Upregulation of Collagen Messenger RNA Expression Occurs Immediately After Lung Damage*

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Background: **Mortality of ARDS still exceeds 50%. Though pulmonary fibrosis is a marker of severe prognosis in the evolution of ARDS, its onset is not yet established. Cardiopulmonary bypass (CPB), usually utilized in patients with a previously normal lung, can cause ARDS and often causes alveolar damage, the earliest lesion observed in ARDS, thus providing a unique opportunity to study the molecular mechanisms of fibrogenesis.**

Objective: **To measure immediately after CPB, at the onset of alveolar damage, the expression of messenger RNAs (mRNAs) for collagen type I.**

Methods: **Pre-CPB and post-CPB lung biopsy specimens were obtained from patients submitted to myocardial revascularization for coronary artery disease. Alveolar damage was characterized by comparison between before and after specimens and quantified by point counting of polymorphonuclear cells (PMN). Type I collagen mRNAs were quantified by scanning densitometry of Northern blot autoradiographs, corrected for RNA loading by 18S ribosomal RNA hybridization.**

Results: **Alveolar damage was characterized by lung interstitial edema and by polymorphonuclear cell infiltration after CPB (PMN pre-CPB 0.010±0.004×PMN post-CPB 0.052±0.022; n=7; p=0.0017, *t* test). Type I collagen mRNA increased 91.1±68.2% (Ln pre-CPB×Ln post-CPB; n=15; p<0.00001, *t* test) immediately after CPB (mean CPB time, 108.8±37.2 min).**

Conclusion: **Fibrogenesis, as measured at the molecular level, is a very early event following diffuse alveolar damage, attributable mainly to resident fibroblast activation.**

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Key words: ARDS; cardiopulmonary bypass; collagen; lung

Abbreviations: CPB=cardiopulmonary bypass; mRNA=messenger RNA; PMN=polymorphonuclear cell; SDS=sodium dodecyl sulfate; SSC=sodium saline citrate; TGF-β=transforming growth factor-β

Despite advances in the critical care of ARDS patients, mortality is still high, exceeding 50%.[1] The cause of death in such cases has been increasingly recognized as fibrosis, in association with irreversible respiratory failure, rising to 40%[1] of the patients. While ventilator parameters and patient oxygenation indexes were not related to death, pulmonary biopsy findings of early fibrosis were significantly related to a poor outcome.[2] Increased levels of type III procollagen in BAL are strongly associated with risk for fatal outcome in patients with ARDS.[3]

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Cardiopulmonary bypass (CPB) is one of the known pulmonary insults able to induce ARDS.[4] Histologically a diffuse alveolar damage picture, with endothelial and pneumocyte type I cell lesions, edema, mitochondrial alterations in type 2 pneumocytes, and focal alveolar hemorrhage, is commonly found, particularly when the CPB clamping period exceeds 150 min.[5] Moreover, collagen secretion without fibroblast proliferation was described to occur as early as 4 days in ARDS patients following CPB.[6] In CPB, the molecular response to injury is immediate, with a significant increase of ICAM 1 and E-selectin messenger RNA (mRNA) levels being observed in the myocardium right after CPB.[7]

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It is thought that in ARDS, fibrosis begins to occur approximately 7 days following the insulting event.[8] Fibrosis, however, was histologically confirmed already after only 1 day of ARDS evolution,[9] suggesting that fibrogenesis has an early onset in this setting. During fibrogenesis, collagen type I accumulates in the lungs, changing the normal type I:III relation from 2:1 to 4:1.[10] The molecular mechanisms of fibrotic reactions have been partially elucidated. Transforming growth factor-β (TGF-β), a key modulator of these reactions, while inhibiting collagen degradation, stimulates fibroblasts to migrate to the site of injury, proliferate, and produce collagen.[11] Since fibrosis is a marker of poor prognosis in ARDS patients and the onset of fibrogenesis is not yet established, we decided to study type I collagen expression in the lung immediately before and after CPB to evaluate how fast such responses can occur in diffuse alveolar damage.

Materials and Methods

Patient Selection

The study was approved by the hospital's ethical committee. All patients gave an informed consent before participating. Inclusion criteria were age younger than 70 years, no prior thoracotomy, absence of previous lung disease, and indication for mammary anastomosis (and therefore pleura opening during revascularization) for treatment of coronary artery disease.

Surgical Procedure

Patients were submitted to general anesthesia with midazolam, fentanyl, pancuronium, and droperidol and ventilated with a tidal volume of 10 to 15 mL/kg and a respiratory rate of 15 breaths/min. CPB equipment consisted of a bubble oxygenator, and moderate hypothermia (28 to 30°C) was induced in all patients.

Tissue Samples

Two lung biopsy specimens were obtained from each patient. The first, pre-CPB, was obtained after desiccation of the mammary artery and before cannulation for the bypass, therefore before heparin administration. Surgery proceeded normally; immediately after cannulation removal, therefore after protamine administration, the post-CPB specimens were collected. Both specimens were immediately frozen and stored in liquid nitrogen until required. Frozen tissue was fixed in Carnoy's solution (ethanol:chloroform:acetic acid 60:30:10) at −70°C for 24 h. Ethanol concentration was increased (70%, 80%, 90%) hourly after that period and finally the specimens were kept in 100% ethanol for another 24 h; 3-μm sections of paraffin-embedded material were obtained and stained with hematoxylin-eosin.

Histopathologic Evaluation

The histologic slides were submitted to descriptive pathologic studies aimed to characterize the degree of pulmonary edema (interstitial and intra-alveolar) as well as the recruitment of inflammatory cells to the alveolar septa.

To further characterize inflammatory changes, quantitative analyses were performed. The numeric density of polymorphonuclear cells (PMNs) adhering to the alveolar walls was determined by morphometric procedures by conventional point-counting procedure, using a reticulated eyepiece (100 points and 50 lines). At a magnification of ×400, the points overlying tissue were computed. This count was corrected by the areal fraction of alveolar tissue under the microscopic field, determined by point counting. This procedure was undertaken in five noncoincident fields, excluding airways and pulmonary vessels.[12]

RNA Extraction and Analysis

Tissue specimens were pulverized under liquid nitrogen. The tissue powder was homogenized in guanidine isothiocyanate (4 mol/L) guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 100 mM β-mercaptoethanol) and phenol/chloroform extracted. Quality of RNA preparations was examined by the integrity of 28 and 18S ribosomal bands in analytical agarose gel electrophoresis. Ten micrograms of total RNA from each pre-CPB and post-CPB sample were denatured with formaldehyde-formamide, separated by electrophoresis on a 1% agarose formaldehyde gel, and transferred to nylon membranes. Northern filters were hybridized under stringent conditions with ^{32}P random oligonucleotide primed labeled probes. Filters were washed twice at room temperature in 2× SSC/0.1% sodium dodecyl sulfate (SDS) for 10 min; once in 1×SSC/0.1% SDS, room temperature, for 10 min; twice in 0.1×SSC/0.1% SDS, at 65°C, for 30 min; and twice in a room temperature 0.1×SSC solution, then exposed to film (X AR-5, Kodak USA) with an intensifying screen at −70°C for 2 or 5 days.

Scanning densitometry of the autoradiographs was carried out to quantify levels of gene amplification and results corrected for RNA loading using 18S ribosomal RNA hybridization.[13]

Probes

For type I collagen gene expression, the probe DNA HF 677, specific for the type I α-1 collagen chain, was used,[14] and for 18S ribosomal RNA, the probe isolated by Arnhein[15] was used.

Statistical Analysis

Morphometric data were analyzed for statistical significance by the paired *t* test. To compare mRNA levels in pre-CPB and post-CPB samples, a paired *t* test on log-transformed values was performed.

Results

Patients

Fifteen patients, 1 woman and 14 men, were included in the study. Mean age was 56.3 years (range, 39 to 69 years). All patients had severe coronary insufficiency and nine had an episode of myocardial infarction during their evolution. All patients had a severe (>80%) occlusion of at least one main coronary artery.

Surgical Procedure

None of the patients presented any morbidity regarding the biopsy procedures. One mammary

artery anastomosis was done in 11 patients and 4 patients received two anastomoses. Mean saphenous bypass number was 2.20±0.86 per case. Mean total CPB time was 108.8±37.2 min and mean aorta clamping time was 59.8±17.8 min. All but two patients received 500 mg of methylprednisolone sodium succinate (Solu-Medrol) during the bypass procedure. None of the patients developed any clinical signs of ARDS or was submitted to >24 h of mechanical ventilation.

Histologic Analysis

In seven patients, the specimens obtained were large enough to allow biochemical and histologic analyses. The lung parenchyma in pre-CPB specimens was normal in all seven cases. The post-CPB specimens disclosed edema of the alveolar septa, the perivascular space, and the interlobular connective tissue. In addition, collapsed areas and interstitial infiltration with PMNs were observed. Mean PMNs per lung parenchyma fields analyzed increased from 0.010±0.004 to 0.052±0.022 (p=0.0017) (Table 1).

RNA Analysis

Hybridization of Northern blot filters with the HF 677 complementary DNA probe for type I α-1 collagen chain revealed a prominent RNA band of 4.8 Kb (Fig 1), in accordance with the pattern described by Chu et al.[14] For densitometric analysis, only the 4.8-Kb band was used since it corresponds to 80% of the total mRNA. Post-CPB specimens showed a 91.1±68.2% mean increase in type I collagen gene expression when compared to pre-CPB specimens (n=15; p<0.00001; Fig 2). There was no correlation between the levels of type I collagen mRNA and either the duration of the CPB or postoperative lung function in these patients.

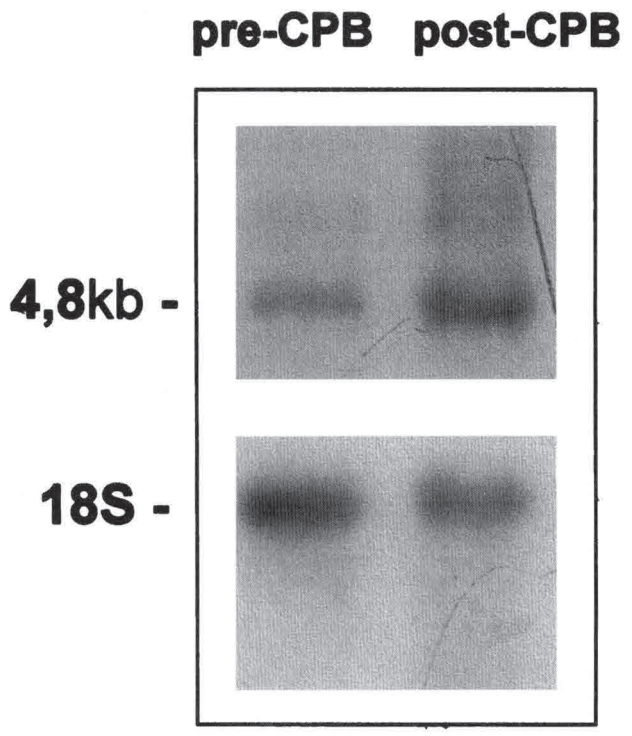

FIGURE 1. Example of Northern blot hybridization with HF 677 for type I α-1 collagen chain. *Top:* characteristic 5.8- and 4.8-Kb bands obtained; *bottom:* correspondent 18S hybridization. Lane 1 is the pre-CPB specimen and lane 2 is the post-CPB specimen.

DISCUSSION

The use of CPB caused, at least in these patients, a pulmonary lesion defined histologically by neutrophil accumulation in the pulmonary parenchyma, interstitial edema, and endothelial lesions. The highly significant increase in neutrophils in the lung parenchyma is in accordance with previous observations[12] and only reinforces the magnitude of the inflammatory response seen after CPB.[5,6] This pattern reflects the rather limited early lung response to injury characterized as alveolar damage, also seen in

Table 1—*Mean PMNs Counted per Field in Lung Tissue Specimens Obtained Pre-CPB and Post-CPB*

Patient	Pre-CPB	Post-CPB
7	0.005	0.038
8	0.011	0.050
9	0.010	0.054
10	0.017	0.026
12	0.009	0.035
14	0.007	0.090
15	0.016	0.073
Mean	0.010±0.004	0.052±0.022*

*p=0.0017 post-CPB×pre-CPB (paired Student's *t* test).

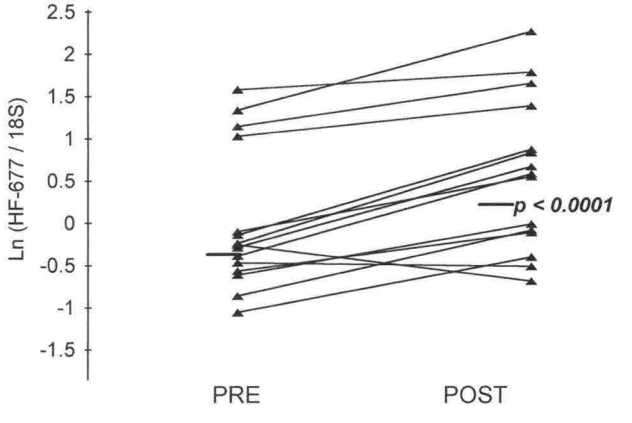

FIGURE 2. Results of the hybridization with HF 677 for type I α-1 collagen chain, corrected for 18S hybridization. Expressed as Neperian logarithm.

the early phases of ARDS, enabling the study of metabolic responses to acute pulmonary lesions.

The appearance of pulmonary edema following CPB could be predicted not only when full perfusion time exceeded 150 min[5] but also by the number of bypass grafts with mammary arteries, transfusions of pooled blood, and presence of a significant lesion of the left main stem and right coronary arteries.[16] Of the above predictors, the only one present in all patients was a graft with a mammary artery, a condition necessary for the biopsy procedures.

After CPB, collagen type I mRNA was significantly increased, with a mean rise of 92% above the pre-CPB period, over a period as short as 108 min. After a delay of 2 h, an increase of type I collagen protein would be improbable. However, since the level of collagen accumulation in the lung appears to correlate with steady-state collagen mRNA,[17] it is possible to assert that collagen production begins very early, almost at the onset of the pulmonary lesion.

To our knowledge, only one study addressed collagen synthesis in the lungs during CPB.[18] In this study, the C terminal peptide of type III collagen was measured, at baseline, in the pulmonary artery and in the left ventricle and a significant increase in the level of the C terminal peptide of type III collagen in the left ventricle was found, demonstrating that collagen synthesis occurs normally in the lung. Following CPB, the difference between measurements was reduced, indicating a decrease in the rate of synthesis. The explanation was that the oxygenator membrane could be trapping the peptide or, more probably, that the surgical trauma induced a reduction in the metabolic activity of the lung.[18] In view of our results, another possible explanation is the dissociation between plasmatic levels of collagen synthesis markers and those verified in the alveolar space, where most of the collagen synthesis occurs after a pulmonary lesion.[19]

Following ARDS, collagen accumulates up to three times over normal controls in the lungs, in a time-dependent fashion, according to data obtained by hydroxyproline quantification.[20] However, a particular problem in such studies is the difficulty in establishing a good unit to express the results, since the wet-to-dry weight relation of the tissue analyzed varies widely as a consequence of the inflammatory response, which also changes other possible units such as the amount of DNA in the tissue. Notwithstanding, biochemical measurements confirmed that total lung collagen was indeed increased in patients dying of ARDS, with the normal 2:1 type I: type III collagen changed to 3:1 and even 4:1.[21] Using monoclonal antibodies, Raghu et al[10] also demonstrated an increase of type I collagen in the lungs of ARDS patients. Serum concentrations of type III procollagen propeptide are significantly increased in patients with ARDS relative to control subjects as soon as 2 days after injury,[19,22] and correlate with indexes of lung function,[22] supporting the concept that early fibrogenesis, with functional consequences, occurs in the evolution of ARDS.

Therefore, the observed increase in type I collagen mRNA is in accordance with the expected lung response to injury, although to our knowledge, such a response has never been demonstrated to take place in human beings as early as 2 h after onset of the injury.

Following CPB, none of these patients developed ARDS. A possible explanation could be that, following activation, fibroblasts might display a form of programmed death signalized by negative growth regulatory peptides.[23] Another explanation could be that the initial extent of the host defense response is not sufficient to sustain the evolution of ARDS.[24]

A putative effector of the observed increase of collagen mRNA could be TGF-β. This cytokine plays a central role in tissue repair, stimulating the production of matrix proteins, inhibiting proteases, and modulating the expression of matrix receptors on the cell surface.[11] Overexpression of TGF-β mRNA in sites of collagen synthesis was demonstrated in idiopathic pulmonary fibrosis.[25] In an experimental model of shock-induced ARDS, increased levels of TGF-β mRNA were demonstrated, but pretreatment with an anti-TGF-β monoclonal antibody completely prevented the histologic changes of ARDS.[26]

The above findings allow us to construct another hypothesis for the derangements seen in the pulmonary parenchyma following diffuse alveolar damage. Instead of fibrosis following mesenchymal cell proliferation, as previously reported, we can think of a model where the fibroblast is activated early and participates in the inflammatory response. In support of this hypothesis, it has been shown that the fibroblast is able to: synthesize interleukin 8,[27] thus recruiting neutrophils; produce TGF-β with an autocrine regulation, as such controlling its own production of matrix proteins;[28] activate alveolar macrophages, mostly through collagen breakdown products,[29] but also inducing macrophage proliferation through macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor synthesis.[30] Fibroblasts derived from an acute lung injury model exhibited in culture features consistent with increased metabolic activity, synthesized more collagen, but had no differences in terms of growth rate, total protein synthesis, or interleukin-1α production, again suggesting that collagen synthesis is not related to fibroblast proliferation.[31] The above data are in conformity with the role played by the

host defense response, characterized by a complex interaction of proinflammatory cytokines, in the installation and progression of ARDS.[24]

In conclusion, we have shown that following acute lung damage, a very fast upregulation of collagen mRNA occurs. Although the role of this increased collagen production in the development of ARDS is not yet clear, early markers of enhanced collagen synthesis might identify patients at risk.

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