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The response of subpleural pulmonary capillary endothelium to hydrothorax in rats*

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Summary. The principal focus of this study was to evaluate the hypothesis that increased interstitial fluid pressures served to stimulate de novo vesicle formation in pulmonary capillary endothelium. Direct measurements of interstitial fluid pressures within the alveolar septa pose great technical difficulty. The pleural space and subpleural capillaries are easily accessible, and thus, provide a more feasible model to test this hypothesis. After hydrostatic pressure of pleural space fluid was increased by periodic saline infusions into the pleural cavity, vesicle numerical densities were significantly increased in portions of the subpleural capillary endothelium. Those segments of the endothelium that directly apposed the interstitium of the visceral pleura displayed de novo vesicle formation. The endothelial segments located immediately adjacent to the alveolar epithelium were not affected by the elevated interstitial fluid pressures. In addition to the increased vesiculation, those same segments of the endothelium were characterized by increased attenuation of their cytoplasmic compartments. These conformational changes in the plasmalemma of portions of the subpleural capillary endothelium provide support to the tentative hypothesis, however, whether the increased numbers of vesicles contribute to a potential transendothelial transport system or expand a possible static network of membrane invaginations remains uncertain.

Key words: Morphometry - Endothelium - Hydrothorax - Plasmalemmal vesicles

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Introduction

Previous investigations from this laboratory provide support for the concept that increased pulmonary capillary filtration and subsequent interstitial fluid accumulation in the alveolar septa are associated with de novo vesicle formation in alveolar capillary endothelium (DeFouw and Chinard, 1983). If it is assumed that interstitial fluid accumulation equates with increases in interstitial fluid pressures (Magno et al., 1980), these tissue pressures may serve to initiate the increased endothelial vesiculation, which is then interpreted as a secondary result of increased capillary filtration rates. That increased endothelial vesiculation is not primarily associated with septal edema formation is consistent with previous studies from this laboratory of edematous lungs at 15° C (Chinard and DeFouw, 1981). Although hydrodynamic edema was induced at 15° C, endothelial vesiculation was not increased, most likely the result of a temperature related decrease in membrane lipid fluidity which limited new vesicle formation.

The purpose of the present study was to test the hypothesis that increased interstitial fluid pressures served to stimulate plasmalemmal vesicle formation in pulmonary capillary endothelium. The subpleural pulmonary capillaries served as the experimental model. The composition of pleural fluid was reported to be generally similar to that of interstitial fluid (Miserocchi and Agostoni, 1971) and pleural fluid pressures were regarded to be comparable to interstitial fluid pressures under normal conditions (Kim et al., 1979). Thus, interpretations of results from the present study are based upon the assumption that increased interstitial fluid pressures surrounding the subpleural pulmonary capillaries would tend to parallel those observed directly in the pleural space. That is, increases in pleural fluid pressures after saline infusion into the pleural space, are associated with fluid accumulation and attendant elevations of fluid pressures in the visceral pleural interstitium, which contains the subpleural pulmonary

capillaries. Under these conditions, the results of the present study are consonant with the concept that increased interstitial fluid pressures are associated with membrane conformational changes in portions of the capillary endothelia, marked by vesicle formation and attenuation of the cytoplasmic compartment. The functional implications of such changes remain to be tested.

Materials and methods

Long Evans rats, approximately 300-400g body weight, were anesthetized with sodium pentobarbitol (I.P., 3mg/100g body weight) and secured in the supine position. After tracheal cannulation, the lungs were ventilated by a small animal respirator operating at 60 cycles/min and delivering 6cm^3 room air per inspiratory stroke. End-expiratory pressure was maintained at 2-3 cm H_2O. The abdominal cavity was opened by a mid-line incision to enable viewing of the lungs through the tendinous portion of the diaphragm. The left eighth intercostal space on the lateral surface of the thorax was carefully exposed and a fluid filled cannula was inserted into the pleural space at the level of the costodiaphragmatic recess. The cannula, which was secured to the point of insertion by a purse string suture, was connected to a strain gauge transducer and associated recorder for continuous monitoring of pleural fluid pressures. The pressures were read at end-expiration at 3 minute intervals and then averaged for each animal preparation. Zero pressure readings were adjusted to the level of the tip of the cannula which was set at the lateral midpoint of the intercostal space.

A group of eight rats served as sham operated controls and pleural fluid pressures were recorded continuously for 60 minutes. Pleural fluid was then aspirated from the costodiaphramatic recess (approximately 0.5 ml was collected from each animal). The pleural fluid samples from each of the eight animals were pooled and the protein concentration of the combined fluid sample was determined by the Lowry method (Lowry et al., 1951). Osmotic pressure (o.p.) was then calculated, according to Landis and Pappenheimer (1963), as:

$$\text{o.p.} = (2.1c + 0.16c^2 + 0.0009c^3) \times 1.356$$

where c is the protein concentration extrapolated to g/100 ml.

In a second group of eight rats pleural fluid hydrostatic and osmotic pressures were modified by infusions of 0.9% saline at approximately 1 ml/6 min over periods of 60 minutes. The saline was infused via 3-way stopcock connected to the pleural space cannula. Periodic infusions served to maintain elevated hydrostatic pressures which tended to dissipate after a single infusion. After 60 minutes, pleural fluid samples were obtained from the costodiaphragmatic recess for subsequent estimates of protein concentrations and osmotic pressures.

In both the sham operated and saline infused groups, a median sternotomy was then performed and the lungs were fixed by tracheal instillation of 2% glutaraldehyde in 0.15M bicarbonate buffer at 20 cm H_20 pressure. Prior to instillation of fixative the location of the cannula in the costodiaphragmatic recess was carefully checked. After overnight fixation, the lungs were sliced into six longitudinal segments for tissue sampling. A single tissue block was taken from six regions dispersed along the pleural surface of each of the six longitudinal segments. The tissue blocks were postfixed with osmium tetroxide in 0.15M bicarbonate buffer, reacted with 1% tannic acid in bicarbonate buffer (Simionescu and Simionescu, 1976), dehydrated in graded series of ethanols and propylene oxide, and embedded in Epon. From the primary sample of 36 tissue blocks, a single block was chosen randomly from each of the six pleural sample regions for ultramicrotomy. A technically perfect thin section (75nm, as judged by the silver-gold interference color) was obtained from each block, stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope operating at 60 Kv. Five micrographs, at 960 original magnification, of the visceral pleura and five micrographs, at 5800 original magnification, of the subpleural capillaries were recorded. The capillaries were demarcated into those portions of the endothelium which apposed the pleural interstitium and those endothelial segments which were closely apposed to the epithelium of the underlying alveoli.

A multipurpose test system was used to collect morphometric data from the subpleural capillaries and a curvilinear test system, which accounts for tisssue anisotropy, was used to obtain stereologic counts from the visceral pleura (Weibel, 1979). The micrographs at 960 original magnification served to provide morphometric estimates of the average thickness of the visceral pleura, including mean thicknesses of the pleura's mesothelium and underlying interstitial compartment. The micrographs of the subpleural capillaries were used to obtain morphometric estimates of mean thicknesses of the capillary endothelium, including percent of the endothelial cytoplasmic compartment comprised of attenuated segments (20-30 nm maximum thickness), and endothelial vesicle numerical densities. Standard stereologic point and line intercept counting procedures, as described previously (DeFouw, 1984), were used to define each of the morphometric parameters.

The morphometric data obtained from each lung were grouped according to category with data from the other seven lungs of each experimental group. An additional group of rats was anesthetized and the lungs were fixed without prior cannulation of the pleural space to provide a morphometric baseline of the normal visceral pleura and subpleural capillaries. Samples of pleural fluid from these rats were also analyzed to determine protein concentrations and osmotic pressures in normal anesthetized rats. Mean values for the morphometric parameters were determined for each group and comparisons of means between the groups were performed with a one-way analysis of variance and Duncan's multiple comparison procedure (Duncan, 1955). Indications of statistically significant differences were accepted at $p < 0.05$.

Results

The visceral pleura of the rat is composed of a mesothelium and an underlying interstitial compartment which is continuous with the lung parenchyma (Fig. 1). The subpleural capillaries are located within this interstitial compartment. They are positioned with portions of their endothelium closely apposed to the epithelial lining of the underlying alveoli (thin sides of the capillaries) with the remaining endothelial segments directly adjacent to the pleural interstitium (thick sides of the capillaries).

Table 1 presents the recorded hydrostatic pressures, protein content, and calculated osmotic pressures of the pleural fluid in each experimental group. The pleural fluid sampled from the sham operated controls presented protein concentrations and thus, calculated osmotic pressures that were identical to values obtained in the normal rats. The negative (subatmospheric) hydrostatic pressures recorded in the pleural space of the controls rats were within the expected range (Agostoni and D'Angelo, 1969). Thus, cannulation of the costodiaphragmatic recess was not associated with recognizable changes in pleural space fluid. After saline infusion into the pleural space, hydrostatic pressure of the pleural fluid was increased significantly. Protein content and calculated osmotic pressures of the pleural fluid, on the other hand, were decreased. Based upon the Starling relationship, it is reasonable to assume that portions of the infusate were absorbed from the pleural space under conditions of decreased osmotic pressure and increased hydrostatic pressure of the pleural fluid. That approximately 35% of the infusate was not recovered from the pleural space is consistent with this assumption.

Further support to the suggestion that portions of the saline infusion were absorbed from the pleural space is presented in Table 2. After saline infusion the overall thickness of the visceral pleura was increased. Average thickness of the mesothelium was not changed, therefore, the increase in pleural thickness was the apparent result of fluid accumulation in the pleural interstitium and consequent increases of interstitial compartmental thickness (Fig. 2). Respective mesothelial and interstitial compartmental thicknesses in the sham operated controls

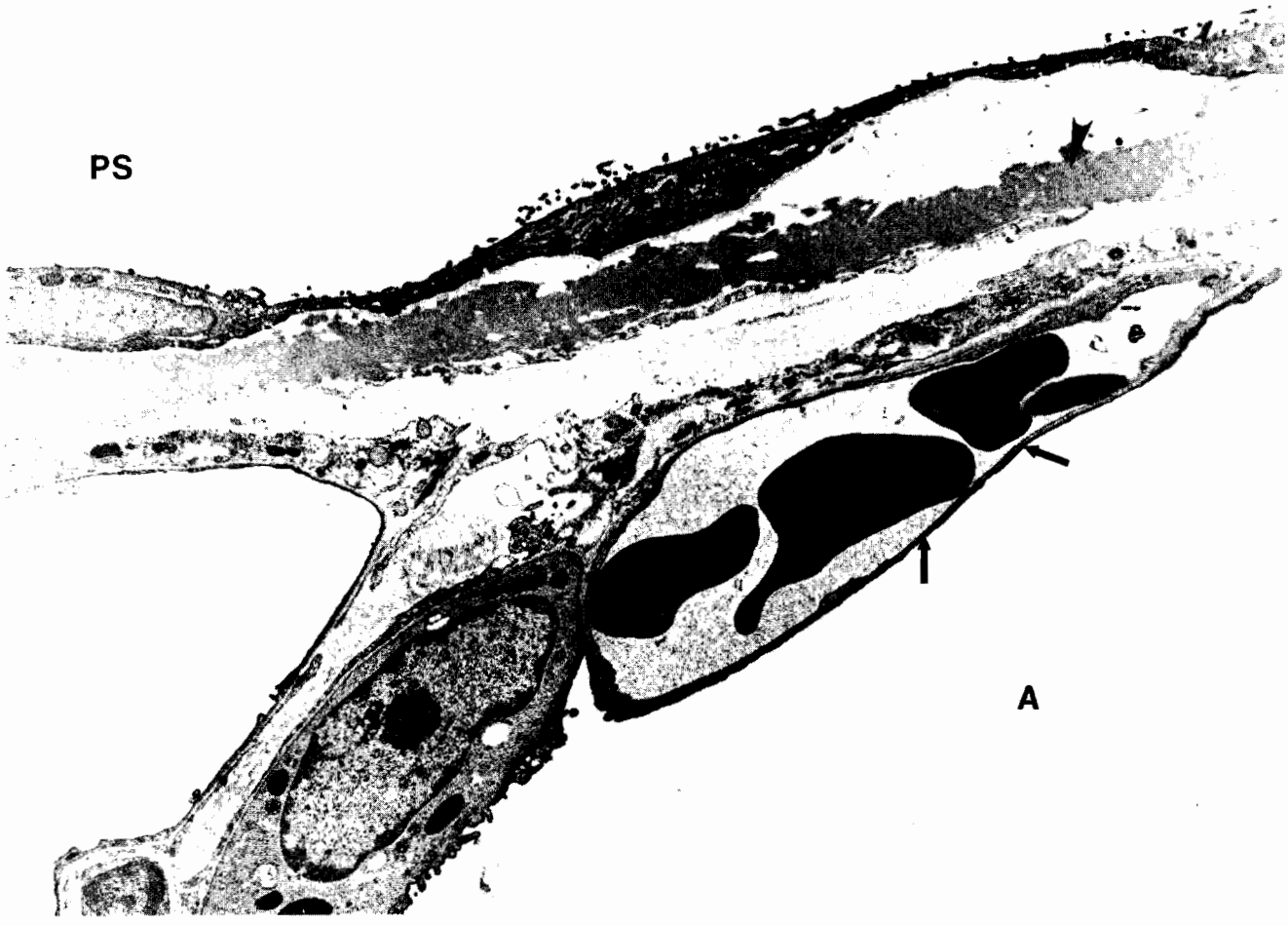

Fig. 1. The ultrastructural architecture of the visceral pleura a normal rat illustrates the presence of subpleural capillaries in the interstitial compartment of the pleura. The pleural interstitum, which also presents a prominent elastic lamina (arrowhead), collagen fibrils, and segments of fibroblastike cells, is continuous with the underlying alveolar septal interstitium. The close apposition of the thin sides (arrows) of the capillary to the alveoli (A) is clearly depicted. The pleural mesothelium provides a continuous cellular lining of the pleural space (PS). x 7, 180

were not different than those recorded for the visceral pleura of the normal rats. Again, the efects of cannulation of the pleural space appeared minimal under the present experimental conditions.

The morphometric data presented in Tables 3 and 4 serve to define the influence of interstitial fluid accumulation on the subpleural capillaries present in the interstitial compartment of the visceral pleura. Portions of the capillary endothelium that directly oppose this interstitial space (thick sides of the capillaries, Fig. 3) are described by the data presented in Table 3. After fluid accumulation in the interstitium, the percentage of the endothelial compartment comprised of thinnly attenuated regions (maximun thickness 20-30nm) was substantially increased. Further, the number of plasmalemmal vesicles within the cytoplasmic compartment was significantly elevated (Fig. 4). The thin sides of the capillaries, comprised of those portions of the endothelium that are separated from the alveolar epithelium by a common basal lamina, were not affected by the accumulation of interstitial fluid (Table 4). That is, neither the number of plasmalemmal vesicles nor the percentage of attenuated endothelial segments was increased.

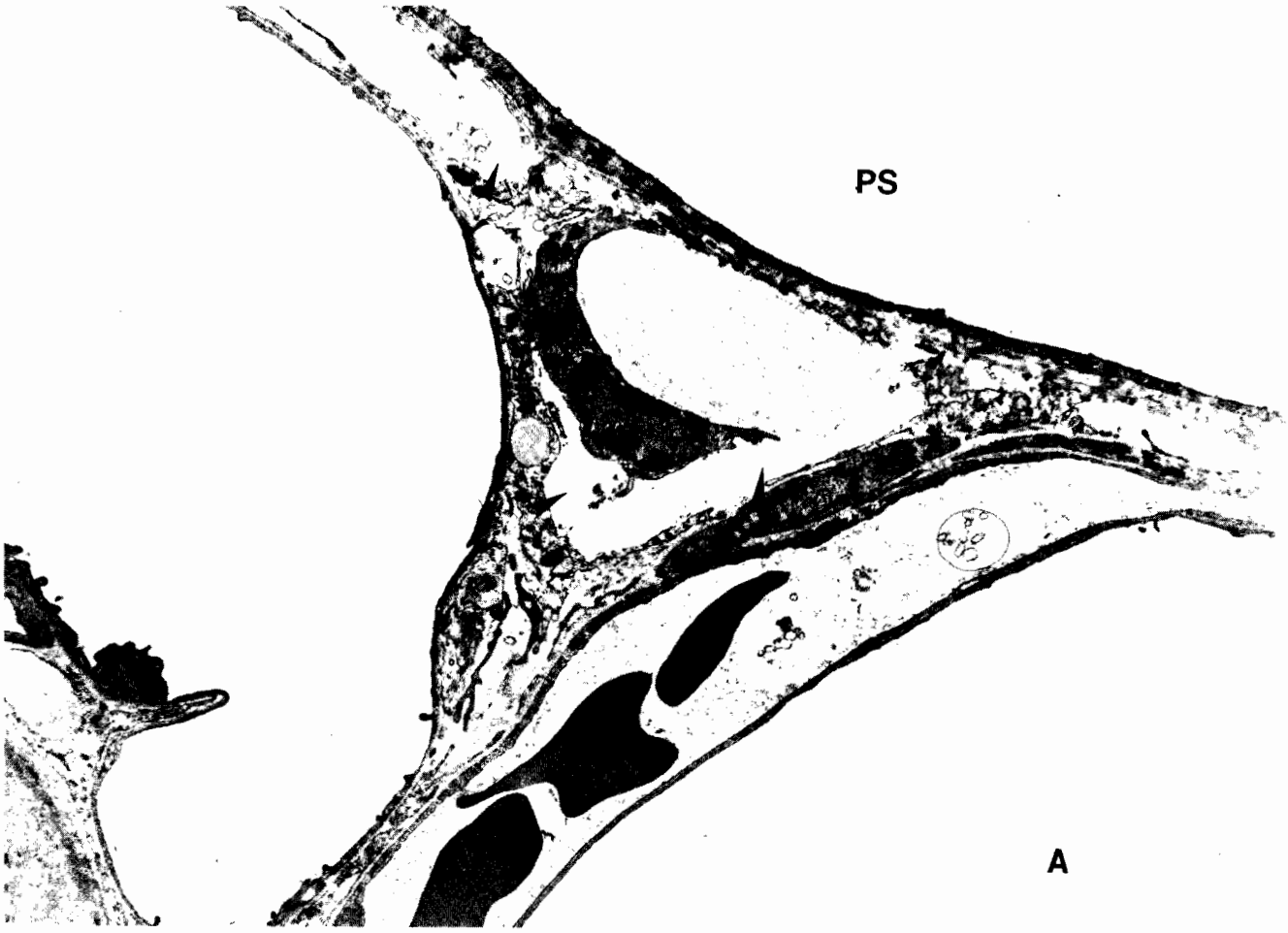

Fig. 2. The arrowheads indicate the presence of fluid accumulation within the pleural interstitium after periodic infusions of saline into the pleural space (PS). Fluid accumulation within the alveolar interstitium is not indicated and the appearance of the pleural mesothelium remains unchanged. x 5,550.

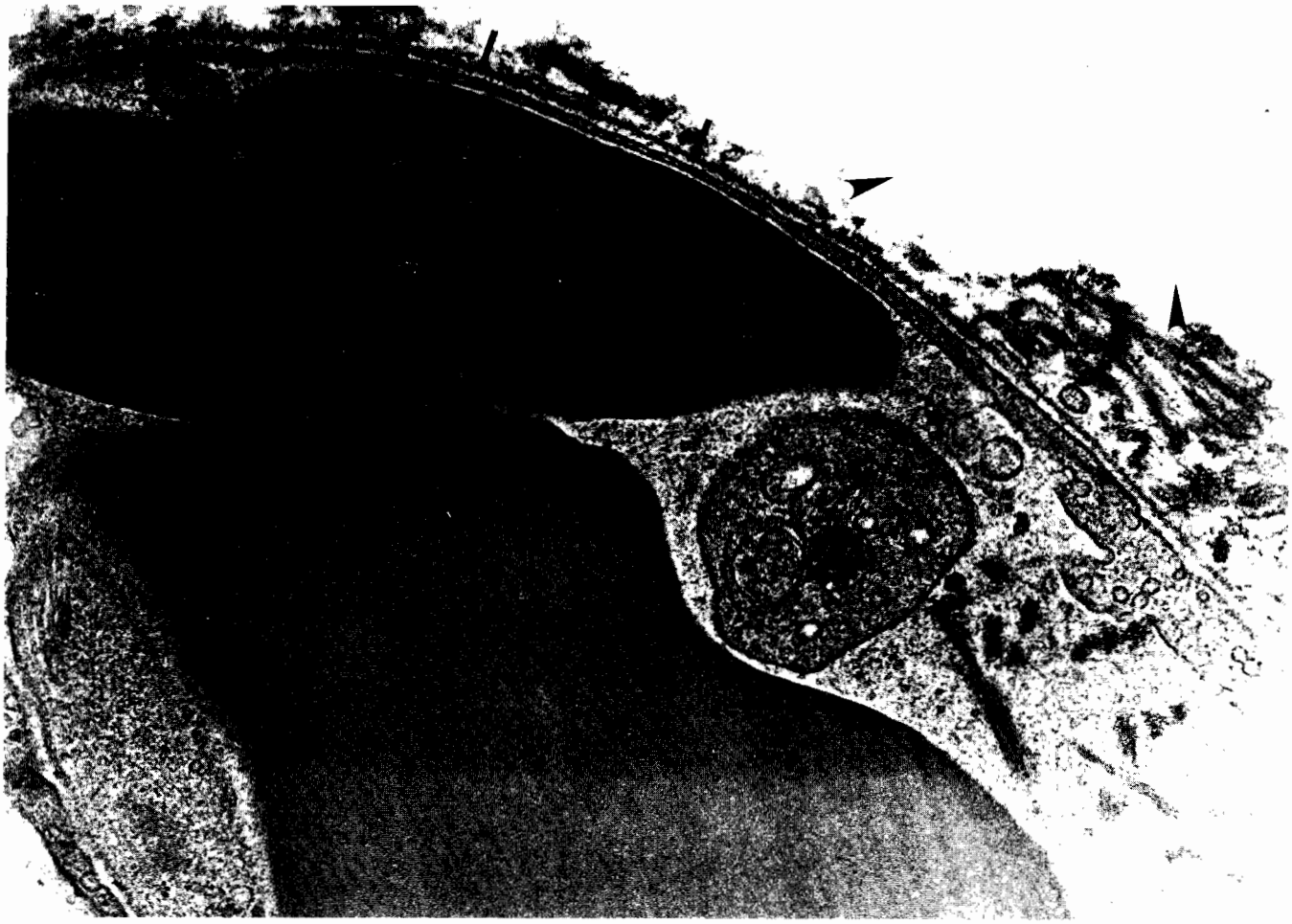

Fig. 3. A segment of the thick side of a subpleural capillary is presented in immediate apposition to the interstitial compartment of the visceral pleura. Thinnly attenuated segments (arrows) serve to characterize this portion of the endothelium after fluid accumulation (arrowheads) in the pleural interstitium. Plasmalemmal vesicles are restricted to the nonattenuated portions of the endothelium. x 38,425

Table 1. Pleural fluid hydrostatic and osmotic pressures.

	NORMAL	CONTROL	SALINE INFUSION
Fluid volume infused per animal (ml)[a]	0	0	10
Fluid volume sampled per animal (ml)	0.5	0.5	6.5
Hydrostatic pressure (cm H_2O)[b]	− 1.0 to − 3.0[c]	− 2.5 ± 0.5	+ 2.7 ± 0.5*
Protein content (g/100 ml)[d]	2.5	2.5	0.67
Osmotic pressure (cm H_2O)	8.7	8.7	2.1

a. volume injected at approximately 1 ml/6min.
b. pressures recorded at end expiration at 3 min intervals and then averaged for each animal.
c. values from Agostoni and D'Angelo (1969).
d. single value determined from pooled pleural fluid samples obtained from individual animals in each group.
* significantly different than control values.

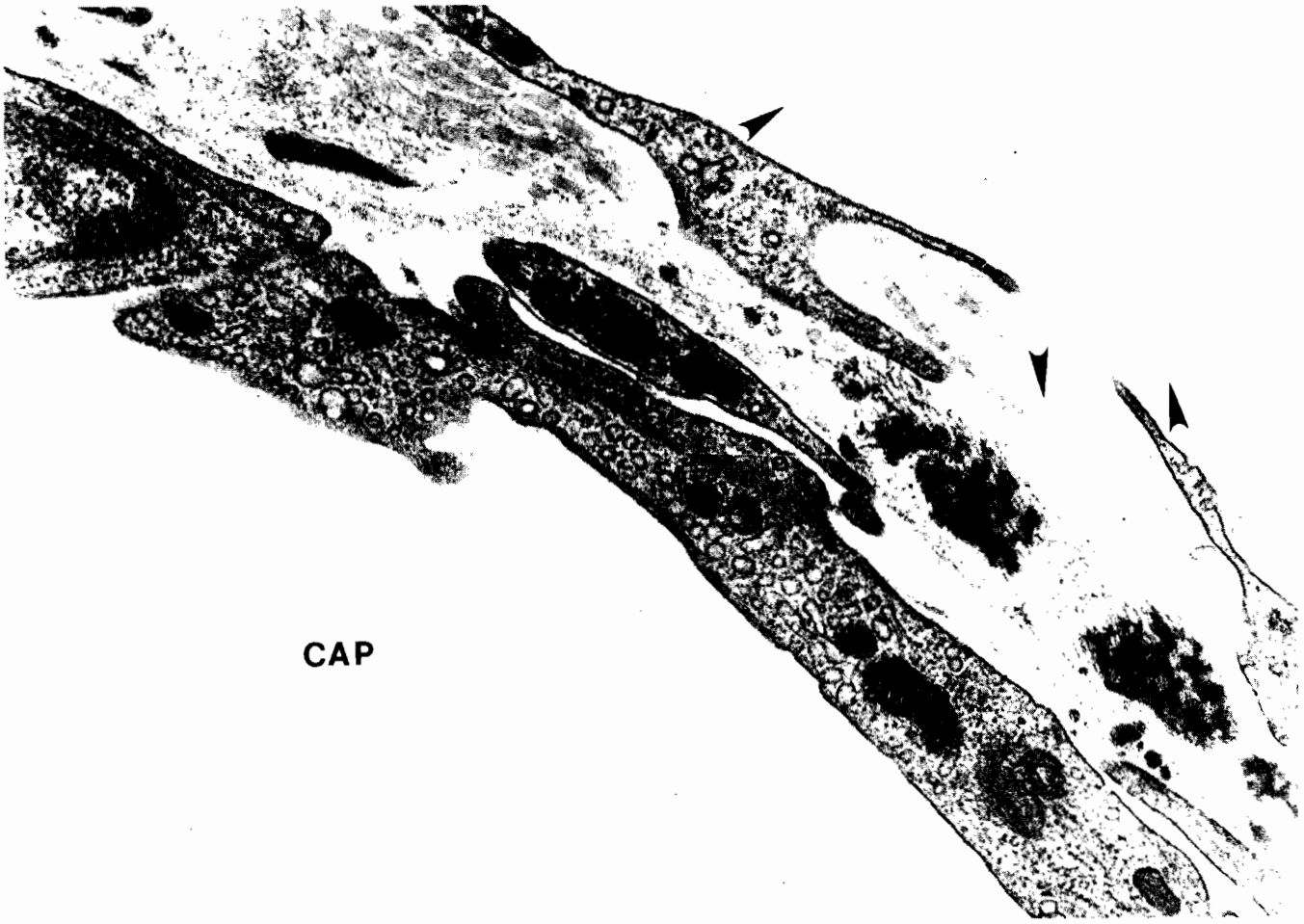

Fig. 4. Fluid accumulation (arrowheads) within the interstitium surrounding a subpleural capillary (CAP) is indicated after saline infusion into the pleural space. Numerous plasmalemmal vesicles are the principal ultrastructural feature of the nonattenuated portions of the endothelium which comprise the thick sides of the subpleural capillaries. x 38,940.

Table 2. Mean thicknesses of the visceral pleura'

	NORMAL	CONTROL	SALINE INFUSION
Total thickness (μm)	1.15 ± 0.07	1.17 ± 0.09	1.55 ± 0.03 *
Mesothelial thickness	0.26 ± 0.02	0.25 ± 0.02	0.23 ± 0.04
Interstitial thickness	0.88 ± 0.05	0.92 ± 0.08	1.29 ± 0.05 *

'data represent mean values ± one standard deviation.

* significantly different than both the normal and control values.

Table 3. Morphometric parameters from the thick sides of the subpleural capillary endothelium'

	NORMAL	CONTROL	SALINE INFUSION
Endothelial thickness (μm)	0.15 ± 0.03	0.16 ± 0.04	0.13 ± 0.02
Percent attenuation	7 ± 4	5 ± 3	21 ± 4 *
Vesicle number per μm^3 cytoplasm	97 ± 9	94 ± 7	135 ± 6 *
Mean vesicle diameter (nm)	88 ± 16	91 ± 8	87 ± 10

'data represent mean values ± one standard deviation.

* significantly different than both the normal and control values.

Table 4. Morphometric parameters from the thin sides of the subpleural capillary endothelium'

	NORMAL	CONTROL	SALINE INFUSION
Endothelial thickness (μm)	0.10 ± 0.01	0.10 ± 0.01	0.08 ± 0.02
Percent attenuation	34 ± 5	38 ± 4	30 ± 6
Vesicle number per μm^3 cytoplasm	118 ± 4	112 ± 10	115 ± 6
Medan vesicle diameter (nm)	82 ± 10	85 ± 5	87 ± 2

'data represent mean values ± one standard deviation.

Discussion

The significance of the results from this study is based upon the premise that direct measurements of fluid hydrostatic pressures in the pleural space serve to indicate interstitial fluid pressures of the visceral pleura. The pleural space and interstitial compartment are separated by a highly permeable mesothelium which provides less of a barrier to water flow than endothelial surfaces (Kim et al., 1979; Kinasewitz et al., 1983). Thus, after saline infusion into the pleural space with concomitant increases in hydrostatic pressure and decreases in oncotic pressure of the pleural fluid, flux of water across the mesothelium from the pleural space would be expected. Expansion of the interstitial compartment of the visceral pleura observed presently and results from previous studies (Stewart and Burgen, 1958; Kinasewitz and Fishman, 1981; Nakamura et al., 1984) provide evidence consistent with this interpretation. Since the parietal pleura provides an extensive arrangement of lymphatic vessels, readily acessible to the pleural space (Wang, 1975), it is reasonable to expect that lymphatic drainage would also serve, in conjuction with the visceral pleural interstitium, to accommodate the saline infusate. The relative contribution of these two mechanisms, however, remains uncertain.

It has been reported that localized infusion of saline into the pleural cavity was accompanied by rapid redistribution of fluid throughout the pleural space (Miserocchi et al., 1983). That mean thickness of the interstitial compartment of the visceral pleura was increased in the present study is consistent with dispersion of fluid throughout this interstitial space after filtration across the pleural mesothelium. Thus, the recorded increases of pleural fluid hydrostatic pressures and the morphometric determinations of fluid accumulation in the pleural interstitium imply that interstitial fluid pressures were also increased. The reported similarity of interstitial and pleural fluid pressures under normal physiologic conditions (Kim et al., 1979) complies with this conclusion.

Previous studies from this laboratory (DeFouw and Chinard, 1983; DeFouw et al., 1985), which contributed to the hypothesis that increased interstitial fluid pressures after septal edema and alveolar flooding served to stimulate alveolar capillary endothelial vesicle formation were deficient in measurements of alveolar septal interstitial fluid pressures. Since technical difficulties preclude measurements of alveolar interstitial pressures, accessibility of the pleural space provided a more feasible means of evaluating effects of interstitial fluid pressures on pulmonary capillary endothelium. Further, it is well established that subpleural capillaries in the rat and other species with a thin visceral pleura arise from the pulmonary circulation (McLaughlin et al., 1961). The present results provide support to the original hypothesis, however,

increased vesiculation was not observed uniformly along the circumference of the pleural capillary endothelium. Portions of the endothelium directly apposed to the pleural interstitium (thick sides of capillaries) responded to increased interstitial fluid pressures with increased segments of cellular attenuation and de novo vesicle formation. This response might represent a cellular defense mechanism against increased interstitial pressures whereby membrane deformation serves to prevent cellular disruption. Since cellular attenuation and potential fusion of increased numbers of vesicles could effectively reduce the diffusion distance from the interstitium to the capillary lumens, the subpleural capillaries might also contribute to removal of excess infusate from the interstitium. Further, this dual response of increased vesiculation and attenuation might serve to represent the fluidity of cell membranes such that attenuated endothelial segments might arise by membrane translocation from those regions destined to become attenuated to the nonattenuated regions for incorporation into the expanded vesicular population. Additional evidence is needed to clarify these conformational changes of the endothelial plasmalemma.

In contrast to the thick sides of the capillaries, those portions of the endothelium that face the alveoli (thin sides of the capillaries) were not affected by the increased interstitial fluid pressures. In edematous lungs the thin sides of the alveolar capillaries were characterized by increased vesiculation (DeFouw and Chinard, 1983), however, alveolar flooding, and presumed elevations in alveolar pressures, were also present. Random microscopic samples from the lungs of the saline infused rats failed to detect septal edema or alveolar flooding, therefore, the thin sides of the subpleural capillaries were likely not directly exposed to elevated interstitial fluid pressures. It should be pointed out that the fraction of the endothelium comprised of attenuated cytoplasmic segments (20-30nm maximum thickness) is consistently greater on the capillaries' thin sides in the normal and the two experimental groups. Since endothelial attenuation is believed to be unique to pulmonary microvessels (Gil, 1982), its predominance on the capillaries' thin sides could contribute to the conditions which facilitate gasseous exchange from the alveoli across the capillaries' thin sides(Simionescu and Simionescu, 1983).

Whether plasmalemmal vesicles provide a mechanism of transendothelial transport (Bruns and Palade, 1968; Simionescu et al., 1978) or represent static elements in a branching system of membrane invaginations (Bundgaard et al., 1983) remains uncertain. Thus, the appearance of increased numbers of plasmalemmal vesicles in response to elevated ambient interstitial fluid pressures might serve to either accentuate a transcellular transport system or to expand a sessile network of membrane invaginations. Since plasmalemmal vesicle number in pulmonary capillary endothelium was also reported to increase in response to ethchlorvynol (Fischer et al., 1977) and to platelet activating factor (Lewis et al., 1983), additional studies are needed to evaluate this issue.

Vesicle numerical densities obtained from rapidly frozen tissues (Mazzone and Kornblau, 1981; Wagner and Andrews, 1985) were considerably less than those obtained from glutaraldehyde-fixed tissues. Therefore, chemical fixation could serve to artifactually increase vesicle numerical densities in the subpleural capillaries. The normal, sham operated controls, and saline infused animals were, however, uniformily fixed with buffered glutaraldehyde and evidence for increased vesiculation was presented only in the latter group. Since the subpleural capillaries would be readily preserved in frozen lung samples, further investigations utilizing frozen tissue would provide an interesting corollary to the present study.

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