

**EVIDENCE THAT MESOTHELIAL CELLS MAY REGULATE THE  
ACUTE INFLAMMATORY RESPONSE IN TALC PLEURODESIS**

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**Abbreviations:**

**IL-8:** interleukin 8

**VEGF:** vascular endothelial growth factor

**TGF $\beta$ -1:** transforming growth factor  $\beta$  1

## Summary

Intrapleural instillation of talc is used to produce pleurodesis in cases of recurrent malignant pleural effusions. The mechanisms by which pleurodesis is produced remain unknown but may involve either injury or activation of the mesothelium. The aim of this study was to assess the response of pleural mesothelial cells to talc in an experimental model in rabbits. A group of ten rabbits were injected intrapleurally with talc (200mg/kg) and undiluted pleural fluid was collected after 6, 24 or 48 h for measurement of IL-8, VEGF and TGF $\beta$ 1. Samples of pleura were studied to assess mesothelial cell viability. **Results:** The pleural fluid IL-8 concentration peaked at 6 h, whereas VEGF and TGF $\beta$ 1 concentrations increased steadily over 48 h. Immunohistochemistry for cytokeratin showed a preserved layer of mesothelial cells despite the intense inflammatory pleural reaction. Thus, we propose that the mesothelial cell, although injured by the talc, may actively mediate the primary inflammatory pleural response in talc-induced pleurodesis.

## Introduction

Malignant pleural effusion is a common complication of advanced cancer (1) and, despite the fact that patients with this malignant complication have a short life expectancy, the prompt control of the effusion is imperative to obviate discomfort of persistent cough and dyspnea. Drainage of the pleural cavity and instillation of a sclerosing agent is the standard method to produce pleural symphysis and prevent fluid re-accumulation (2-4). Talc, instilled either by slurry or by thoracoscopy (5-7), has been shown to produce more than 90% success in the control of the recurrent malignant pleural effusion (8, 9). The mechanism by which talc produces a pleurodesis remains unclear. Talc may injure the mesothelial layer or may stimulate monocytes or mesothelial cells to produce a local reaction mediated by major inflammatory cytokines (10, 11). Among the cytokines involved in the acute inflammatory response, interleukin 8 and VEGF are the main mediators of leukocyte activation and vascular capillary response to inflammation, respectively (12, 14), whereas TGF- $\beta$ 1 and FGF are involved in the activation of fibroblasts to produce and deposit collagen (15, 16).

In animal and clinical models of pleurodesis, it is difficult to isolate the response of the mesothelial cells to the sclerosing agent because of the active multicellular population found in the pleural space. The purpose of this study was to determine the inflammatory response of the pleural space and the viability of the mesothelial cells in the inflammatory pleural infiltrate injured by talc used in clinical pleurodesis. Our report indicates that mesothelial cells have the potential to be the major contributor to the inflammatory response in the pleural space in talc-induced pleurodesis.

## Methods

This study was approved by the Ethics Committee of the Heart Institute (InCor), University of Sao Paulo Medical School, which oversees research involving both animals and humans.

**Pleural Injection:** Ten New Zealand white male rabbits weighing 2.5kg were anesthetized and injected intrapleurally with 3mL of talc (200mg/kg) currently used to produce pleurodesis in clinical practice (Magnesita, Bahia, Brazil; mean length 25.4 $\mu$ m, range 6.4 to 50.5 $\mu$ m). The talc was suspended under sterile conditions in an endotoxin-free saline solution. The protocol of pleural injection has been described in details in previous studies (17, 18). After 6, 24 or 48 h, the animals were sacrificed, and after exposure of the diaphragm via a mid abdominal incision, the pleural fluid was aspirated and processed for cytokine measurements.

## Cytokine Analysis

Interleukin-8 (OptEIA, rabbit IL-8 set, Pharmingen, San Diego, CA), vascular endothelial growth factor (VEGF; R&D System, Inc., Minneapolis, MN) and transforming growth factor beta 1 (TGF- $\beta$ 1; R&D System, Inc.) were measured by ELISA (enzyme-linked immunoabsorbent assay) as described previously (13).

## Tissue Samples

Samples of visceral pleural tissue were fixed in 10% formalin for 48 h and processed for histological analysis. The slides were stained by immunohistochemistry for cytokeratin (AE1/AE3, Dako Cytomation, Produktionsvej, Denmark) following the manufacturer's directions.

**Statistical Analysis:** Data are expressed as mean  $\pm$  one standard deviation. One way analysis of variance was used to compare differences among subgroups and the Tukey test was used to perform multiple comparison procedures, using statistical computer software (SigmaStat, SPSS, San Rafael, CA). A p value  $< 0.05$  was accepted as significant.

## Results

### *Cellular response (Table 1)*

**WBC:** Total white blood counts were significantly greater at 6 and 24 hours in comparison to 48 hours (respectively,  $19,430 \pm 1,750$  and  $18,810 \pm 5,660$  vs.  $11,270 \pm 1,190$  cells/mm<sup>3</sup>;  $p < 0.05$ ).

**%N:** Similar to WBC levels, neutrophil percents were increased in the first 6 hours ( $74 \pm 6$ ) in comparison to 24 hours ( $60 \pm 5$ ;  $p < 0.05$ ) and 48 hours ( $24 \pm 8$ ;  $p < 0.001$ ).

### *Cytokine Production (Table 2)*

**IL-8:** Concentrations of IL-8 increased as soon as 6 h ( $797 \pm 335$  pg/mL) after the pleural space was exposed to talc and remained elevated until 24 h ( $665 \pm 125$  pg/mL), decreasing significantly after 48 h ( $134 \pm 33$  pg/mL;  $p < 0.001$ ).

**VEGF:** Unlike IL-8, VEGF levels increased with time. At 48 h VEGF concentration ( $689 \pm 115$  pg/mL) was significantly greater than 6 h ( $164 \pm 64$  pg/mL;  $p < 0.001$ ) and 24 h ( $336 \pm 188$  pg/mL;  $p < 0.05$ ).

**TGFβ1:** Similarly to VEGF, the concentrations of TGFβ1 also increased over time. At 48 h and 24 h the levels were significantly greater than 6 h (respectively,  $1135 \pm 223$  and  $931 \pm 60$  vs.  $385 \pm 104$  pg/mL;  $p < 0.001$  and  $p < 0.05$ ).

### *Correlations of the WBC and Neutrophil Percent with the Cytokine IL-8*

Although WBC and neutrophil percent had the same behavior of the cytokine IL-8 in the pleural fluid after talc instillation, no correlation was found among these parameters (data not shown).

### *Microscopic Analysis of the Pleural Tissue*

Microscopic samples of the visceral pleura showed an acute inflammatory reaction as early as 6 h after intrapleural talc injection. The histological analysis of the slides stained by immunohistochemistry for cytokeratin revealed in several zones throughout the slides a preserved brown-stained monolayer of pleural mesothelial cells underlying an intense inflammatory process characterized by a multi cell population (**Figure 1**).

## Discussion

The mechanisms by which talc produce pleurodesis remain poorly understood. The regulation of the pleural acute inflammatory response following the injection of a sclerosing agent is crucial to understanding the mechanism of pleurodesis. The findings of this study indicate that pleural mesothelial cells may actively contribute to modulate the inflammatory process in talc induced pleurodesis.

Our findings are in accordance with previous reports that have shown that mesothelial cells exposed to talc can actively produce pro-inflammatory IL-8 and MCP-1 (11, 13), VEGF (13), TGF- $\beta$  (15) and bFGF (16) cytokines.

In addition, a recent study evaluating submicroscopic features of active pleural remodeling associated with talc pleurodesis stated that talc acutely induces a prominent injury to the mesothelial cells and mesothelial cell–mesothelial basement membrane union. However, focal remesothelialization of the denuded areas were documented (19), showing an active role of mesothelial cells in the healing process in talc pleurodesis. Our results indicate that WBC, neutrophil percent and IL-8 levels were increased in the first twenty-four hours, whereas VEGF and TGF- $\beta$ 1 levels were initially lower and increased with time.

Because the population of active inflammatory cells found in the pleural space in talc-induced pleurodesis is diverse, the specific contribution of the mesothelial cell response in the pleurodesis induced by talc still poses a challenge. Our results indicate that mesothelial cells may account for the inflammation seen *in vivo*.

We used a dose of talc comparable to that used in human pleurodesis. If we consider the surface of both pleural membranes in the rabbit (200cm<sup>2</sup>) and in humans (2000 cm<sup>2</sup>), the amount of talc used *in vivo* is approximately the same; in rabbits, 200 mg/kg x 2.5 kg/200 cm<sup>2</sup> = 2,500 ug/cm<sup>2</sup> and in humans 5 g/2000cm<sup>2</sup> = 2,500 ug/cm<sup>2</sup>.

The experimental finding that mesothelial cells may play a major role in the mechanism of pleural inflammation and contribute to an effective sclerosis in talc-induced pleurodesis may help explain why malignant pleural effusions with a high tumor burden and a low pleural fluid pH and glucose concentration may be less responsive to talc pleurodesis than malignant effusions with less malignant involvement of the pleural space (18-22). However, this is not absolute because effective pleurodesis can be achieved using talc by thoracoscopy despite the findings of low pleural fluid pH (23). In conclusion, our findings indicate that the acute inflammatory process in talc pleurodesis may have the active contribution of the mesothelial cells.

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**Table 1:** Results of pleural fluid WBC (cells/mm<sup>3</sup>) and neutrophil (%) in talc 200mg/kg injected rabbits after 6, 24 and 48 hours.

	WBC	% N
6 h	19,430 ± 1,750	74 ± 6
24 h	18,810 ± 5,660	60 ± 5*
48 h	11,270 ± 1,190*	24 ± 8 <sup>†#</sup>

**WBC:** 6 and 24 hours > 48 hours (\*p< 0.05)

**%N:** 6 hours > 24 hours (\*p< 0.05) and 48 hours (<sup>†</sup>p< 0.001); 24 hours > 48 hours (<sup>#</sup>p< 0.001)

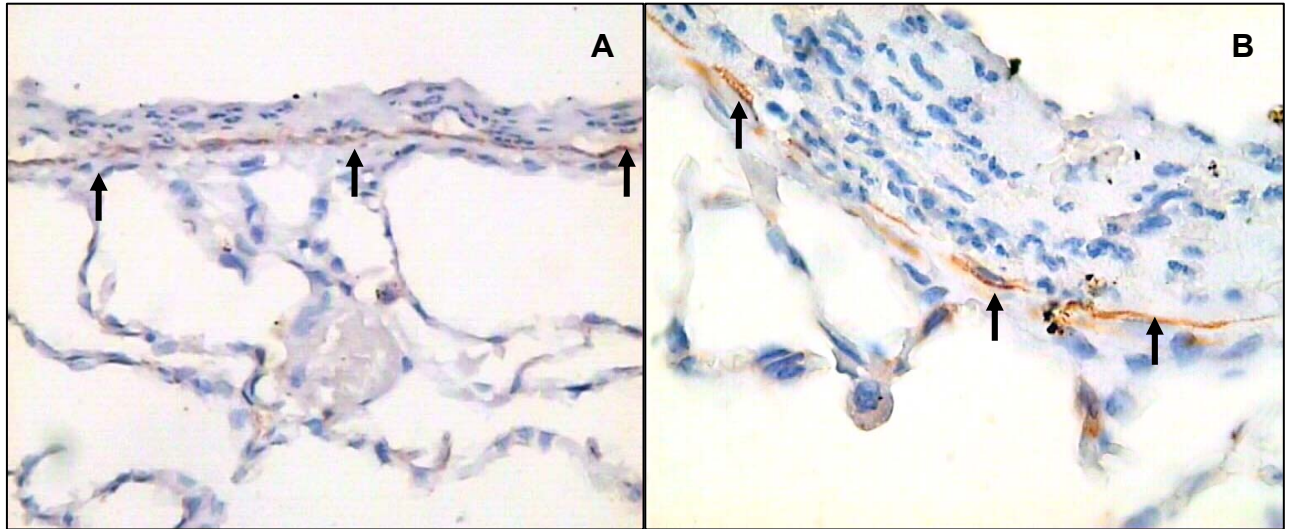
**Table 2:** Results of pleural fluid IL-8, VEGF and TGF-β1 (pg/mL) in talc 200mg/kg injected rabbits after 6, 24 and 48 hours.

	IL-8	VEGF	TGF-β1
6 h	797 ± 335	164 ± 64*	385 ± 104 <sup>*†</sup>
24 h	665 ± 125	336 ± 188 <sup>†</sup>	931 ± 60
48 h	134 ± 33*	689 ± 115	1135 ± 223

**IL-8:** 6 and 24 hours > 48 hours (\*p< 0.001)

**VEGF:** 48 hours > 6 hours (\*p< 0.001) and 24 hours (<sup>†</sup>p< 0.05)

**TGF-β1:** 48 hours > 6 hours (<sup>\*</sup>p< 0.001); 24 hours > 6 hours (<sup>†</sup>p< 0.05)



**Figure 1:** Microscopic findings: acute pleural inflammatory reaction in rabbits injected with talc 200mg/kg after 24 hours showing a preserved brown-stained cytokeatin positive monolayer of pleural mesothelial cells (arrows) (A: x400 and B: x1000).

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