Histol Histopathol (2006) 21: 1309-1319

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Morphogenesis of rat experimental pulmonary emphysema induced by intratracheally administered papain: changes in elastic fibres

L.M. Pastor[1], F. Sánchez-Gascón[2], J.C. Girona[2], C.M. Bernal-Mañas[1], E. Morales[1], E. Beltrán-Frutos[1] and M. Canteras[3]

[1]Department of Cellular Biology, Aging Institute, Medical School, University of Murcia, Spain, [2]Department of Internal Medicine, Medical School, University of Murcia, Spain and [3]Department of Statistic, Medical School, University of Murcia, Spain

Summary. The ultrastructural changes of elastic fibres in emphysematous lungs have been studied in men, but few works exist on this topic in experimental emphysematous animals. In this paper, the morphogenesis of emphysema and alterations of the elastic fibres produced by the instillation of papain are described by light and electron microscopy. Wistar rats were instilled through the trachea with papain at a rate of 3 mg/100 g animal weight. The animals were sacrificed 12 h, 3 days, 10 days and 60 days after enzyme instillation. The "Mean Linear Intercept" (MLI), the "Number of fenestrations/respiratory units" (NF) the "Number of macrophages per mm of alveolar wall" (NM) and the "Number of respiratory unit/mm^2" (RU), both in the control and experimental groups were studied. Two months after treatment, the experimental group showed a strong increase in the MLI ($p<0.001$) and NF ($p<0.001$), and a diminished number of RU ($p<0.05$) compared with the control group. Partial correlation analysis showed a positive correlation only between MLI and NF. Twelve hours after papain instillation an inflammatory response was observed, the elastic fibres were ruptured, while the microfibrilar component remained. New formations of eulanin elastic fibres were observed three days post papain instillation. After ten days the interalveolar oedema had disappeared and the elastic fibres were of normal morphology although irregular groups of strips of elastic fibres were evident. A mixed pattern of panlobular, centrilobular and normal lung zones were observed. Two months after papain instillation abundant accumulations of elastic fibres of irregular outline were observed associated to collagen fibres. In conclusion, the morphometric parameters studied showed a significant progression of the emphysema. The strong correlation between NF and MLI suggested that papain-induced emphysema is principally caused by breaches of the alveolar walls. The results seem to point to a very abnormal remodelling process associated with elastic fibre regeneration, although there were no signs of destruction of these new fibres formed in emphysematous rat lung induced by papain.

Key words: Papain, Emphysema, Elastic fibres, Ultrastructure

Offprint requests to: Prof. Dr. Luis Miguel Pastor García, Department of Cell Biology, Medical School, University of Murcia, 30100 Espinardo, Murcia, Spain. e-mail: bioetica@um.es

Introduction

Since 1965 the study of pulmonary emphysema has made much progress following the instillation of elastolytic enzymes in animal models (Gross et al., 1965). Subsequent studies, first with proteolytic papain enzyme and then with elastase, produced emphysema in animals three weeks after instillation and suggested that the disease could be a consequence of the destruction and abnormal reparation of the elastic fibres (EF) by the proteolytic enzymes that had been used (Johanson et al., 1973; Kuhn et al., 1976).

Before this, several studies had suggested that a lack of balance between proteolytic enzymes and elastolytic inhibitors inside the lung was the probable cause of disease (Laurell and Eriksson, 1963; Gadek et al., 1980). However, apart from the results of the first morphological studies, little attention was paid for many years to the morphogenic mechanisms of this disease either in man or in experimental models. The few data known had been obtained mainly in experimental emphysema produced by elastase (Kuhn et al., 1976), and it seemed that panlobular and centrilobular emphysema were the result of two different morphogenic processes: the former due to enlargement of the alveolar ducts and the latter to destruction of the alveolar septa. In this way, panlobular emphysema would be produced by a distensive mechanism, as happens in the congenital lobar emphysema, with few

fenestrations and with a gradual diminution in the thickness of the alveolar septa. In centrilobular emphysema, the alteration would be produced by the development of fenestrations in the alveolar walls (Kuhn et al., 1976). In elastase-induced emphysema in rat, the production mechanism could be the same as in human panlobular emphysema, namely, enlargement of the air spaces. In the case of papain-induced emphysema, little is known about the morphogenic production mechanism. Parra et al. (1980) asserted that the morphogenic mechanism produced by the papain seemed to differ from the elastase-induced mechanism observed by Kuhn et al. (1976). The literature that exists concerning the precise structural alteration in the EF that appear in the emphysematous human lung is scant, as is the literature on the alteration and possible reparation that occurs in the lung fibres of animals in which emphysema has been produced experimentally. This is surprising, taking into account that morphological and biochemical studies strongly suggested that lesion of the elastic fibre could be the main cause of the formation of the emphysema. Since the papers by Kuhn et al. (1976) and Kobrle et al. (1982), who described the experimental models produced by elastase and papain, the idea that the emphysema established in the lung as a result of the destruction and subsequent repair of the EF, which would have non-functional characteristics, has gained support. However, this has not been thoroughly checked from a morphological point of view. In recent years, diverse studies suggest that an aberrant complex remodelling process, which may or may not be initiated by elastin degradation, is involved in emphysema pathogenesis. The inflammatory-repair hypothesis, which is based on the tissue rebuilding due to local collagen (seldom elastin fibres) focal accumulation (Sulkowska and Sulkowski, 1997), or biomechanical stress versus proteinase imbalance (Stehbens, 2000), would modify the theory of the elastase/anti-elastase hypothesis pathogenesis of emphysema, which considers that elastin degradation by the unopposed action of elastase underlies alveolar destruction (Snider et al., 1986).

In this paper, we study the morphogenic mechanisms and histological alterations of the EF in emphysematous lung of rat produced by papain since, to date, no results fully explain a) the type of morphogenesis that forms the emphysema after repair (at 60 days), (Valentine et al., 1983) and b) whether histological alterations of the EF and collagen exist in this emphysema that can be compared with the more recent data suggesting that remodelation of the fibres may be an important factor involved in the development of emphysema.

Materials and methods

Induction of emphysema

Emphysema was induced in female Wistar rats (N=80) weighing 200-250g by the intratracheal administration of papain with an activity of 1:350 (egg albumin) and 12000 U/g (gelatine); each animal was given a dose of 3mg/100 g of body weight, and the solution was prepared in 1 ml of 0.9% (w/v) NaCl immediately before administration. The animals were anaesthetized by inhaling ethylic ether and then divided into the following experimental groups: Group 1 (n=10). Rats in which no experimental procedure was realized, and sacrificed after 60 days. Group 2 (n=10). Rats given 1 ml of 0.9 % (w/v) NaCl intratracheally and sacrificed 12 hours later. Group 3 (n=10). Rats given 1 ml of 0.9 % (w/v) NaCl intratracheally and sacrificed 3 days later. Group 4 (n=10). Rats given 1 ml papain solution intratracheally and sacrificed 12 hours later. Group 5 (n=10). Rats given 1 ml papain solution intratracheally and sacrificed 3 days later. Group 6 (n=10). Rats given 1 ml papain solution intratracheally and sacrificed 10 days later. Group 7 (n=20). Rats given 1 ml papain solution intratracheally and sacrificed 60 days later.

Before sacrifice, the animals were anaesthetized with sodium pentobarbital with a dose of between 30-50 mg/kg animal weight.

Fixation and tissue preparation

After being anesthetized, 60% of the lungs in each group were fixed by instillation and the remaining 40% by perfusion. In the case of instillation, a tracheotomy was performed and the lungs were connected to a reservoir and instilled with a 2.5% glutaraldehyde solution with 0.1 M sodium cacodylate buffer pH 7.2-7.3, at a pressure of 20 cm H_2O. Before instillation, the abdominal aorta was cut in order to allow the blood to exit from the lungs. In the case of perfusion fixation, a 0.5% solution of 0.1 M tannic acid in 0.5% buffered sodium cacodylate was administered with 2.5% glutaraldehyde to help identify the amorphous substance of the EF by transmission electron microscopy (Cotta-Pereira et al., 1976). For this, a fine catheter was inserted in the pulmonary artery after sectioning the left atrium to check that the fixation fluids had been expelled. Later, the different lobules were sectioned and samples were obtained from the hiliar, media and subpleural regions in both cases.

For light microscopy the samples were postfixed in 10% formol for a week before introducing the tissue samples in an automatic Shandon® processor and embedding in paraffin. Then 5 µm sections were cut with a Leitz 1512® microtome and stained with hematoxylin-eosin. The sections were observed in a Leitz-Dialux® photomicroscope, using both photonic and fluorescent light (to identify the EF by autofluorescence, Carvalho and Taboga, 1996; Borges et al., 2005).

Samples for transmission electron microscopy (TEM) of $1mm^3$ were cut and fixed in 2.5 % glutaraldehyde in 0.1 M buffered cacodylate or 0.5% solution of 0.1 M tannic acid 0.5% buffered sodium cacodylate with 2.5% glutaraldehyde, postfixed in 1%

osmium tetroxide, dehydrated in acetone and embedded in Epon 812. Semithin sections were obtained and stained with toluidine blue. Five ultrathin sections per animal of alveolar ducts and alveoli portions were cut using a Reichert-Imy Ultracut ultramicrotome and stained with uranyl acetate and lead citrate. Electron microscopy was performed with a Zeiss EM 10C® apparatus. Samples for Scanning Electron Microscopy (SEM) were dehydrated in acetone, critical point dried (CPDO2 Balzers Union), gold sputtered BIO-RAD POLARON DIVISION (200 A)® and observed in a Jeol-JSM T300 microscope.

Morphometric analysis

Several morphometric parameters are used to evaluate the extent and evolution of emphysema (Thurlbeck, 1967). In this study, groups 1, 5, 6 and 7 were analysed morphometrically. In the group sacrificed after 12 hours it was impossible to obtain any measurements because of the initial lung damage. To calculate the different parameters, a Leitz Dialux® microscope with a x 40 lens was used.

The following parameters were analysed:

Number of respiratory units per mm^2 (RU). Two sections of each animal (3 animals per group) were taken and in each section we counted the number of RU included in an area of 0.0273 mm^2. 100 areas were measured per section. The results were later transformed to RU/ mm^2. The alveoli and alveolar ducts were considered as a respiratory unit, and those completely included in the area and also those which were only partially contained in it were counted as whole units.

Mean Linear Intercept (MLI). The number of interalveolar walls that were intersected were counted in two sections from each lung (3 animals per group), by a straight line segment with a total length of 7.6 mm. 50 random fields were used for the MLI measurement, avoiding areas containing airways and large vessels. The quotient between the total length and the number of intersections gave the MLI.

Number of macrophages per mm in an alveolar wall (NM). We counted the number of macrophages per respiratory unit, considering respiratory units with no fracture as circumferences. Two sections per animal (two animals per group) were examined. Later we calculated the length of a hypothetical alveolar circumference with the data obtained from the MLI corresponding to each animal, according to the formula $L=2\pi R$ (where L is the length of the circumference and R is the radius). The data obtained are the parameter expressed as the number of macrophages per mm of alveolar wall.

Number of fenestrations or discontinuities per respiratory unit (NF). Fenestration or discontinuities in alveolar wall (zones with broken alveolar wall) were counted. We considered the fenestration which appeared between the alveolar ducts as one discontinuity, and two discontinuities when the fenestrations appeared between two alveoli. Two sections in each animal (two animals per group) were examined, counting 500 respiratory units. The parameter was expressed as discontinuities per respiratory unit.

Statistical analysis

The means for each observation (in the case of RU, NM, MLI and NF) with respect to the time elapsed since emphysema induction were analysed by a one way ANOVA. This analysis was completed with comparisons between pairs of averages by means of a statistical distribution according to Student's t test. The data were conveniently transformed as follows: square root was applied to the MLI, RU and NM parameters, and log (1 + X) was applied to the number of discontinuities (NF) per respiratory unit.

The parameters were analysed with a simple linear correlation. To analyse the dependence between two parameters, neutralizing the dependence of a third parameter, a partial correlation analysis was carried out in the case of MLI, RU and NF. In all these analyses we used the overall means of the observations obtained in each animal.

Results

Light microscopy

The lungs of the animals designated as controls showed the normal appearance of the species, with thin pleura, no interlobular septa and no inflammatory infiltration (Fig. 1a). In the perfused rats, the alveoli were collapsed and the spaces observed were the alveolar ducts, while the capillaries were dilated and the airways presented a lower diameter. With fluorescent light the EF were observed as smooth strips on the walls of the alveolar ducts (Fig. 1b).

Twelve hours after papain instillation, the lungs appeared oedematous with focal subpleural and intraparenchymal haemorrhages. In addition, we observed peribronchial and perivascular interstitial oedemas, with polymorphonuclear leucocytes, lymphocytes and macrophages. The lymphatic vessels were dilated with lymphocytes in their interior. The septum architecture was preserved and there were laminar bodies in the alveoli. Macrophages with phagocytised erythrocyte residues were evident in the alveolar lumen and interstitial enlargement resulted from the oedema (Fig. 2a). Fluorescence pointed to a reduced intensity in the septa and in the alveolar walls, suggesting destruction of the elastic fibres (Fig. 2b). Three days after instillation, the haemorrhage and the interstitial oedema had diminished, alveolar macrophages were present and the alveolar walls showed more fractures, with dilation of initial portions of the alveolar ducts and also cellularity had increased in the alveolar interstitium. Centrilobular emphysema was already established.

At ten days, no oedema was observed in the

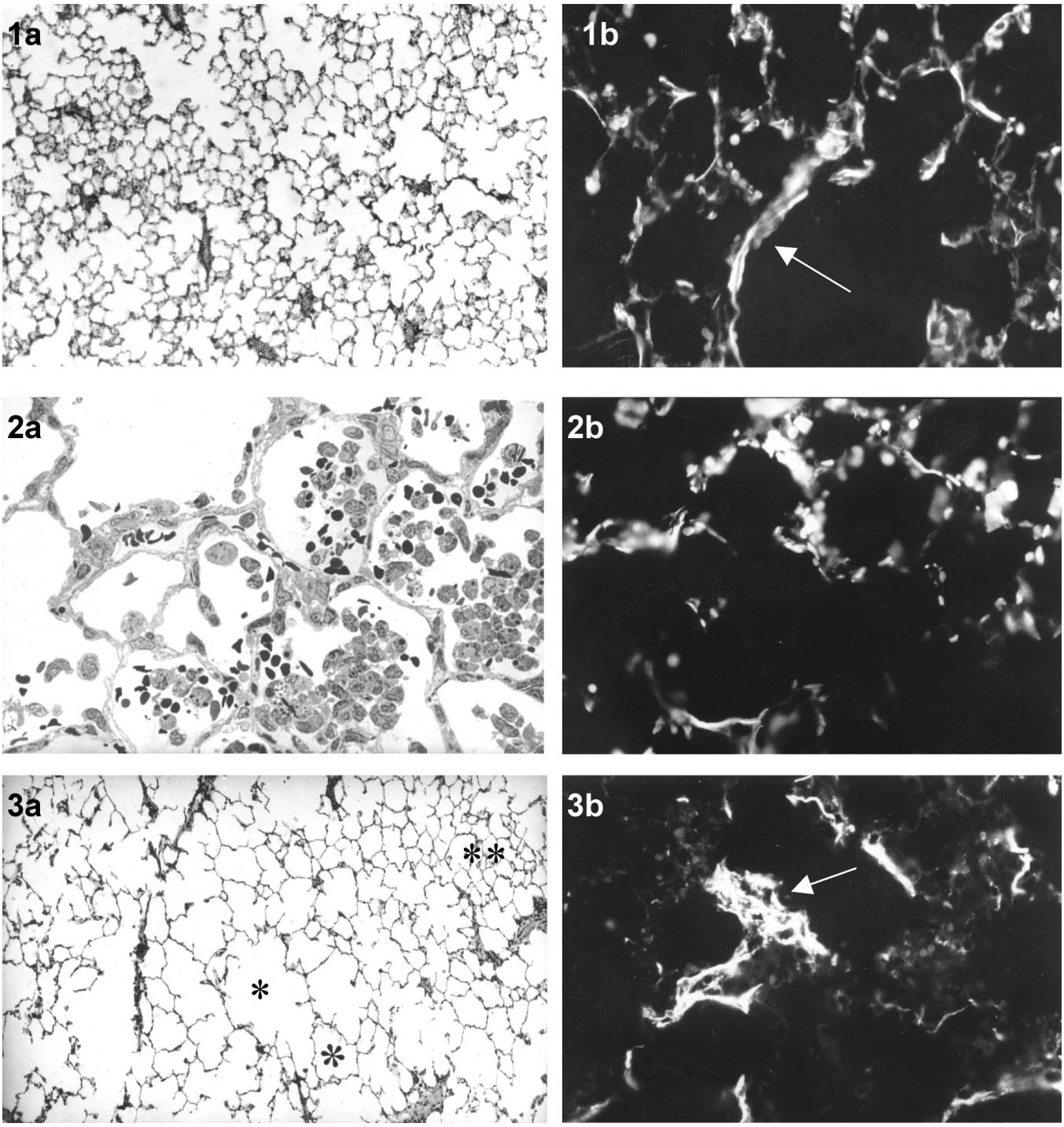

Fig. 1.a. Normal lung parenchyma in which several alveolar ducts together with alveoli groups can be seen. H&E, x 63. **b.** Clumps of EF and smooth strips (arrow) in the alveolar duct portion next to the terminal bronchioles. Fluorescence microscopy, H&E, x 400

Fig. 2.a. Thickened alveolar walls probably due to the interstitial oedema, twelve hours after papain instillation. Toluidine blue, x 400. **b.** Normal areas together with other zones from where EF have disappeared in the alveolar sacs. Fluorescence microscopy, H&E, x 400

Fig. 3.a. Two months after administering papain, areas with alveolar ducts and dilated alveoli (asterisk) alternate, together with others with less affected alveoli (double asterisk). H&E, x 63. **b.** Large and irregular accumulations of EF (arrow), two months after administering papain. Fluorescence microscopy, H&E, x 400

interstitial inflammatory infiltrate or in the interior of the alveoli, while the size of the alveoli and also of the fenestrations had increased. A mixed pattern of panlobular, centrilobular and normal zones was observed. Irregular groups of strips of EF were evident.

Two months after instillation, panlobular emphysema had spread over the whole lung (Fig. 3a). The diameter of the alveoli and the alveolar ducts were greater than in the control animals (Fig. 1a). Fenestrations had increased in size and number in the alveolar walls. The EF formed large irregular accumulations in the alveolar ducts, which were dilated (Fig. 3b).

Morphometric analysis

Table I summarizes the results obtained. The NF was greater both 10 days and 60 days after papain instillation than in the control group and was also greater at 60 days than at 3 days after instillation ($p<0.025$). The RU significantly ($p<0.001$) decreased as time elapsed in all groups with respect to the control, and also between the 3 and 10 day groups on one hand and the 60 day group on the other ($p<0.001$). The MLI increased as time elapsed and was significantly greater than in the control group ($p<0.001$) and also in the 3 day group compared with the 10 day group ($p<0.005$) and the 60 days group ($p<0.001$). There was also a significant difference between the 10 and the 60 day groups ($p<0.001$). The NM was greater in the 3 and 10 day groups than in the control group ($p<0.01$), but had returned to normal values 60 days after instillation. The fall in the NM was statistically significant between days 3 and 60 ($p<0.01$), and between days 10 and 60 ($p<0.02$). No correlation was observed with NM with the other parameters. The NF, on the other hand, was negatively correlated with the RU ($p<0.01$; $r= 0.603$ and 74% of dependence) and positively correlated with the MLI ($p<0.001$; $r=0.9500$, 90.3% of dependence). The MLI was also negatively correlated with the RU with $p<0.005$, $r=0.8784$ and 77.2% of dependence.

To determine the influence that NF and RU had on the MLI, the significance was studied when one of these variables was kept constant. No correlation was observed between RU and MLI when the NF was constant; but a correlation was observed between NF and MLI, when the RU was constant ($p<0.05$, $r=0.7976$).

Transmission electron microscopy

In the control animals, the alveolar epithelium was formed by type I, II and III pneumocytes. Collagen and EF were seen in the pulmonary interstitium, the latter showing a regular pattern in the cross-section, with a smooth boundary and scarce vacuolated spaces in their interior (Fig. 4a).

Twelve hours after papain instillation, the alveoli showed an exudate with infiltrations of neutrophilic leucocytes, erythrocytes and macrophages, the last being particularly numerous (Fig. 4b). Type I pneumocytes were unchanged except for the presence of a double membrane structure in their cytoplasm. In the pulmonary interstitium, there were many neutrophils with abundant lysosomes in their cytoplasm. The collagen fibres showed a normal ultrastructure. In the EF, electrodense amorphous substance was ruptured (Fig. 5a) and the amorphous substance tended to disappear, while the microfibrilar component remained. New formations of eulanin EF, together with digested EF and normal collagen fibres, were observed three days post papain instillation (Figs. 5b, 6a).

Ten days after papain instillation, the interalveolar oedema had disappeared and normal type I and II pneumocytes were observed. The collagen fibres were

Table 1. Values of morphometric analysis in control group and 3, 10 and 60 days post intratracheal administration of papain in Wistar rats.

Parameters studied	RU	MLI	NM	NF
Control animals	260.19±23	0.030±0.0016	1.82±0.36	0.333±0.060
3 days	172.27±17[1,2]	0.038±0.004[3,4,5]	10.16±1.31[7,8]	0.747±0.141[12]
10 days	158.23±16[1,2]	0.042±0.003[3,6]	7.79±1.46[7,9]	1.585±0.330[10]
60 days	113.43±13[1]	0.059±0.033[3]	1.64±0.22	1.826±0.358[11]

Values represent means ± SEM. Abbreviations: RU, Number of respiratory units per mm^2; MLI, Median linear intercept; NM, Number of macrophages per mm in an alveolar wall; NF, Number of fenestrations or discontinuities per respiratory unit; 1 Significant differences in the number of respiratory units per mm^2 between the different groups and the control group ($p<0.001$), 2 Significant differences in the number of respiratory units per mm^2 between the different groups and the 60 days group ($p<0.001$), 3 Significant differences in median linear intercept between the different groups and the control group ($p<0.001$), 4 Significant differences in median linear intercept between the 3 days group and the 10 days group ($p<0.005$), 5 Significant differences in median linear intercept between the 3 days group and the 60 days group ($p<0.001$), 6 Significant differences in median linear intercept between the 10 days group and the 60 days group ($p<0.001$), 7 Significant differences in the number of macrophages per mm in an alveolar wall between the 3 and 10 days groups and the control group ($p<0.01$), 8 Significant differences in the number of macrophages per mm in an alveolar wall between the 3 days group and the 60 days group ($p<0.01$), 9 Significant differences in the number of macrophages per mm in an alveolar wall between the 10 days group and the 60 days group ($p<0.02$), 10 Significant differences in the number of fenestrations or discontinuities per respiratory unit between the 10 days group and the control group ($p<0.002$), 11 Significant differences in the number of fenestrations or discontinuities per respiratory unit between the 60 days groups and the control group ($p<0.001$), 12 Significant differences in the number of fenestrations or discontinuities per respiratory unit between the 3 days group and the 60 days group. ($p<0.025$).

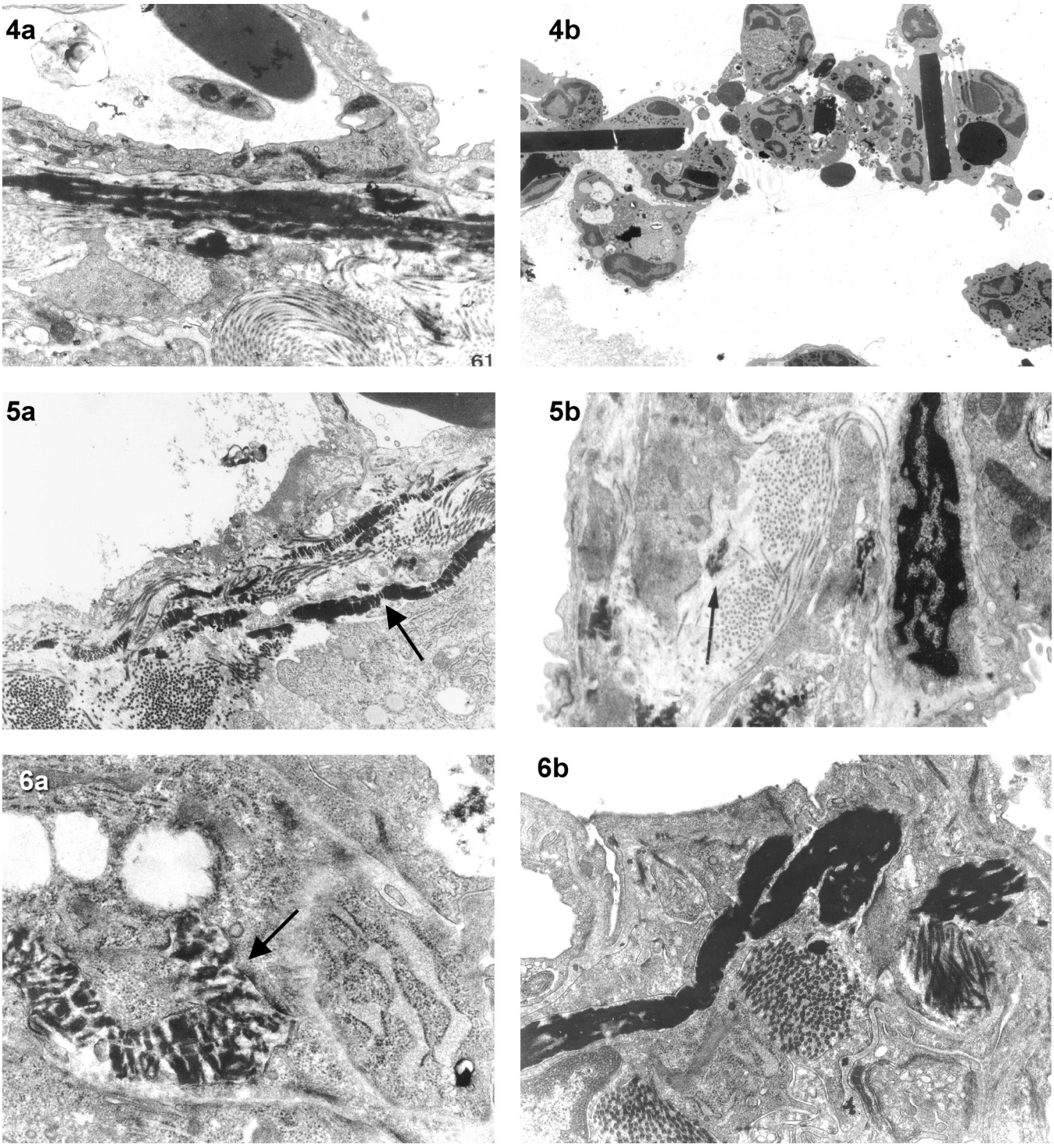

Fig. 4.a. In control animals, some EF surrounded by different collagen fibres were observed. TEM, x 18,200. **b.** At 12 hours, the PMN had phagocytised the haemoglobin crystal, and cellular detritus in the intraalveolar space was observed. TEM, x 3,300

Fig. 5.a. At 12 hours the EF fibres stained with tannic acid present a contrasted amorphous substance showing a strong degree of disintegration (arrow). Tannic acid. TEM, x 13,000. **b.** 3 days after instillation papain, elaunin fibres (arrow) appear near a fibroblast, indicating elastin neosynthesis. Tannic acid. TEM, x 10,200

Fig. 6.a. At 3 days EF with electrodense amorphous substance in destruction process (arrow). Tannic acid. TEM, x 30,200 **b.** EF at 10 days. The fibres present smooth outline and normal aspect. Tannic acid. TEM, x 23,100

normal. The matured EF appeared with normal morphology (Fig. 6b). Numerous fenestrations were present (Fig. 7), and the thinness of the alveolar walls was evident (Fig. 8).

Two months after papain instillation, the alveolar epithelium was apparently normal but had abundant fenestrations and zones with a thin alveolar wall. No elaunin EF were observed. With tannic acid, the EF showed high electrodensity, a small diameter and regular morphology, and were short; we also observed abundant accumulations of EF associated with abundant collagen fibres, principally in the wall alveolar ducts (Fig. 9). In these, the EF showed an irregular boundary in longitudinal sections (Fig. 10). Neither interstitial nor intralveolar signs of an inflammatory process could be observed. Only alveolar macrophages without neutrophils were observed in the alveoli.

Scanning electron microscopy

In the control animals the alveoli showed a rounded polyhedral morphology (Fig. 11) with a smooth inner surface with some protuberances (blood vessels) and, infrequently, small Kohn pores joining neighbouring alveoli (Fig. 12).

Twelve hours after administering the enzyme, fracture of the alveolar walls was observed and erythrocyte and fibrin nets appeared in many alveoli

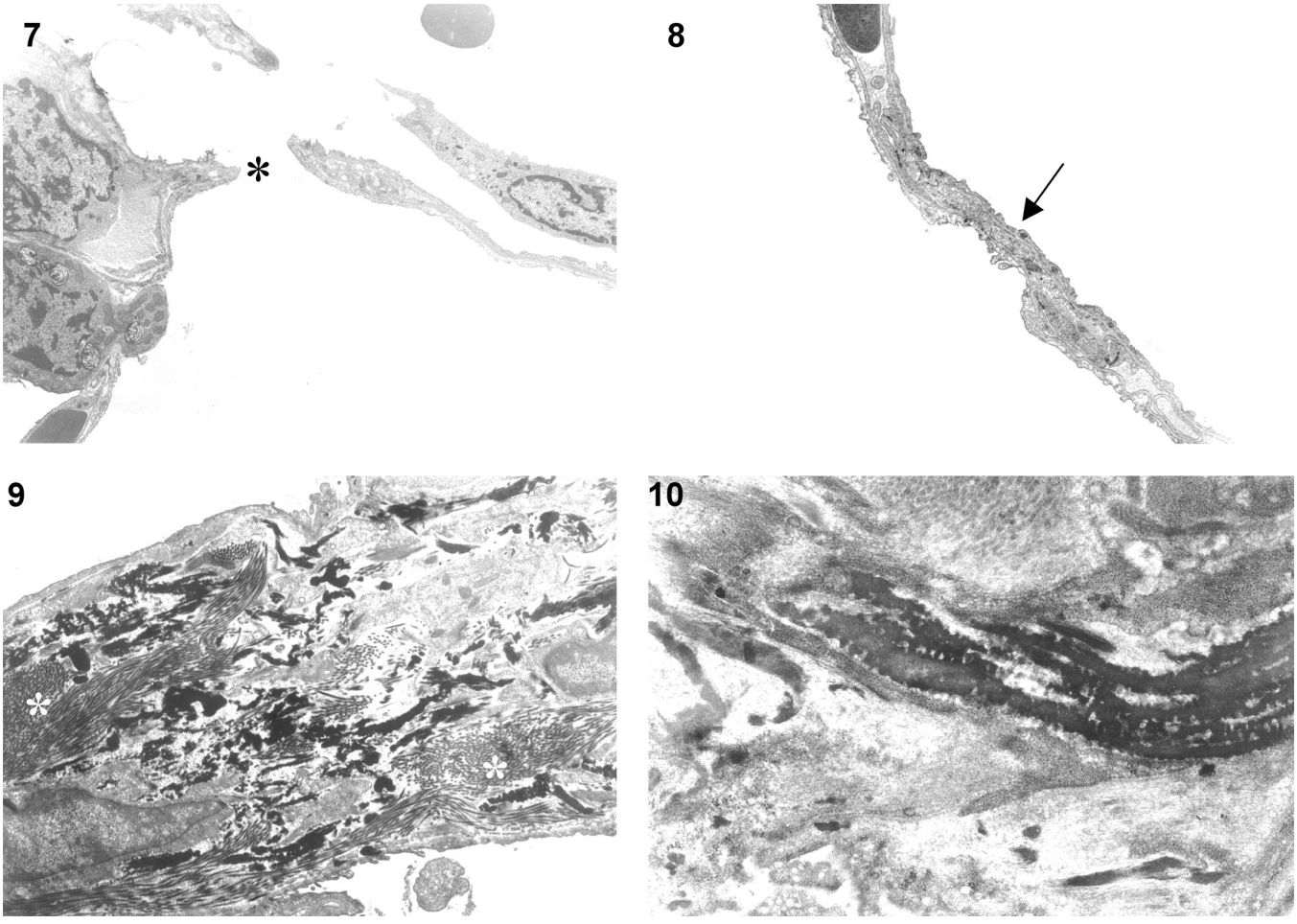

Fig. 7. At 10 days after papain instillation, Kohn pores are observed (asterisk). TEM, x 5,100

Fig. 8. Detail of the alveolar wall, which is very thin (arrow). TEM, x 8,900

Fig. 9. Accumulations of EF of varying size sometimes intermingle with high collagen fibre concentrations (asterisk) in lungs two months after papain instillation. Tannic acid. TEM, x 8,200

Fig. 10. EF with irregular boundary two months after papain instillation. TEM, x 23,000.

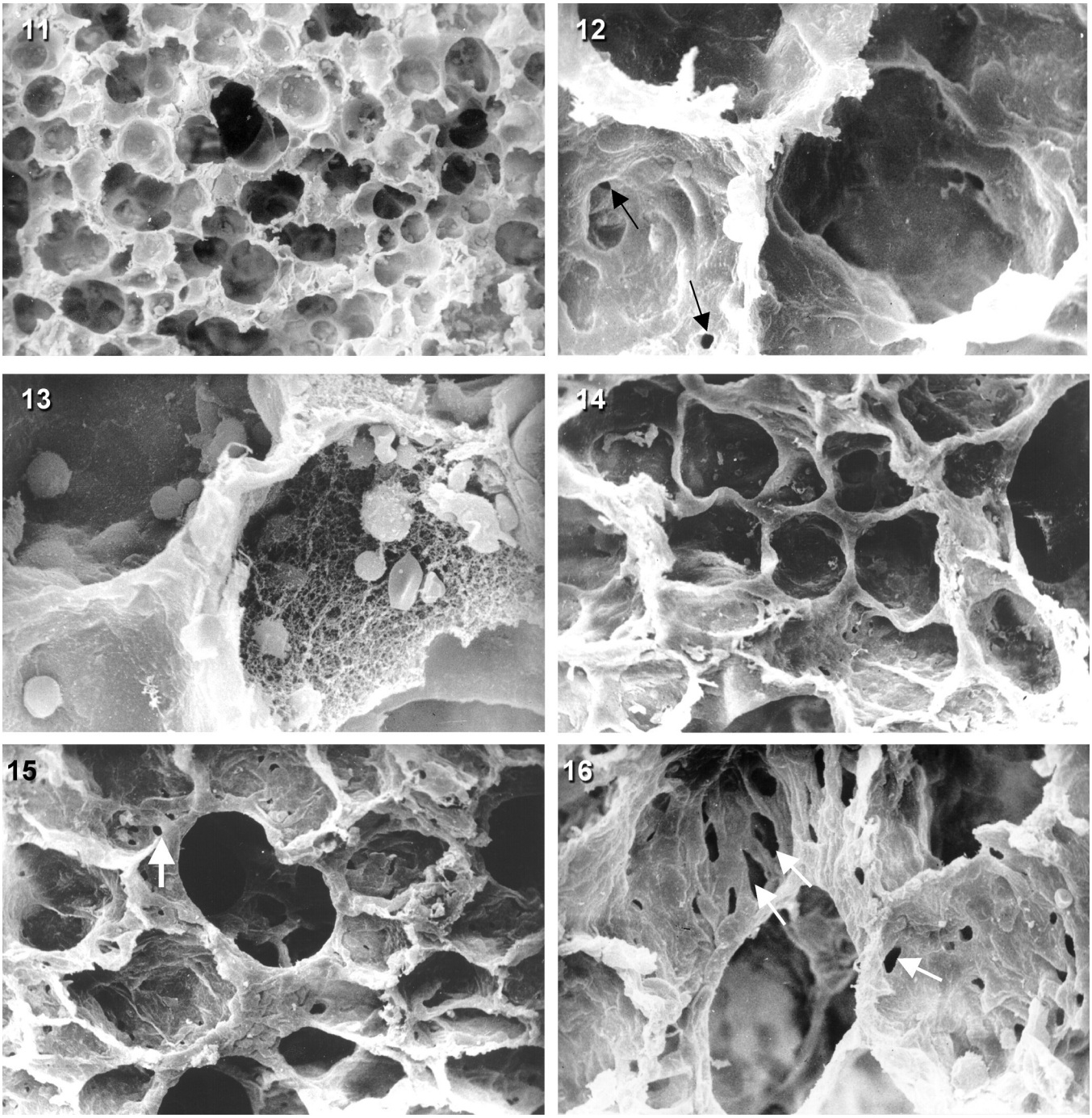

Fig. 11. Normal alveolar structure of control animals. The more or less spherical morphology can be observed. SEM, x 250

Fig. 12. Lung alveoli. The surface appears wrinkled and some Kohn pores (arrows) are observed in control animals. SEM, x 2,000

Fig. 13. Erythrocytes and phagocytes joined to the intraalveolar exudate can be observed twelve hours after papain instillation. SEM, x 2,500

Fig. 14. At 3 days after the administration of papain the alveolar ducts are dilated. SEM, x 250

Fig. 15. Two months after papain instillation, several fenestrations (arrow) can be distinguished in the alveoli. SEM, x 650

Fig. 16. Several fenestrations can be seen in the same alveolar wall (arrows), two months after papain instillation. SEM, x 1,500

(Fig. 13). Three days post papain instillation dilated alveolar ducts and thickening of the initial portion of the walls (Fig. 14) were observed.

Two months after instillation, the following signs of emphysema could be observed: a) many fenestrations of variable diameter (Fig. 15); b) the disappearance of alveolar walls as a result of the fenestrations coalescing (Fig. 16).

Discussion

The histological alterations related with the inflammatory response to the administration of papain observed in our study can be summarized in the following way: a) strong alveolar and interstitial oedema without destruction of the epithelial pneumocytes after twelve hours, b) no destruction of collagen fibres by papain, c) the presence of numerous erythrocytes and fibrin clumps, and d) infiltration of interalveolar neutrophils and macrophages. In a second phase, there was a sharp increase in the number of macrophages and an apparent decrease in the number of neutrophils after 3 days, although the inflammatory response remitted after 10 days.

Studies of the first few hours after papain instillation are scarce (Johanson et al., 1971; Parra et al., 1980), but are abundant after elastase administration (Morris et al., 1981; Busch et al., 1984; Snider et al., 1986). To date, it has been accepted that some parallelism exists between both inflammatory responses. Our study further correlates both responses because, previously, erythrocytes with crystalline forms and the presence of structures with double membrane in the pneumocytes I had only been described in animals treated with elastase (Morris et al., 1981, 1986). In short, after enzyme administration an acute inflammation is produced in the lung and gradually remits. This inflammation, as with many others that occur in the lung, is accompanied by both intralveolar and interstitial oedematous changes and the presence first of a strong infiltrate of polymorphonuclear neutrophils, which are later substituted by alveolar macrophages (Hayes et al., 1975; Parra et al., 1980; Bowden, 1984).

The emphysema produced by the papain develops progressively; during the first few days, a centrilobular emphysema is localized in the initial portions of the alveolar duct, associated, to a certain extent, with remodelling of the walls and disappearance of the alveolar septa and the alveoli. This is followed by the simultaneous enlargement of the middle and final portions of the alveolar duct. The diameters of the alveolar sacs increase, and this increase is associated with the formation of numerous discontinuities between the alveolar walls. In the animals sacrificed two months after instillation, scanning and transmission electron microscopy show that there are numerous fenestrations in the alveolar sacs. These qualitative results are confirmed by morphometric results, which indicate that as early as the third day after papain instillation the RU is altered, as is the MLI (95% greater after two months that in the control animals); later, too, the RU is altered (57% decrease at two months). Of note is the fact that the MLI is more strongly correlated with the NF than with the RU.

The morphogenesis of the emphysema produced by papain has been scarcely studied. Many authors think that such emphysema is initially of a centriacinar and then of panacinar (Gross et al., 1965) or simple panacinar (Chyczewski and Sulkowski, 1988) type. On the other hand, for Johanson et al. (1971), there is only the centriacinar type and no histopathological progression after the fourth week post papain injection.

As regards the histological mechanisms of papain emphysema formation, only some isolated data in the literature can be found. Parra et al. (1980) observed both alveolar fenestrae and dilated air spaces with tissue remodeling, and considered that both histological alterations are caused by the same morphogenesis process. For Caldwell (1971), the principal effect of the alveolar instillation of papain is the rupture of alveolar walls, resulting in damage of the alveolar parenchyma. Our study corroborates these isolated results and shows for the first time the morphogenetic mechanism of rat papain emphysema. In accordance with our results, the first pathological alteration could be the centriacinar emphysema that develops a few days after papain instillation and which remains. At the same time, discontinuities and fenestrations are gradually formed with dilatation of the distal air spaces. A panacinar emphysema is established, together with zones of normal alveolar parenchyma. Since the emphysematous lesion increases with papain enzyme concentration (Hayes et al., 1975) and since our study used a similar concentration to Parra et al. (1980), it is probable that the results of Johanson et al. (1971) were caused by the low papain concentration used, which only damaged the initial portion of the alveolar duct, with no subsequent evolution. This would explain why our MLI at two months was higher (95%) than that observed by Johanson et al. (1971) (28%). In our model of emphysema, the MLI is strongly correlated with NF. This result supports the qualitative observations, whereby the increased size of air spaces is mainly caused by discontinuities and fenestrations and not by dilatation of the air spaces.

The several re-synthesis stages of EF observed in our study are similar to those observed by other authors in emphysema produced by elastase (Khun et al., 1976), the papain model not having been previously described. As in the elastase model, new elaunin fibres are formed, which become more mature EF with a greater diameter and electrodense amorphous substance, as can be seen from our study at 10 days. This seems to be the same as occurs in the elastase model, in which newly formed fibres can be seen at 8 days (Morris et al., 1981). As already mentioned, several authors have suggested that emphysema progresses in both experimental models and in man through a morphological alteration of the EF,

although the elastine content of emphysematous lungs may be normal (Fonzi and Lungarella, 1980; Valentine et al., 1983). There are two theories that relate EF damage and the formation of a given type of emphysema. Some authors suggest that panlobular-type lesions of the EF in emphysematous lungs due to a deficit of α-1-antitrypsin consist of digested abnormal fibres, as observed by Fukada et al. (1989) in man. For this author, this type of damaged fibre is directly related with human panlobular emphysema. Other authors maintain that, although the beginning of emphysema results from digestion of the EF, the pathology is produced by EF re-synthesis in an abnormal morphological organization with abnormal functional properties. This has been demonstrated in elastase-treated animals, which developed panlobular emphysema (Mercer and Crapo, 1992). In these lungs, the re-synthesised EF show a defect, mainly the presence of discontinuities in the EF surrounding the extremes of the alveolar septa. In humans, too, Fukada et al. (1989) found types of abnormal EF in centrilobular emphysema, which were probably related with remodelling of the alveolar ducts. For Fukada et al. (1989), centrilobular emphysema in human is not only related with EF damage but also with the neo-formation of EF and processes of fibrosis in the remodelled zones. In the case of papain-produced emphysema, the data referring to EF in emphysematous lungs are scarce, although for Johanson et al. (1971) the EF are normal after the repair process, while Kobrle et al. (1982) suggested that non-functional elastin and accumulations of collagen are formed. The findings of our study based on a papain model show that, contrary to the assertions of Johanson et al. (1971), alterations do exist in the EF and these show some similarity with those observed by Fukada et al. (1989) in the human centrilobular emphysema. The EF show an uneven pattern, are tortuous and, in the initial zones of the alveolar ducts (remodelled zones), form accumulations next to collagen fibres; no fibres in the process being digested can be observed at two months. The fibres are also numerous just where the alveolar ducts are inserted, where they are of a large diameter. According to Fukada et al. (1989) and Kuhn et al. (1976), these alterations would represent the beginning of centrilobular emphysema as a result of remodelling and fenestration. The presence of clumps of collagen has been demonstrated in elastase-produced panlobular emphysema, in human centrilobular emphysema (Finlay et al., 1996) and in experimental papain-induced emphysema in rat (Sulkowska and Sulkowski, 1997). These observations lend weight to the theory that collagen deposition and subsequent remodelling is a significant feature in the pathogenesis of emphysema and also support the inflammation/repair hypothesis. The probable cause of this increase in collagen synthesis would be the peptides derived from elastogenesis and factors derived from pneumocytes II and macrophages, which increase in size or number following elastase or papain installation, as seen in our study (Finlay et al., 1996; Sulkowska and Sulkowski, 1997). As in elastase-produced emphysema (Mercer and Crapo, 1992), our morphological results point to an aberrant remodelling process, rather than endogenous enzymes, as being responsible for the emphysematous lesions. This process would not only involve an increase in collagen deposition but also the abnormal repair of the EF. Just as Lang et al. (1994) observed remodelling both in human centrilobular and elastase-produced panlobular emphysema, so we observed the same process in both types of emphysema, although accompanied by the neo-formation of aberrant EF. This strongly suggests that the distinction proposed by Cardoso et al. (1993) concerning two different processes being involved in centrilobular (inflammation/repair) and panlobular emphysema (protease/antiprotease imbalance) is not totally exact, since a phenomenon of tissue repair exists in the pathogenesis of both types, whereby not only the collagen fibres participate but also the EF, as observed in our model.

Acknowledgements. We wish to thank Mª Carmen González Ulloa (University of Murcia) for her technical assistance.

References

Borges L.F., Taboga S.R. and Gutierrez P.S. (2005). Simultaneus observation of collagen and elastin in normal and pathological tissues: analysis of Sirius-red-stained sections by fluorescence microscopy. Cell Tissue Res. 320, 551-552.

Bowden D.H. (1984). The alveolar macrophage. Environ. Health Perspect. 55, 327-341.

Busch R.H., Lauhala K.E., Loscutoff S.M. and McDonald K.E. (1984). Experimental pulmonary emphysema induced in the rat by intratracheally administered elastase: morphogenesis. Environ. Res. 33, 497-513.

Caldwell E.J. (1971). Physiologic and anatomic effects of papain on the rabbit lung. J. Appl. Physiol. 31, 458-465.

Cardoso W.V., Sekhon H.S., Hyde D.M. and Thurlbeck W.M. (1993). Collagen and elastin in human pulmonary emphysema. Am. Rev. Respir. Dis. 147, 975-981.

Carvalho H.F. and Taboga S.R. (1996). Fluorescence and confocal laser scanning microscopy imaging of elastic fibers in haematoxylin-eosin stained sections. Histochem. Cell Biol. 106, 587-592.

Chyczewski L.S.T. and Sulkowski S.T.L. (1988). The effect of activation of macrophages on the advancement of experimental lung emphysema. Exp. Pathol. 34, 41-50.

Cotta-Pereira G., Gerra-Rodrigo F. and David-Ferreira J.F. (1976). The use of tannic acid-glutaraldehyde in the study of the elastic and elastic related fibers. Stain Technol. 51, 7-11.

Finlay G.A., O'Donnell M.D., O'Connor C.M., Hayes J.P. and FitzGerald M.X. (1996). Elastin and collagen remodeling in emphysema. A scanning electron microscopy study. Am. J. Pathol. 149, 1405-1415.

Fonzi L. and Lungarella G. (1980). Correlation between biochemical and morphological repair in rabbit lungs after elastase injury. Lung 158, 165-171.

Fukuda Y., Masuda Y., Ishizaki M., Masugi Y. and Ferrans V.J. (1989). Morphogenesis of abnormal elastic fibers in lungs of patients with

panacinar and centriacinar emphysema. Hum. Pathol. 20, 652-659.

Gadek J.E., Hunninghake G.W., Fells G.A., Zimmerman R.L, Keogh B.A. and Crystal R.G. (1980). Evaluation of the protease-antiprotease theory of human destructive lung disease. Bull. Physiopathol. Respir. (suppl). 16, 27-40.

Gross P., Pfitzer E., Tolker E., Babyak M.A. and Kaschak M. (1965). Experimental emphysema. Its production with papain in normal and silicotic rats. Arch. Environ. Health. 11, 50-56.

Hayes J.A., Korthy A. and Snider G.L. (1975). The pathology of the elastase induced panacinar emphysema in hamsters. J. Pathol. 117, 1-14.

Johanson W.G., Pierce A.K. and Reynolds R.C. (1971). The evolution of papain emphysema in the rat. J. Lab. Clin. Med. 78, 599-607.

Johanson W.G., Reynolds R.C., Scott T.C. and Pierce A.K. (1973). Connective tissue damage in emphysema. An electron microscopic study of papain induced emphysema in rats. Am. Rev. Respir. Dis. 107, 589-595.

Kobrle V., Hurych J. and Holusa R. (1982). Changes in pulmonary connective tissue after a single intratracheal instillation of papain in the rat. Am. Rev. Respir. Dis. 125, 239-243.

Kuhn C., Yu S.Y., Chraplyvy M., Linder H.E. and Senior R.M. (1976). The induction of emphysema with elastase. Changes in connective tissue. Lab. Invest. 34, 372-380.

Lang M.R., Fiaux G.W., Gillooly M., Stewart J.A., Hulmes D.J.S. and Lamb D. (1994). Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs. Thorax 49, 319-326.

Laurell C.B. and Eriksson S. (1963). The electrophoretic alpha1-globulin pattern of serum in alpha1-antitrypsin deficiency. Scand. J. Clin. Invest. 15, 132-140.

Mercer R.R. and Crapo J.D. (1992). Structural changes in elastic fibres after pancreatic elastase administration in hamsters. J. Appl. Physiol. 72, 1473-1479.

Morris S.M, Stone P.J., Snider G.L., Albright J.T. and Franzblau C. (1981). Ultrastructural changes in hamster lung four hours to 24 days after exposure to elastase. Anat. Rec. 201, 523-535.

Morris S.M., Kagan H.M., Stone P.J., Snider G.L. and Albright J.T. (1986). Ultrastructural changes in hamster lung 15 min. to 3 hours after exposure to pancreatic elastase. Anat. Rec. 215, 134-143.

Parra S.C., Gaddy R. and Takaro T. (1980). Early ultrastructural changes in papain induced experimental emphysema. Lab. Invest. 42, 277-289.

Snider G.L., Lucey E.C. and Stone P.J. (1986). Animal models of emphysema. Am. Rev. Respir. Dis. 133, 149-169.

Stehbens W.E. (2000). Proteinase imbalance versus biomechanical stress in pulmonary emphysema. Exp. Mol. Pathol. 69, 46-62.

Sulkowska M. and Sulkowski S. (1997). The contribution of type II pneumocytes and alveolar macrophages to fibroplasia processes in the course of enzymatic lung injury. Histol Histopathol. 12, 111-122.

Thurlbeck W.M. (1967). Internal surface area and other measurements in emphysema. Thorax 22, 483-496.

Valentine R., Rucker R.B., Chrisp C.E. and Fisher G.L. (1983). Morphological and biochemical features of elastase-induced emphysema in strain A/J mice. Toxicol. Appl. Pharmacol. 68, 451-61.

Accepted July 7, 2006