelium of small arteries ($P = .001$, .008, .001, and .02, respectively; nonparametric Mann-Whitney test). In both control and ECMO-treated CDH groups, no HSP 70 expression was observed in pulmonary arterial endothelium.

Treatment with ECMO was associated with significantly lower HSP 70 expression in medial SMCs of large ($P = .008$) and small ($P = .004$) arteries and small-artery endothelium ($P = .04$) in patients with CDH as com-

IMMUNOHISTOCHEMISTRY

Paraffin sections of lung tissues were cut at 6 µm and mounted on coated glass slides. Immunohistochemistry was performed by means of a standard avidin-biotin complex method. Slides for HSP 27 were incubated for 20 minutes in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase, then cooked for 15 minutes at 100°C, rinsed with phosphate-buffered saline solution, and placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn, England). After preincubation with 10% normal goat serum for 15 minutes, slides were incubated at room temperature for 45 minutes with mouse monoclonal antibody to HSP 27 (dilution, 1:750) and for 1 hour with mouse monoclonal antibody to HSP 70 (dilution, 1:25) (both from NeoMarkers, Fremont, Calif).

After rinsing with phosphate-buffered saline solution, the slides were incubated for 30 minutes with biotinylated secondary antibody (Multilink, 1:75 dilution, Biogenex, San Ramon, Mo). Slides were rinsed again and incubated for 30 minutes with peroxidase-conjugated streptavidin for HSP 27 and with alkaline phosphatase–conjugated streptavidin for HSP 70, with a dilution of 1:50 for both (Biogenex). The HSP 27 slides were colored with 0.025% 3,3-diaminobenzidine (Sigma-Aldrich Corp, St Louis, Mo) in 0.01-mol/L phosphate-buffered saline solution, containing 0.03% hydrogen peroxide. The HSP 70 slides were rinsed with 0.2-mol/L Tris hydrochloride, pH 8.0, incubated with levamisole to block the endogenous alkaline phosphatase activity, then stained with 0.3% new fuchsin/Tris hydrochloride (Sigma-Aldrich Corp) and briefly counterstained with Mayer hematoxylin. Positive controls consisted of breast carcinoma tissue specimens. Negative controls consisted of omission of the primary antibody.

SEMIQUANTITATIVE ANALYSIS

Before screening by 2 independent observers, sections were coded so that both observers were unaware of the clinical group of the case under study. Expression of HSP 27 and HSP 70 was analyzed semiquantitatively, by means of an arbitrary visual scale with score ranging from 0 to 4: grade 0 represented no staining, grade 1 represented focal staining, and grades 2, 3, and 4 represented diffuse weak, moderate, and strong staining, respectively.[18] Sections were graded from 0 to 4 for the intensity of expression signal of HSPs in the bronchial epithelium, as well as in the endothelium and medial smooth muscle cells (SMCs) of small (external diameter, 50-200 µm) and large (external diameter, >200 µm) pulmonary arteries. Intra-acinar arteries, representing the group of resistance arteries in the pulmonary vascular bed, have external diameters ranging from 50 to 200 µm, while preacinar arteries have external diameters not less than 200 µm.[3,19]

STATISTICAL ANALYSIS

The immunostaining score for the expression of HSPs was calculated from the 3 groups, and the results were expressed as mean ± SEM. Statistical analysis was performed after ranking by means of the nonparametric Mann-Whitney test, which is appropriate for the compared groups of the study. Significance of the results was accepted at $P \leq .05$.

pared with the non–ECMO-treated CDH group (Figure 1, C and D).

There were no statistical differences in expression levels of HSP 70 in pulmonary arteries between the controls and ECMO-treated neonates with CDH.

IMMUNOLOCALIZATION OF HSP 27

In control cases, HSP 27 was localized in the bronchial epithelium and medial arterial SMCs. In the CDH group, the highest expression score was observed in the medial SMCs of small arteries (2.2 ± 0.14), followed by the bronchial epithelium (2.0 ± 0.15), medial SMCs of large arteries (1.83 ± 0.14), and endothelium of large (1.2 ± 0.18) and small (1.03 ± 0.11) arteries. The CDH group showed higher levels of expression of HSP 27 than controls (**Figure 2**, A and B).

Statistical analysis of HSP 27 expression scores between controls and non–ECMO-treated CDH groups showed significantly higher levels of expression ($P \leq .05$) in the medial SMCs of large ($P = .02$) and small ($P = .01$) arteries by means of the nonparametric Mann-Whitney test. Also, endothelium showed significantly higher expression in both large ($P = .003$) and small ($P = .001$) arteries. Although the mean value of expression in bronchial epithelium was higher in the CDH group, this difference did not reach statistical significance. No HSP 27 expression was found in the endothelium of arteries of the control group and of the large arteries of the ECMO-treated group.

Treatment with ECMO was associated with a significant decrease in levels of HSP 27 expression in medial SMCs of small arteries ($P = .03$) and in endothelium of both large and small arteries ($P = .007$ and .008, respectively), as compared with the non–ECMO-treated CDH group (Figure 2, C and D).

There were no statistical differences in expression levels of HSP 27 in pulmonary arteries between the controls and ECMO-treated neonates with CDH.

For both HSP 27 and HSP 70, we did not find differences in the expression levels between the 4 patients who died in the first hour of life and the other patients with CDH who did not receive ECMO treatment and were subjected to ventilatory therapy for longer periods until death. Additionally, we did not observe such differences when we compared the patients with CDH who had PH as the primary cause of death and those who died of extreme lung hypoplasia. The same holds true for the patients who received ECMO, regardless of the duration of ECMO treatment, who died at a mean age of 339 hours after successful decannulation from ECMO.

COMMENT

In the present study, we found increased expression of HSP 70 and HSP 27 in hypoplastic lungs of infants with

Demographic Criteria of Patients in Various Study Groups*

Variable	CDH, No ECMO	ECMO	Control
No. of cases	20	4	5
Gestational age, wk	38.6 ± 0.4	39.0 ± 2.0	37.8 ± 1.2
Birth weight, g	2655 ± 138.0	3045 ± 256.0	3180 ± 244.0
Age at death, h	29.0 ± 2.8	339.0 ± 123.7	11.8 ± 4.0
Pre-ECMO ventilator settings			
Fio_2	1.0	1.0	1.0
PEEP, cm H_2O	5	5	5
PIP, cm H_2O	34	34	31.4
Frequency, cycles/min	60	80	60
Time of pre-ECMO ventilation, h	NA	18	NA
ECMO duration, h	NA	270	NA
Post-ECMO ventilator settings			
Fio_2	NA	1.0	NA
PEEP, cm H_2O	NA	5	NA
PIP, cm H_2O	NA	20	NA
Frequency, cycles/min	NA	50	NA

**Values are presented as mean or mean ± SEM from the number of cases indicated in each group. CDH indicates congenital diaphragmatic hernia; ECMO, extracorporeal membrane oxygenation; Fio_2, fraction of inspired oxygen; PEEP, positive end-expiratory pressure; PIP, peak inspiratory pressure; and NA, not applicable.*

CDH. The HSP 70 expression was highest in the bronchial epithelium, followed by the small-artery medial SMCs, while HSP 27 expression was more pronounced in medial SMCs of pulmonary arteries than in bronchial epithelium. Immunoreactivity for both HSPs was mainly cytoplasmic, with a minor degree of nuclear positivity, as has been reported previously.[13,20]

Expression of HSPs could not be attributed to ventilatory trauma alone, since the controls were subjected to a similar ventilatory treatment and duration with regard to fraction of inspired oxygen, peak inspiratory pressure, and volume changes. More importantly, no difference in the expression levels was found in the 4 patients with CDH who died in the first hour after birth because of unsuccessful resuscitation in any of the examined tissues and that of other neonates with CDH who did not receive ECMO treatment. Also, the significant difference in expression was found mainly in the pulmonary arteries, not in the bronchial epithelium, which might reflect the direct effects of ventilation and barotrauma. The enhanced pulmonary expression of HSPs in CDH indicates a state of stress, and HSPs may be regarded as molecular markers of pulmonary stress.

Although the number of control cases was small, we believe that the neonates with birth asphyxia are good controls. They were screened by histological examination and showed normal lung architecture, except for some

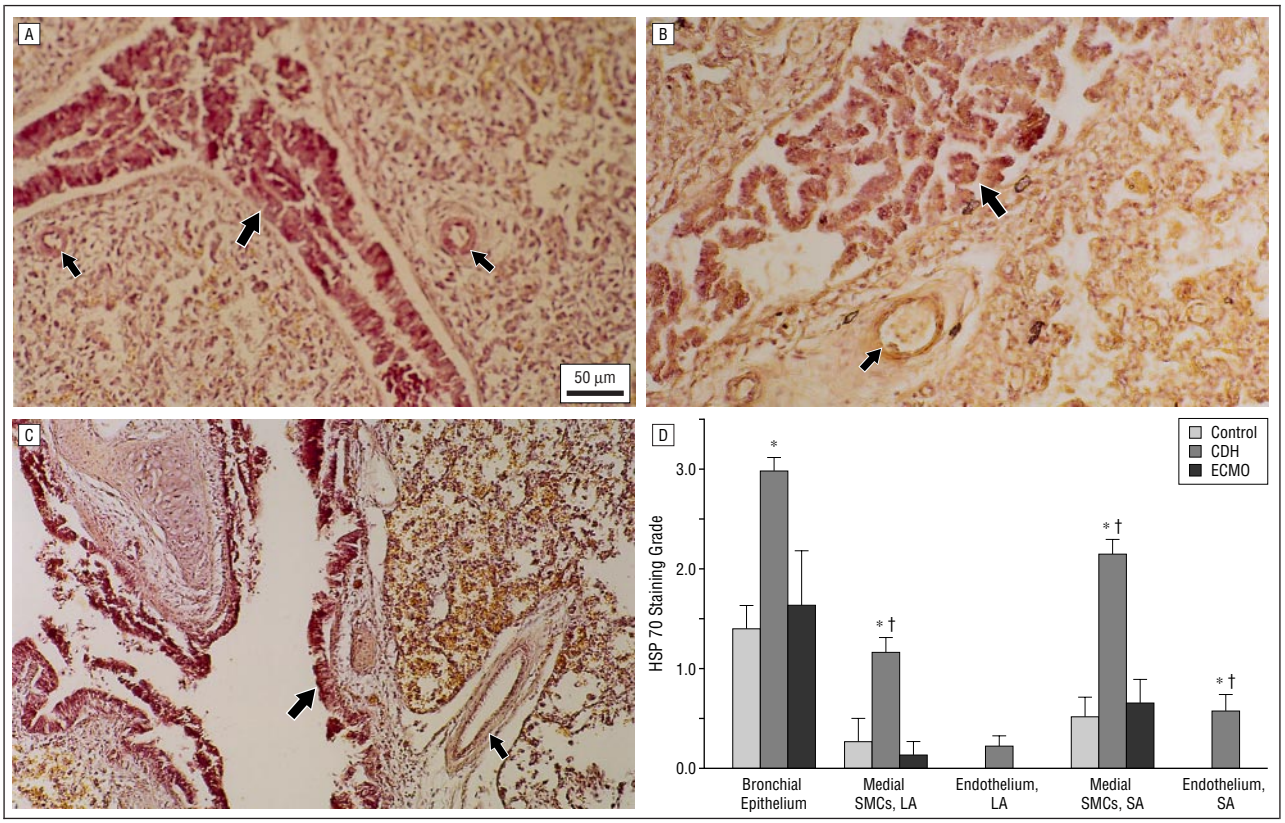

Figure 1. *Immunohistochemical localization of heat shock protein 70 (HSP 70) of lung sections cut at 6 µm and stained by the alkaline phosphatase method. A, Note the immunoreactivity for HSP 70 in bronchial epithelium (large arrow) and medial smooth muscle cells (SMCs) of small artery (small arrow) in a patient with congenital diaphragmatic hernia (CDH). B, Bronchial epithelium (large arrow) and medial SMCs of small artery (small arrow) in control case. C, Bronchial epithelium (large arrow) and medial SMCs of small artery (small arrow) in a patient with CDH treated with extracorporeal membrane oxygenation (ECMO). D, Histogram showing the difference in expression grade of HSP 70 according to the semiquantitative scale for CDH, control, and ECMO-treated groups, in bronchial epithelium, medial SMCs and endothelium of large arteries (LA), and medial SMCs and endothelium of small arteries (SA). Values are represented as mean ± SEM. Statistical significance was accepted at* $P \leq .05$ *for CDH vs controls (asterisk) and for CDH vs ECMO (dagger) by Mann-Whitney nonparametric test.*

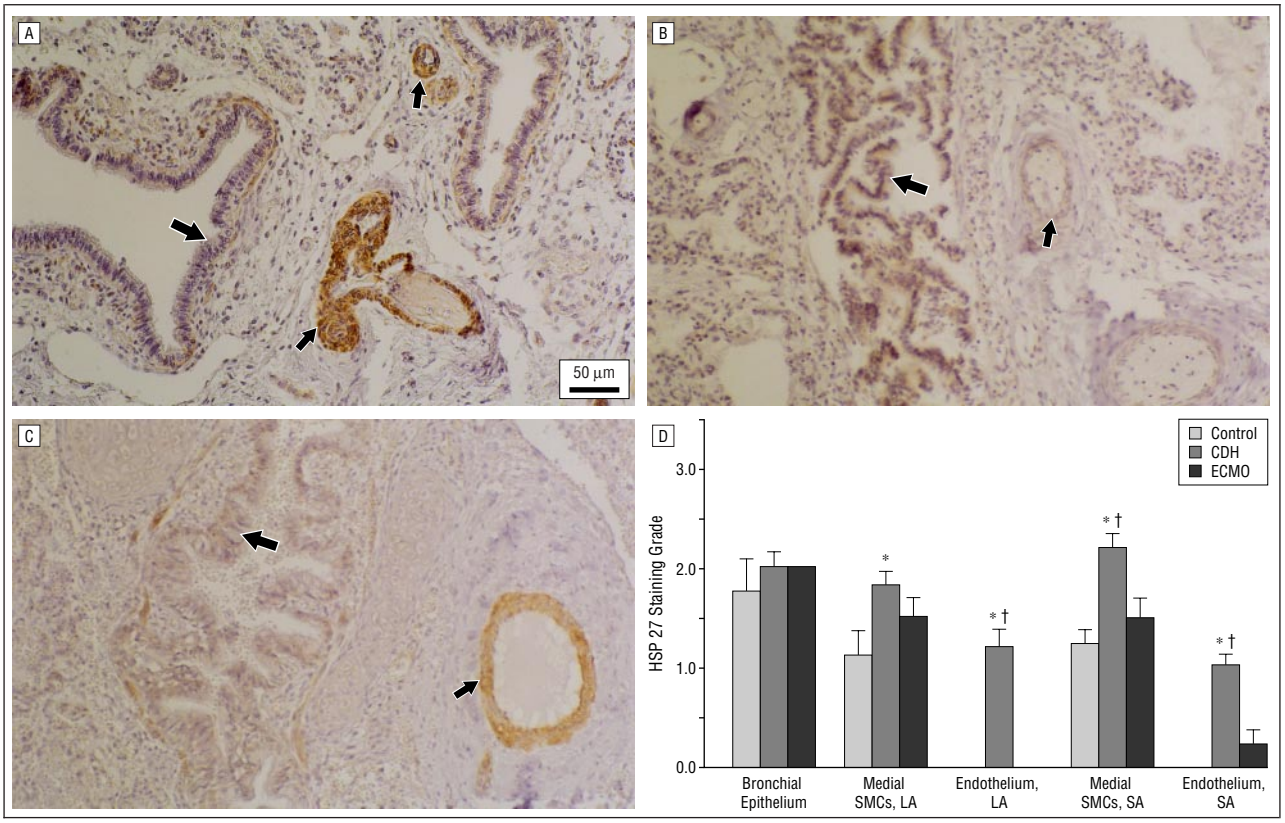

Figure 2. *Immunohistochemical localization of heat shock protein 27 (HSP 27) of lung sections cut at 6 µm and stained by the peroxidase method. A, Note the immunoreactivity for HSP 27 in bronchial epithelium (large arrow) and medial smooth muscle cells (SMCs) of small artery (small arrow) in a patient with congenital diaphragmatic hernia (CDH). B, Bronchial epithelium (large arrow) and medial SMCs of small artery (small arrow) in control case. C, Bronchial epithelium (large arrow) and medial SMCs of small artery (small arrow) in a patient with CDH treated with extracorporeal membrane oxygenation (ECMO). D, Histogram showing the difference in expression grade of HSP 27 according to the semiquantitative scale for CDH, control, and ECMO-treated groups, in bronchial epithelium, medial SMCs and endothelium of large arteries (LA), and medial SMCs and endothelium of small arteries (SA). Values are represented as mean ± SEM. Statistical significance was accepted at* $P \leq .05$ *for CDH vs controls (asterisk) and for CDH vs ECMO (dagger) by Mann-Whitney nonparametric test.*

congestion and edema, and a normal vascular pattern. Moreover, these patients were used in comparable studies for the same reason.[21]

Several reports confirmed the notion that the expression of HSPs is up-regulated in a wide variety of stress-associated conditions.[7,8,20,22] Possibly, the enhanced expression of HSPs in the CDH group reflects a neonatal attempt to establish a protective mechanism against stress, as shown earlier for antioxidant enzyme activity in a rat CDH model.[23]

It has been well documented that the small-diameter pulmonary arteries are the most important vessels in the regulation of pulmonary blood flow and pressure.[2,3,19] Structural changes have been described in pulmonary arteries of patients with CDH complicated by PH.[16,24] Increased expression of HSP 27—which is reported to be enhanced in an earlier developmental stage[15]—in the cases of CDH reported herein may even confirm the stunted growth condition of the pulmonary vasculature, because our patients were nearly full-term.

Our results of increased HSP expression in small-diameter pulmonary arteries indicate that the cellular stress may act especially in the pulmonary pressure–regulating arteries. This partially answers the question raised by DeMello and Reid[25(p771)]: "An intriguing question is how ECMO in some cases allows resolution of pulmonary hypertension of the newborn and whether this occurs by allowing growth or by resting the microcirculation and the small resistance arteries to avoid exposure to blood pressure is not clear."

In our series, ECMO treatment was associated with a significant reduction in the expression levels of HSPs, especially in the medial SMCs of pulmonary arteries. Although the ECMO group was small, ECMO probably has a role in alleviating the PH associated with CDH,[26] as reported in a recent review.[27] The HSP 27 has been reported to be related to smooth muscle cell contraction, as it constitutes the main phosphoprotein[28] and has been observed to be enhanced in vessel walls in response to varying types of stress.[29] Our finding of increased expression in small-artery SMCs of pulmonary vasculature in patients with CDH points to the same. We could not measure messenger RNA levels in this study, since our archival materials were formalin fixed and paraffin embedded, but, interestingly, data from ongoing studies in our laboratory showed a similar significant decrease of HSPs after partial liquid ventilation at both messenger RNA and protein levels in a rat CDH model (Tadaharu Okazaki, MD, Hari S. Sharma, PhD, John Vlot, PhD, et al, unpublished data, 1998). Thus, our current results probably present one of the molecular mechanisms involved in the documented beneficial effect of ECMO in the management of the PH in patients with CDH.[5,6,21,25-27] However, it is unpredictable in the indi-

vidual patient whether it is possible to achieve complete resolution of the pulmonary vascular abnormalities after institution of ECMO. Our findings indicate that a state of pulmonary stress in CDH appears to be ameliorated under ECMO treatment. Decreased pulmonary arterial stress may be a factor by which ECMO results in improvement of the PH associated with cases of CDH.

Additional multicenter studies with increased patient numbers, including other stress genes, are needed for a complete understanding of the underlying molecular mechanisms.

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