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EXTRACELLULAR MATRIX AND SCHISTOSOMIAIS

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The schistosome egg retained within host tissues secretes materials that are potent stimulators of fibroblast proliferation and deposition of extracellular matrix. Liver slices containing schistosome granulomas can produce 16 times more collagen than similar slices from normal liver (Dunn et al., 1977). The egg-derived material is part of the so-called soluble egg antigen (SEA) and its fibrogenic factor can act either directly upon the cells involved in fibrogenesis or through an antigenic mechanism involving sensitized *T* lymphocytes and macrophages. Since the hepatic periovular granuloma can be easily obtained in mice, can be isolated in large amounts and can even be produced *in vitro*, it is nowadays the most studied and most understood type of granuloma. Being an essentially fibrogenic lesion, it has also become a model for studies about extracellular matrix and to study soluble factors involved in fibroblast stimulation and collagen synthesis (Wyler et al., 1986), the sequence of deposition of genetically different types of collagens (Andrade & Grimaud, 1986), and the pathophysiology of extracellular matrix degradation (Andrade & Grimaud, 1986; 1988).

The present article will analize the formation and degradation of connective tissue, especially its extracellular matrix, in experimental and human schistosomiasis. The approach will be a morphological one and an attempt will be made to inquire how basic studies on this subject relate to human schistosomiasis, a condition in which fibrosis appears as an outstanding factor in pathogenesis.

FIBROSIS IN PERIOVULAR GRANULOMAS

Fibroblast proliferation and deposition of concentric collagen fibers first appear as a mild change at the periphery of granulomas formed during the early period of infection, but tend to increase and to involve the entire lesion with time. Ultrastructural changes are impressive in this regard. One can see fusiform cells with well developed endoplasmic reticulum, some of them dilated, forming cisternae filled with amorphous material, and showing prominent Golgi structures and mitochondria. These fusiform cells are aligned in parallel rows separated by an extra-cellular substance containing abundant amorphous, fibrillar and granular material. Collagen fibrils with their typical striated structure are at first loosely arranged, but soon assume a more oriented and dense pattern, forming parallel fibers separated by several cell types, mainly fibroblasts, eosinophils, lymphocytes, neutrophils, a few myofibroblasts and some rare mast-cells. These ultrastructural features of periovular granuloma are consistent with active synthesis by the cells forming extracellular matrix.

Biochemical and immunocytochemical methods have disclosed that the periovular granuloma matrix in early infection contains large amount of type III collagen. Type I collagen is present in lesser amounts, but as the infection matures its quantity gradually increases. In mice infected with 50 cercariae/8 weeks the absolute amount of type I collagen increased 11 times while that of type III increased 22 times (Wu et al., 1982). Type IV collagen is scarse or absent (Wu et al., 1982).

As for the collagen-associated glyco-proteins, fibronectin is abundant during the more active stages of the granuloma. It has been suggested that fibronectin may serve as a marker for granuloma stage. In human material it has been observed that early granulomas contain mainly fibronectin, intermediate granulomas show fibronectin and type III collagen, and late granulomas are formed predominantly by type I collagen, while hyalinized ones do not contain types I and III collagens, nor fibronectin (Al Admani, 1985). Morcos et al. (1985) also observed that granulomas from an 8-week old infection are more rapidly resorbed after chemotherapy than those from a 39-week infection. They believe that the genetically different types of collagen predominating in each case (type III in early and type I in old granulomas) can account for this difference. However, such has not been our experience with experimental material, since the

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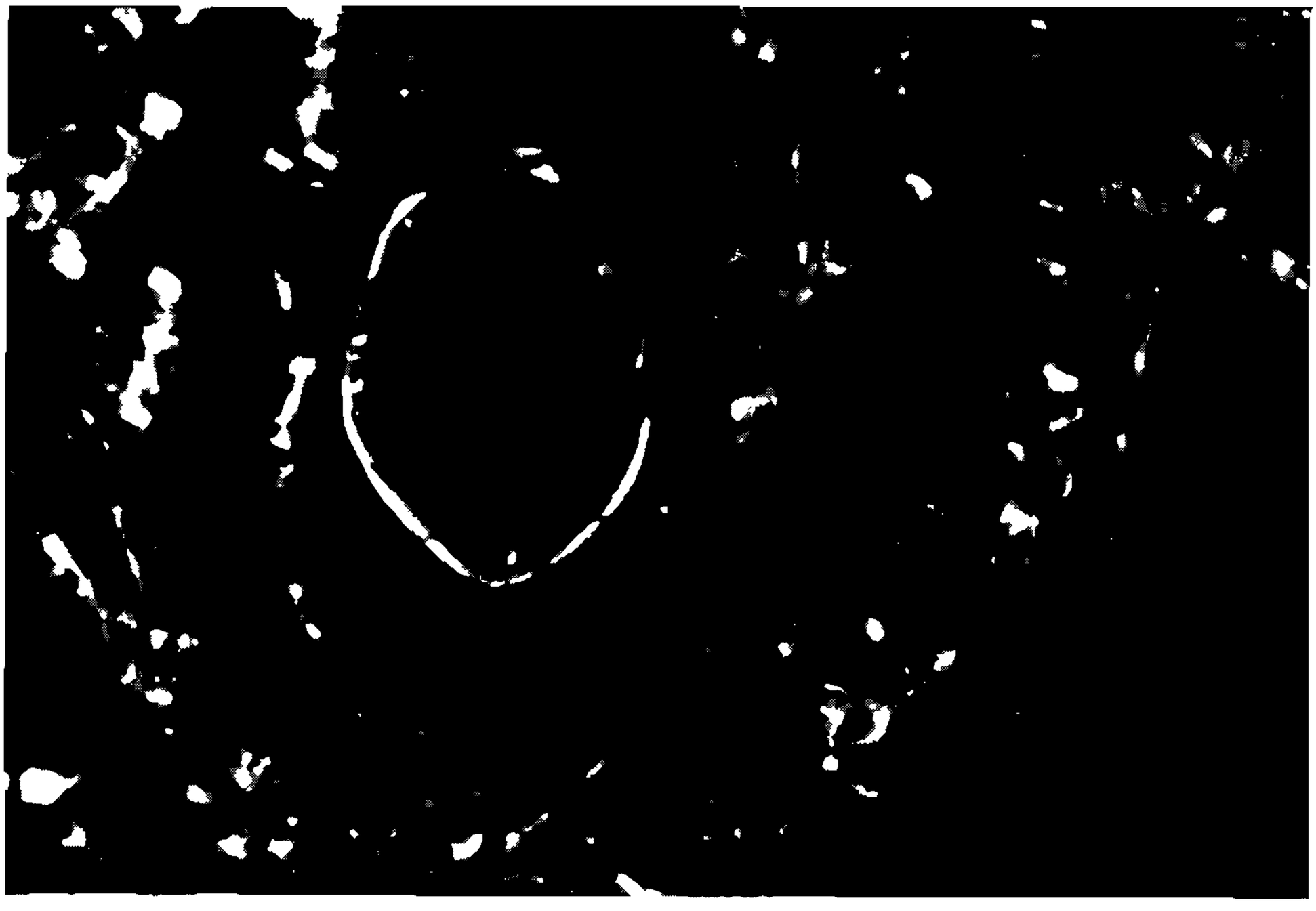

Fig. 1: collagen fragmentation in involuting schistosomal granuloma. Fibers fluorescent for type III collagen appear fragmented and irregularly distributed around the central egg shell in a periovular granuloma. Liver of a mouse with a 10-week old infection, 2 months after treatment. 250 X.

presence of both types I and III collagens were detected in all type of granulomas, early and late. Four and half months after cure of schistosomiais both type I and type III collagens could be detected in residual scars (Andrade & Grimaud, 1986; 1988) (Fig. 1). As for fibronectin, we also noted that it decreased considerably during granuloma involution. It has been suggested that secretion of fibronectin by SEA-stimulated lymphocyte macrophages is a key factor for attraction and stimulation of fibroblasts in periovular granulomas (Wyler et al., 1986), and is a good example of the interaction between inflammatory cells and fibrosis. On the other hand, laminin is scanty regardless of the evolutional stage of the granuloma (Andrade & Grimaud, 1988).

Proteoglycans are abundant during the active stage of the granuloma, but their amounts gradually decreases as the infection becomes chronic, at least in the mouse, either infected with *Schistosoma japonicum* (Olds et al., 1986) or *S. mansoni* (El Meneza, 1989). There are only a few studies concerning proteoglycans and schistosomal granulomas. In the liver it seems that dermatan sulfate is the only glycocosaminoglycan present (Junqueira et al., 1986). This finding may implicate the fat-storing cells (Ito cells) in the genesis of hepatic periovular granuloma, since they are a known source of dermatan and chondroitin sulfate glycosaminoglycans, while hepatocytes synthesize heparan sulfate only (Gressner & Zerbe, 1987).

Elastin, another component of the extracellular matrix, is absent from the granulomas (Andrade & Grimaud, 1986; Junqueira et al., 1986). Curiously, elastic hyperplasia is a prominent finding in portal fibrosis of man ("pipe-stem fibrosis"), as will be discussed later.

An important step in the study of the relationship between granuloma and fibrosis came when Wyler (1978) used the supernatant of a serum-free medium containing isolated liver granulomas to stimulate fibroblasts *in vitro*. Granuloma extracts increased collagen synthesis *in vitro* 16 times, while extracts from normal liver gave negative results. Fibrogenic material secreted by macrophages in the granulomas was seen to differ from SEA and IL-1 and to require protein synthesis for its secretion (Wyler et al., 1983). The materials produced in the granulomas can also stimulate vascular smooth muscle, but the stimulation of endothelial cells seems to depend on a different factor(s) (Wyler et al., 1987).

We can expect that in the near future the factors in periovular granulomas responsible for mesenchymal cell proliferation, for synthesis of collagen, proteoglycan and fibronectin, and for changes in smooth muscle and endothelial cell will be chemically defined and cloned. This will add considerable strength to the studies on extracellular matrix biology and pathology.

THE DEGRADATION OF FIBROUS TISSUE IN PERIOVULAR GRANULOMAS

The schistosomal periovular granuloma passes through a cycle of growth, maturation and degradation. The main commanding factors for these cyclic changes derive from the miracidium and the host immunological status. The miracidium secretes substances (SEA included) that oozes out from the micropores in the egg-shell and induces inflammation. A mature miracidium can stay alive for a maximum of 15-18 days within the tissues of a susceptible host. Following miracidium death a dramatic change in the granuloma occurs within one to two weeks (Andrade & Grimaud, 1986), which leads to involution of the lesion. When miracidial destruction is brought about by chemotherapy, involuting changes appear in every granuloma. During active infection all stages of the granuloma cycle can be observed side by side.

The first well documented report on the reversibility of hepatic fibrosis in schistosomiasis came from Warren (1962). He demonstrated that therapeutic cure of mice was followed by disappearance of inflammatory cells and resorption of the fibrous tissue in the liver. He followed the lesions up to 25 weeks after treatment and was able to observe an almost total regression of the lesions by then. These findings were soon confirmed (Cameron & Ganguly, 1964: Schiller & Haese, 1973). However, sometime later, Warren & Klein (1969) found that hepatic fibrosis due to prolonged schistosomal infection was irreversible. There are differences in the rate of resorption in recent and late fibrosis. The maturation of the collagen tissue with time renders it more resistant to collagenase digestion. Maturation seems to involve the development of intra and inter-molecular collagen cross-linkings, that may block strategic sites of enzymatic digestion. This probably means that degradation of old scars may take more time to be accomplished, rather than that it had become impossible. In favor of this conclusion are the recent findings showing regression of the lesions of pipe-stem fibrosis in mice following treatment of schistosomiasis (Andrade, 1987). In that study, portal fibrosis resembling human pipe-stem fibrosis was produced in mice by means of prolonged (16-25 weeks) and mild (one/two worm pairs) infections. Treatment with oxamniquine caused considerable decrease of fibrosis in the portal spaces three months following treatment.

Early changes in the involuting granuloma include a decrease in the number of inflammatory cells, especially at the periphery of the lesion, while the collagen seems more compact and shrunken. This means that the granuloma becomes small, discrete and dense. At the ultrastructural level early involuting changes are striking. There are extracellular beakdown of collagen and internalization of collagen fragments into fibroblasts, myofibroblasts and macrophages (Fig. 2). The broken extracellular collagen fragments exhibit variable diameters, different sizes and loss of cross-striation. They are distributed at randon in the middle of an increased amorphous matrix. Internalized collagen fragments appear in membrane-bound vacuoli and show variable degrees of disintegration (Fig. 3). These changes are similar to those described for the classical models of collagen degradation, such as the involuting pregnant uterus of the rat, the metamorphosing tadpole tail, the carrageenin-induced granuloma and the early carbon tetrachloride-induced cirrhosis of the rat following discontinuation of the drug. However, after a period of approximately one month these ultrastructural changes subside and the shrunken granulomas in the liver of a mouse submitted to curative chemotherapy seem to reach a state of irreversibility. Actually the involution continues at a slower pace and most of the granulomas would eventually disappear by 4-6 months following treatment (Warren, 1962; Cameron & Ganguly, 1964; Andrade & Grimaud, 1988). The situation may not be the same in *S. japonicum* infection, where degradation of collagen does not seem to be so prominent (Cheever & Deb, 1989).

Changes indicative of chronic collagen degradation have ultrastructural features that differ from those of the "acute" period. Focal areas of degradation now appear in the middle of collagen fibers or bundles. These areas are represented by small empty spaces of focal collagen lysis and by granular and fibrillar changes which form dark (electron dense) areas within the collagen fibers (Fig. 4).

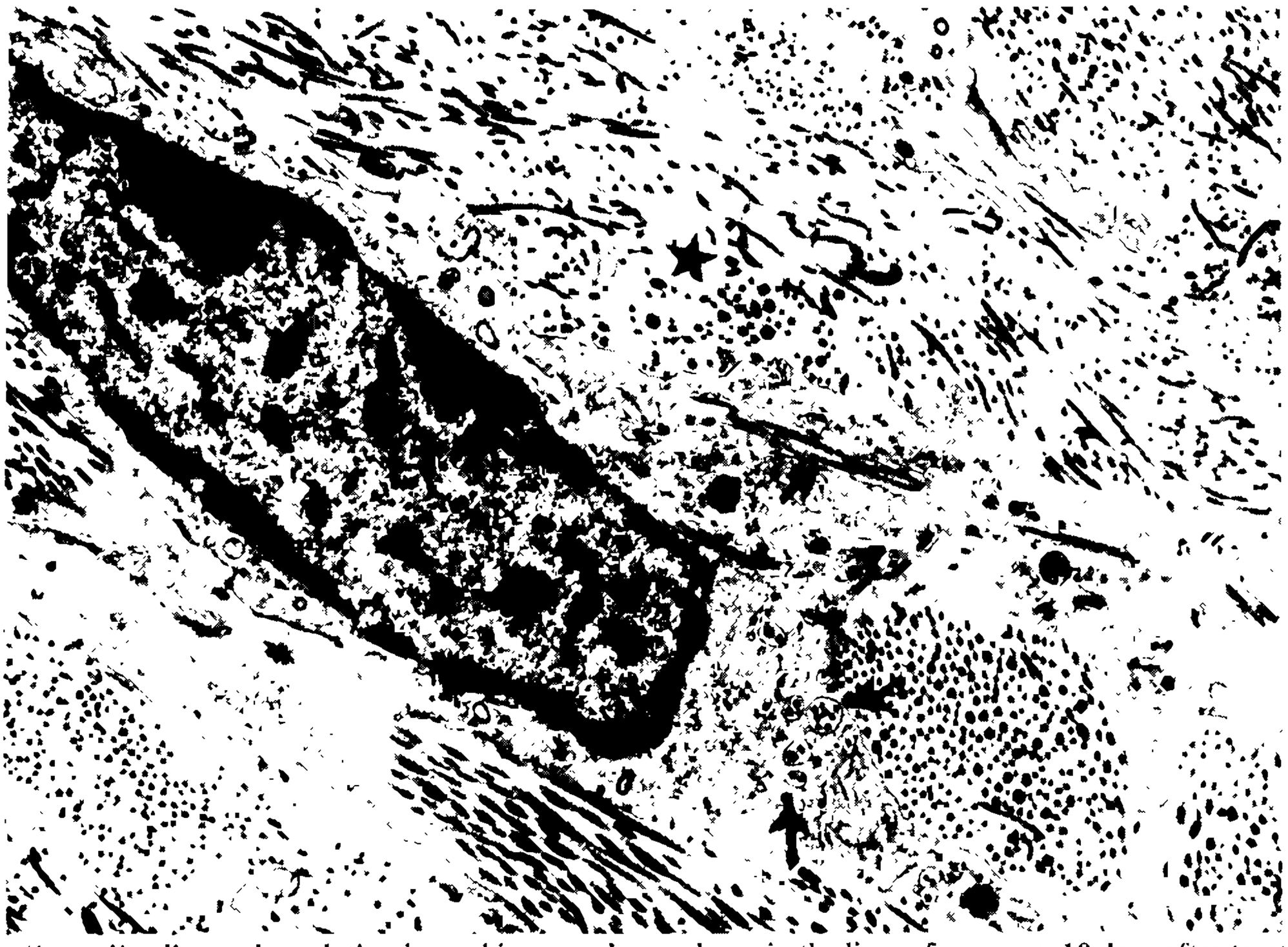

Fig. 2: "acute" collagen degradation in a schistosomal granuloma in the liver of a mouse, 10 days after treatment. The central part of the picture is occupied by a fibroblast which contains collagen fragments internalized in its cytoplasm (arrows). Extracellular collagen breakdown can be seen around the fibroblast. The fibrils appear fragmented and exhibit different sizes and diameters. ★ Electron micrograph. 52,000 X.

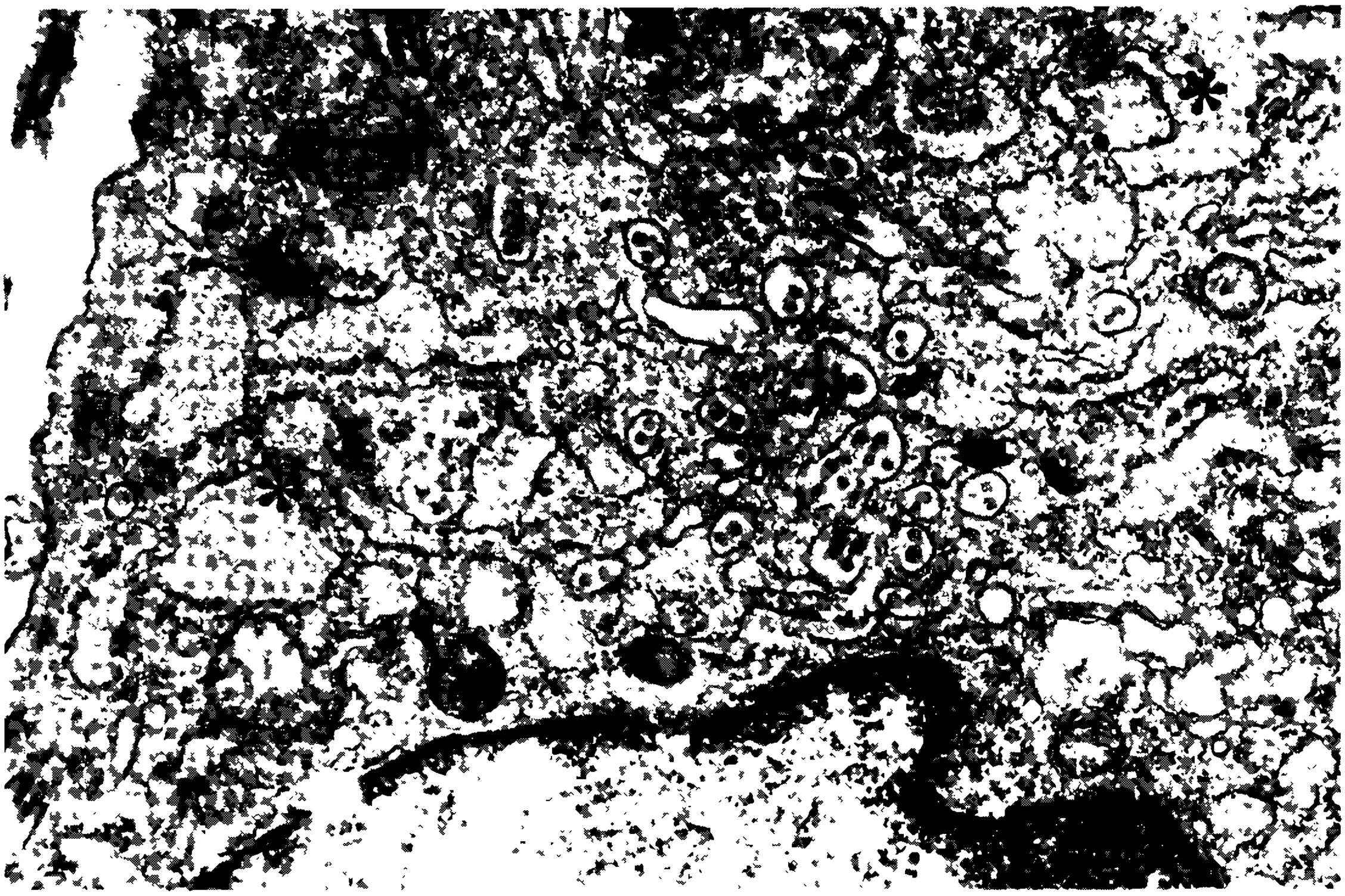

Fig. 3: internalized collagen fragments within membrane-bound vacuoli (arrow) in the citoplasm of a connective tissue cell. There is also dilatation of the endoplasmic reticulum (✤), forming cisternae. From a hepatic granuloma of a mouse, 10 days after treatment. Electron microscopy, 60,000 X.

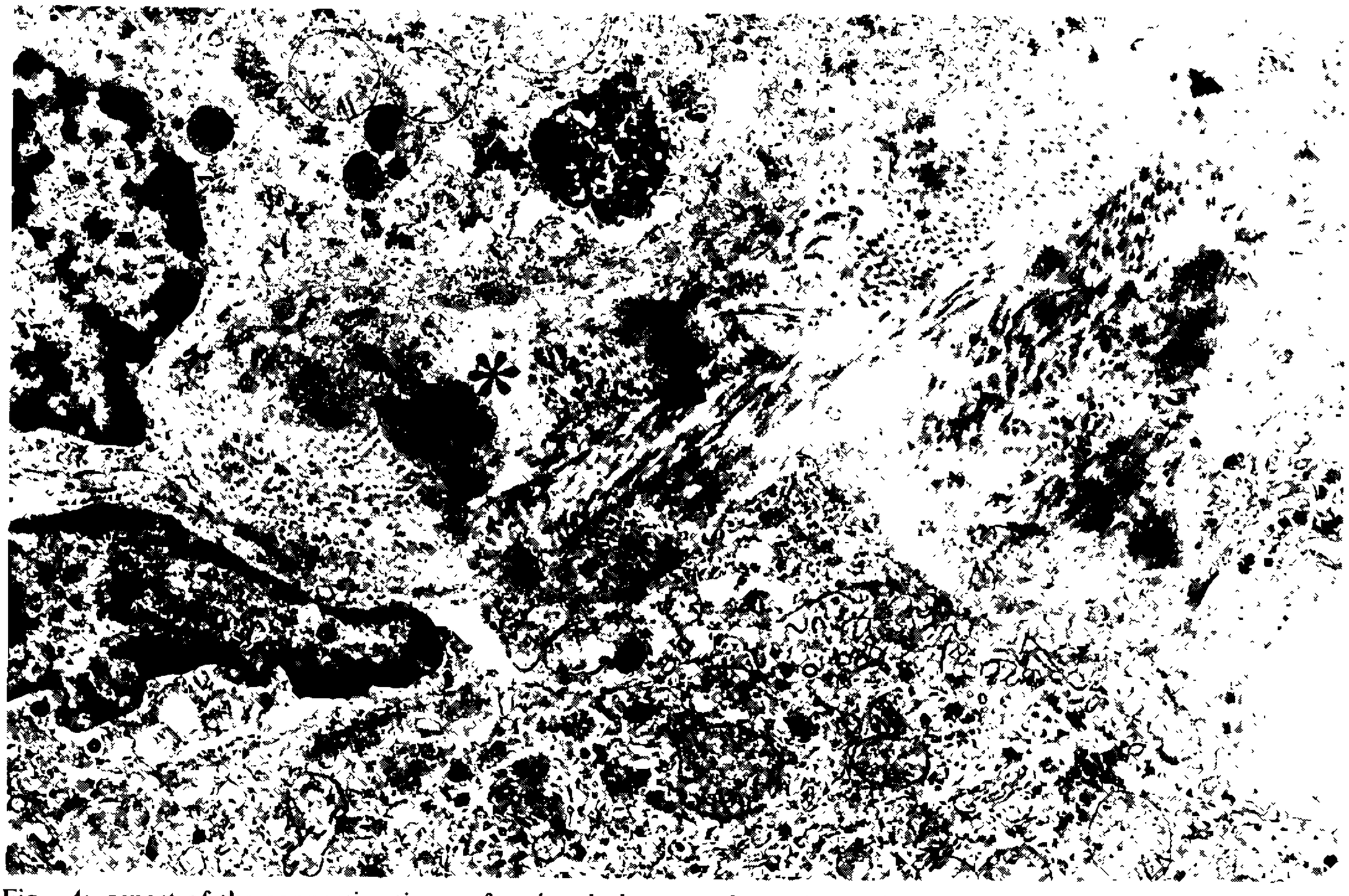

Fig. 4: aspect of the connective tissue of an involuting granuloma 4 months and half after treatment. Bundles of collagen fibrils show focal areas of dissolution (lytic changes ✱) and finely granular dark areas (electron dense changes ➧). The small star (★) marks a deposit of schistosomal pigment. Electron microscopy, 20,000 X.

Since all models so far utilized to study collagen degradation last from hours to a few days or at most one month, they may represent instances of "acute" collagen degradation. The picture seen in the late stages of schistosomal granuloma involution is different and may represent a form of "chronic" collagen degradation. The importance of this concept is not only academic. It implies that the effects of the mechanisms operating for collagen degradation (or extracellular matrix removal) may need much more time to be observed than is usually expected. Also, the factors involved in this form of degradation may not be the same as for the more acute changes. Although new and promising advances are being made in the field of extracellular matrix degradation (Arthur, 1990), with better understanding of the biochemistry of the metalloproteinase family, and increased knowledge about interstitial collagenases, gelatinases and stromelysin, there are only few studies made in schistosomiasis in this specific area.

Fibroblasts and macrophages, among other cells, can elaborate collagenases. It has been demonstrated that marked increases in collagenase activity occur together with increased collagen synthesis in murine schistosomiasis (Takahashi et al., 1980).

Emonard & Grimaud (1989) observed that both active and latent collagenase activity increased considerably 5 days after curative treatment of infected mice, and then decreased gradually up to 72 days. On the other hand, liver hydroxyproline contents were similar for treated and untreated animals 20 days after treatment, showing a marked decrease only after day 30. The presence of hydroxyproline in urine increased 5 days after treatment and reached a maximum between days 10 and 45, decreasing thereafter. Biempica et al. (1983) observed the presence of collagenase bound to collagen by immuno-electron microscopy of the schistosomal granuloma, but did not discriminate between the latent and active forms. The subject becomes even more difficult because within the granuloma there may be inhibitory collagenase factors. Truden & Boros (1988) identified two such factors as alfa-2 macroglobulin and alfa-1 protease inhibitor. Although the level of protease was the same in granulomas from recent and late infection, supernant of cultures of adherent macrophages isolated from granulomas of mice with acute infection contained levels of protease inhibitor several times higher than those of similar preparations obtained during chronic infection. A complex and delicate balance seems to regulate fibrogenesis and fibrolysis in periovular granulomas.

HUMAN STUDIES

Clinical aspects — For sometime the experimental studies on reversibility of fibrosis due to schistosomiais were considered only as a model for basic studies on collagen formation and degradation. Its relevance to problems of human disease was not properly appreciated. Pipe-stem portal fibrosis seen in patients with hepatosplenic schistosomiais, with all its complex intrahepatic vascular changes, was not considered potentially reversible. Fibrous tissue itself was thought of as a stable tissue that once formed would stay in place indefinitely.

The report that hepatosplenic patients living in an endemic area were seen to undergo an apparent spontaneous cure when re-examined 10 years later, did not provoke much concern among investigators (Katz & Brener, 1966). The same was true for the few isolated reports on the reversibility of hepatosplenic disease that appeared thereafter (Lees, 1968).

However, with the advent of new effective curative drugs and the treatment of numerous patients, the possibility of reversibility of hepatosplenic schistosomiais became evident. Bina (1977) demonstrated that chemotherapy could be curative and preventive for hepatosplenic schistosomiais. Six years after curative treatment all his treated patients were seen to be re-infected, but no evolution toward more severe forms of the disease occurred, whereas four hepatosplenic patients had reversed to normal. On the other hand, in the untreated control group 22 patients evolved toward the hepatosplenic form of the disease. Later, Bina & Prata (1983) treated 23 patients with early hepatosplenic disease and found complete reversion in 26% and marked improvement in 78.3% of them. Dietze & Prata (1986) used oxamniquine to treat 70 patients in an endemic area where transmission had been interrupted and re-examined them 6, 18 and 24 months afterwards. Reversibility occurred in 28 patients (40%) Domingues (1986) treated hepatosplenic patients with praziquantel and found reduction of hepatomegaly in 80.95%, reduction of splenomegaly in 78.79% and total reversion of the hepatosplenic form in 18.52% of them, 12 months after treatment.

The involution of hepatosplenic disease after chemotherapy can now be followed in the field thanks to ultrasonography (Homeida et al., 1988).

Schistosomal fibrosis outside the liver may also profit from anti-schistosome treatment. One patient with a colonic tumor caused by schistosomiaisis (pseudo-neoplastic form) showed regression of the mass and clinical cure after treatment (Coutinho et al., 1984). Colonic diffuse polyposis disappeared from three Egyptian cases 3 months after administration of oxamniquine (Bassily et al., (1978), and 12 out of 17 were cured of their intestinal polyps after receiving niridazole (Farid et al., 1974).

Reactive connective tissue, such as it appears in chronic inflammation, posseses mechanisms for both formation and degradation of extracellular matrix. Accumulation of fibrous tissue results when synthesis exceeds degradation (Perez-Tamayo, 1982).

When the cause of inflammation can be completly removed, the mechanisms leading to degradation and removal of excessive matrix can apparently operate without opposition. Several findings seem to indicate that nature always tries to maintain an ajusted proportion between stroma and parenchyma. During experimental starvation an animal may lose 30% of its body weight and 50% of its normal liver weight, but this massive reduction occurred without significant change in the ratio of parenchyma to stroma (Perez-Tamayo, 1965). Once the cause of fibrosis is removed, the natural tendency toward a normal parenchyma/stroma ratio ensues. However, the rate of degradation still depends on several known and unknown factors. The presence of collagenase inhibitors in the tissues (Truden & Boros, 1988) and the degree of collagen maturation (cross-linkings) are two of them. The collagen that is rapidly formed is more prone to degradation (Andrade et al., 1990). Also, the degree and type of distortion of the normal architecture of the organ caused by fibrosis seem important. As an example, portal fibrosis in schistosomiasis should be more amenable to regression than the fibrous bands and septa in hepatic cirrhosis. Furthermore, it has been shown that the peritoneal macrophages from a schistosome-infected animal can secrete both the fibrogenic factor and IL-1, but those from the granulomas secrete only the fibrogenic factor (Wyler et al., 1983). One can assume that a lymphocyte in a granuloma can elaborate more or less factors that may influence the extracellular matrix. One of them, gama-interferon, was demonstrated to inhibit collagen deposition in murine schistosomiais (Czaja, 1989).

Morphological aspects — In spite of the existing clinical and experimental data just discussed, morphological studies on extracellular matrix degradation in human schistosomiasis are scarse or non-existent. The data to be presented below belong to an investigation in progress (Andrade & Peixoto, to be published). Since last year, Dr Ediromar Peixoto, surgeon of the Central State Hospital in Salvador, has provided us with wedge liver biopsies from cases of hepatosplenic schistosomiasis. These patients had not received anti-schistosomal treatment and were operated upon to alleviate the dangerous consequences of portal hypertension. They were adults, 10 males and 4 females, and their ages varied from 18 to 53 years (average: 32.6). The biopsy materials were submitted to histological, ultrastructural and immuno-cyto-chemical studies. At first, the interest in examining such material was to obtain a base-line for comparative studies with treated patients later on. However, some of the patients did not have parasite ova in the sections examined and all of them presented morphological evidences of focal matrix degradation. Our impression is that one could hardly expect to see more in treated patients.

Focal degradation of collagen in these specimens was first noted at the ultrastructural level (Fig. 5). It resembled the changes associated with chronic collagen degradation described in the mouse model that have been labelled "lytic" and "electron dense" changes (Andrade & Grimaud, 1988).

Focal lytic disappearance of collagen fibrils was more frequently seen than focal "electron dense changes", where dark fibrillar and granular areas where noted within groups of collagen fibrils (Fig. 6). Both changes were more frequently observed in septal than in portal fibrosis. Although some collagen breakdown was often observed in the vicinity of mesenchymal cells, focal degenerative changes appeared in the middle of collagen fibrils apparently unrelated to the presence of cells. No internalization of collagen fibrils was ever seen.

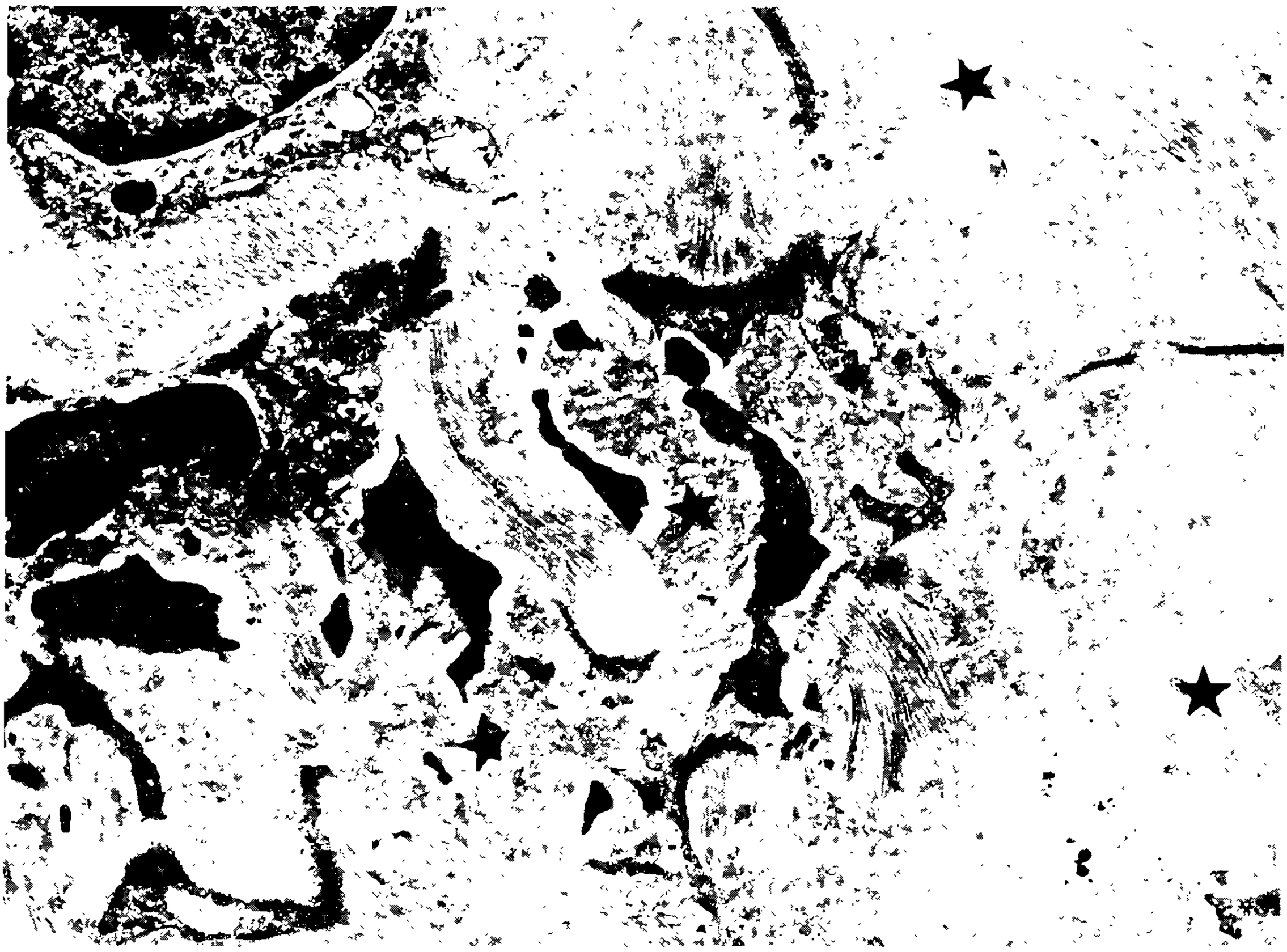

Fig. 5: human pipe-stem fibrosis. No history of previous treatment. Bundles of collagen fibrils in the portal spaces are separated by the cytoplasmic prolongations belonging to connective tissue-cells. There are areas of matrix degradation (★) where the collagen fibrils are replaced by amorphous or granular electron dense material. Electron microscopy, 4,400 X.

Fluorescence studies showed that type III collagen (identified by either anti-type III or pro-III collagen antibodies) was most abundant, with type I collagen coming next. Similar to what has been observed in experimental material, type IV collagen was scanty. Several fibers appeared strongly fluorescent for anti-type V antibodies both in portal and septal areas. As expected, type IV collagen and laminin stained the basal lamina of the numerous blood bessels present in the fibrotic areas. These vessels were also well demonstrated with the use of anti-actin, which stained both the preserved and destroyed muscular coat (Figs 7a, 7b). Dissociated muscle cells, isolated or forming small clusters, were seen "buried" in the portal fibrous tissue (Fig. 8). These two elements, the muscular cells and the endothelial cells, may have an important role in the matricial changes in schistosomiais. One important aspect is that they may synthesize elastin (Davidson, 1987).

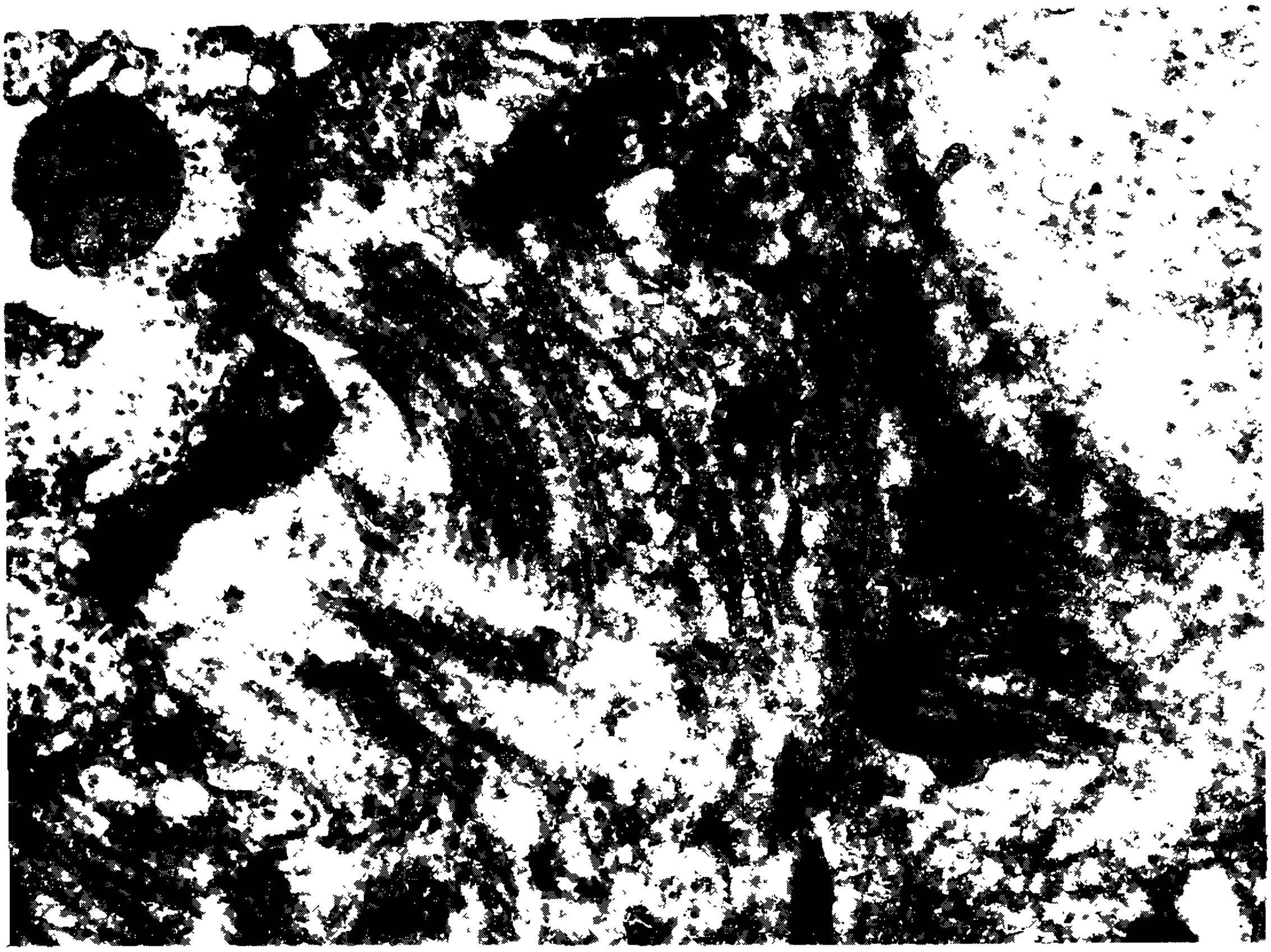

Fig. 6: detail of the precedent microphotograph showing the irregular and fragmented appearance of the collagen, with focal areas of "electron dense" changes. Electron microscopy, 20,000 X.

Elastin was found in great amount in the human hepatic fibrosis due to schistosomiasis examined by us. We used a monospecific anti-elastin antibody as well as the classical Weigert stain. Elastic tissue was abundant in the enlarged portal spaces, and less so in the areas of septal fibrosis (Fig. 7c). The most abundant accumulation of elastica (elastin) appeared either around destroyed portal vessels (Fig. 7d), associated with marked vascular proliferation or in subcapsular areas. The staining of desmin helped in demonstrating the rich vasculature of the fibrotic portal spaces (Fig. 7e) and also marked the Ito cells or perisinusoidal cells within the hepatic lobule (Fig. 7f).

These findings seem to implicate muscle cells and endothelial cells in the production of elastic tissue, since elastin is conspicously absent in the periovular granuloma (Andrade & Grimaud, 1986; Junqueira et al., 1986) (Figs 9, 10).

After collecting all the data with fluorescent and ultrastructural methods, we decided to re-examine histological sections to see if it was possible to find

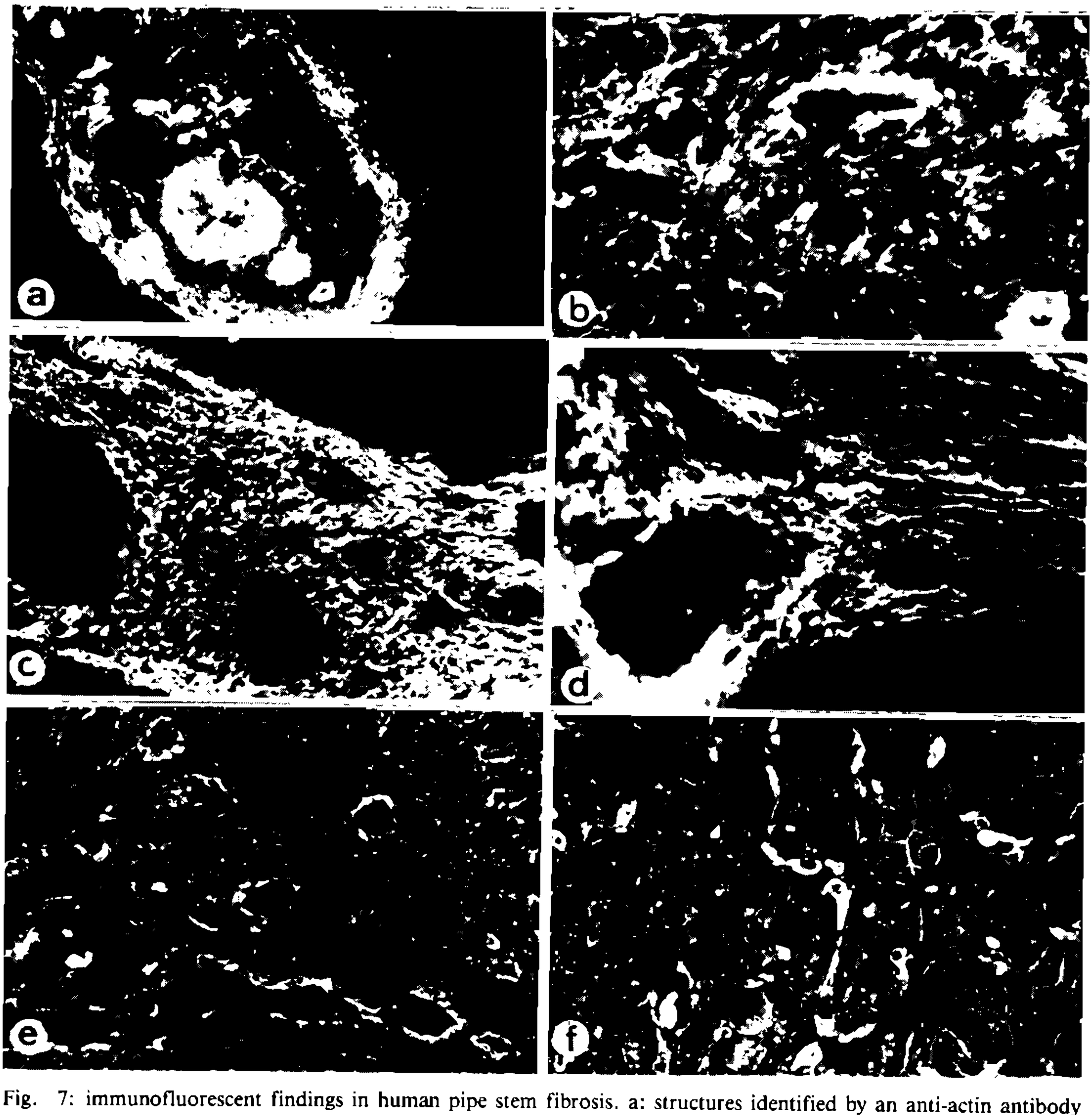

Fig. 7: immunofluorescent findings in human pipe stem fibrosis. a: structures identified by an anti-actin antibody in a fibrotic portal space include the wall of blood vessels and some muscular fibers dispersed within the fibrous tissue. 250 X. b: the actin staining allows the identification of a portal veins with part of their muscular wall destroyed. 400 X. c: specific positive fluorescence ofor elastin disclosing a rich network of fibers in an enlarged portal space. 250 X. 7d: elastin. Numerous parallel fibers extending from the wall of a portal vein into the portal fibrous tissue 400 X. e: presence of desmin-positive endothelial cells in numerous blood vessels in portal space with pipe stem fibrosis. 400 X. f: fluorescent anti-desmin antibodies stain elongated cells in between cords of hepatocytes (Ito Cells). 400 X

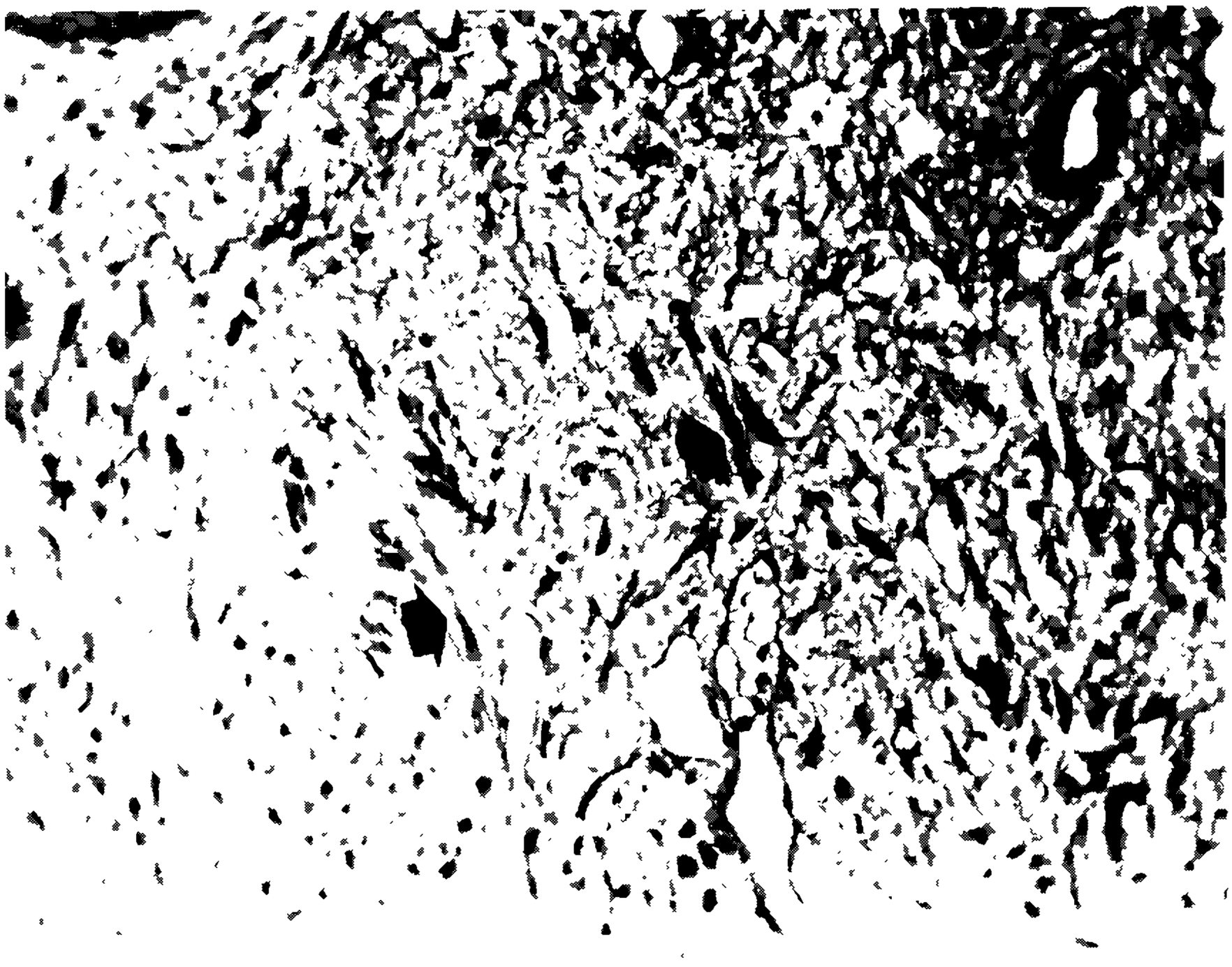

Fig. 8: portal space with fibrosis containing some muscle cells "buried" within it (arrows). The fibrous tissue is well vascularized and in some focal areas assumes a loose and reticulated appearance. Hematoxylin & Eosin, 250 X.

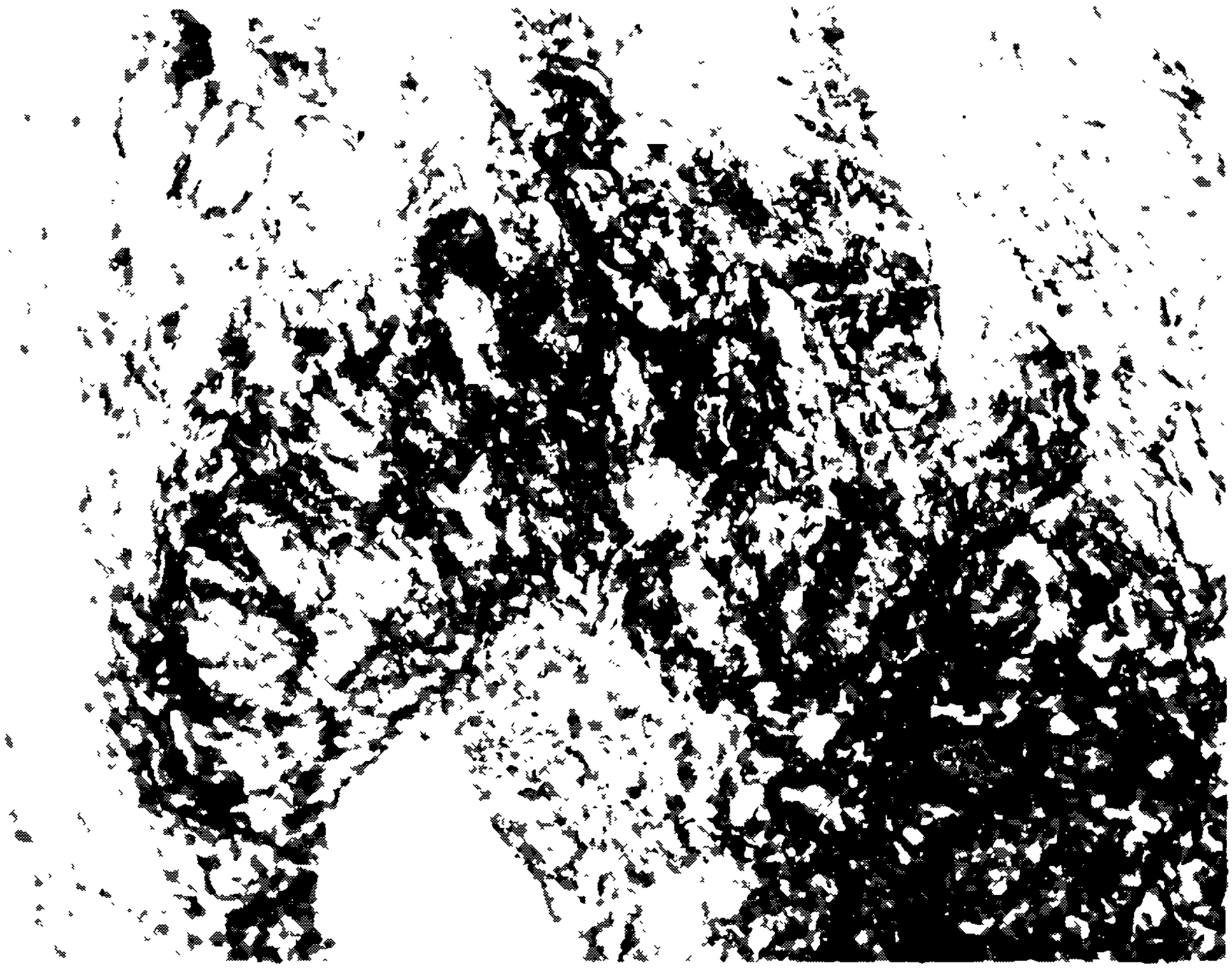

Fig. 9: numerous elastic fibers appear in connection with a sclerotic portal vein. Human hepatosplenic schistosomiais. Weigert stain, 400 X.

evidence of connective tissue degradation by light microscopy. To our surprise we found that it was. Collagen in the schistosomal human liver, especially when stained by picrosirius red and examined with or without polarized light, showed an essentially irregular appearence, with areas of variable densities. In the middle of a compact array of fibers, one could see light areas of variable sizes and shapes. A closer look revealed fragmentation as well as irregularities in size and staining affinity of the collagen fibers. Some areas looked edematous and fibrillar, probably due to a relative or absolute increase in amorphous matricial components (proteoglycans) (Fig. 11).

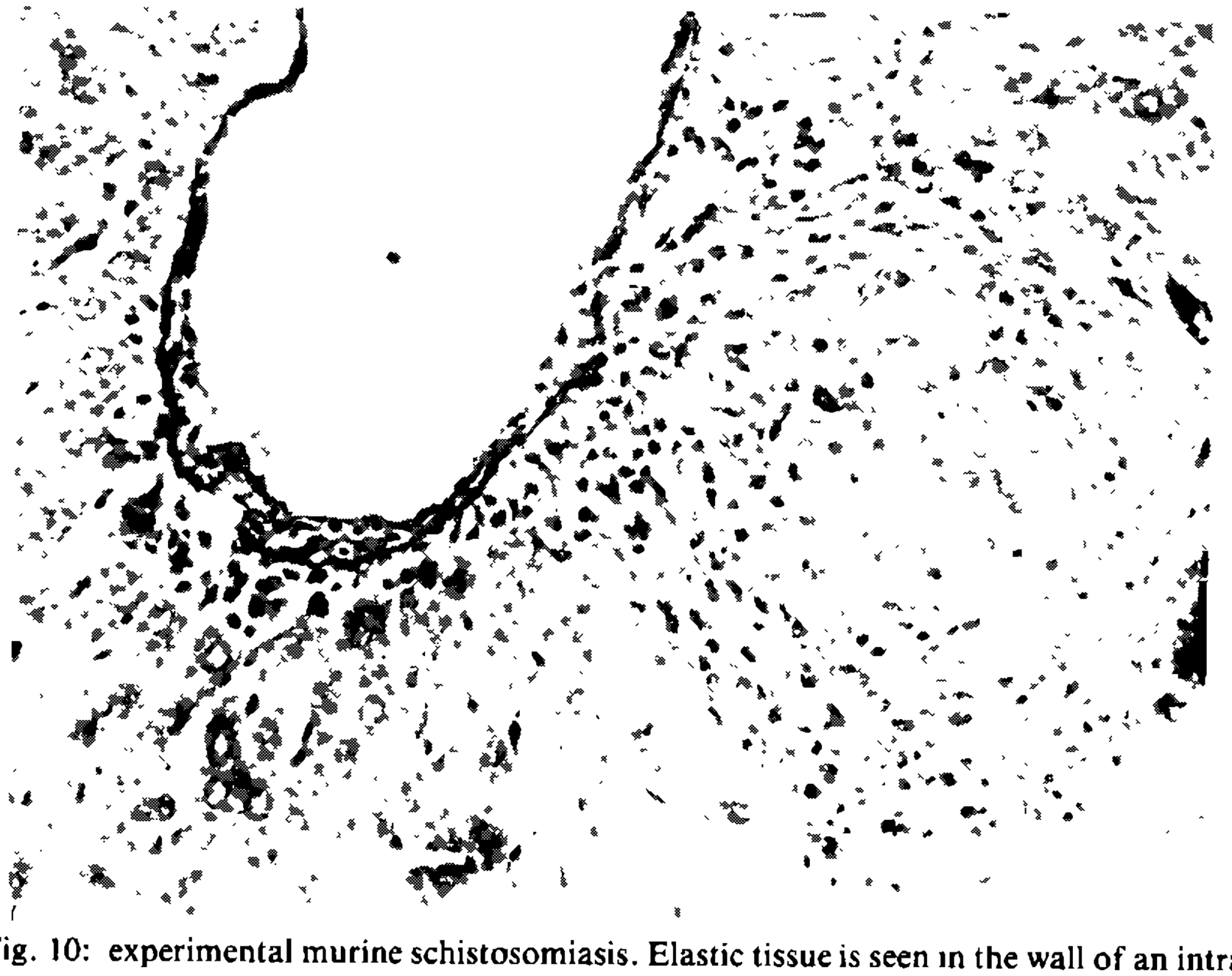

Fig. 10: experimental murine schistosomiasis. Elastic tissue is seen in the wall of an intrahepatic branch of the portal vein, but not in the nearby schistosomal granuloma. Weigert stain, 120 X.

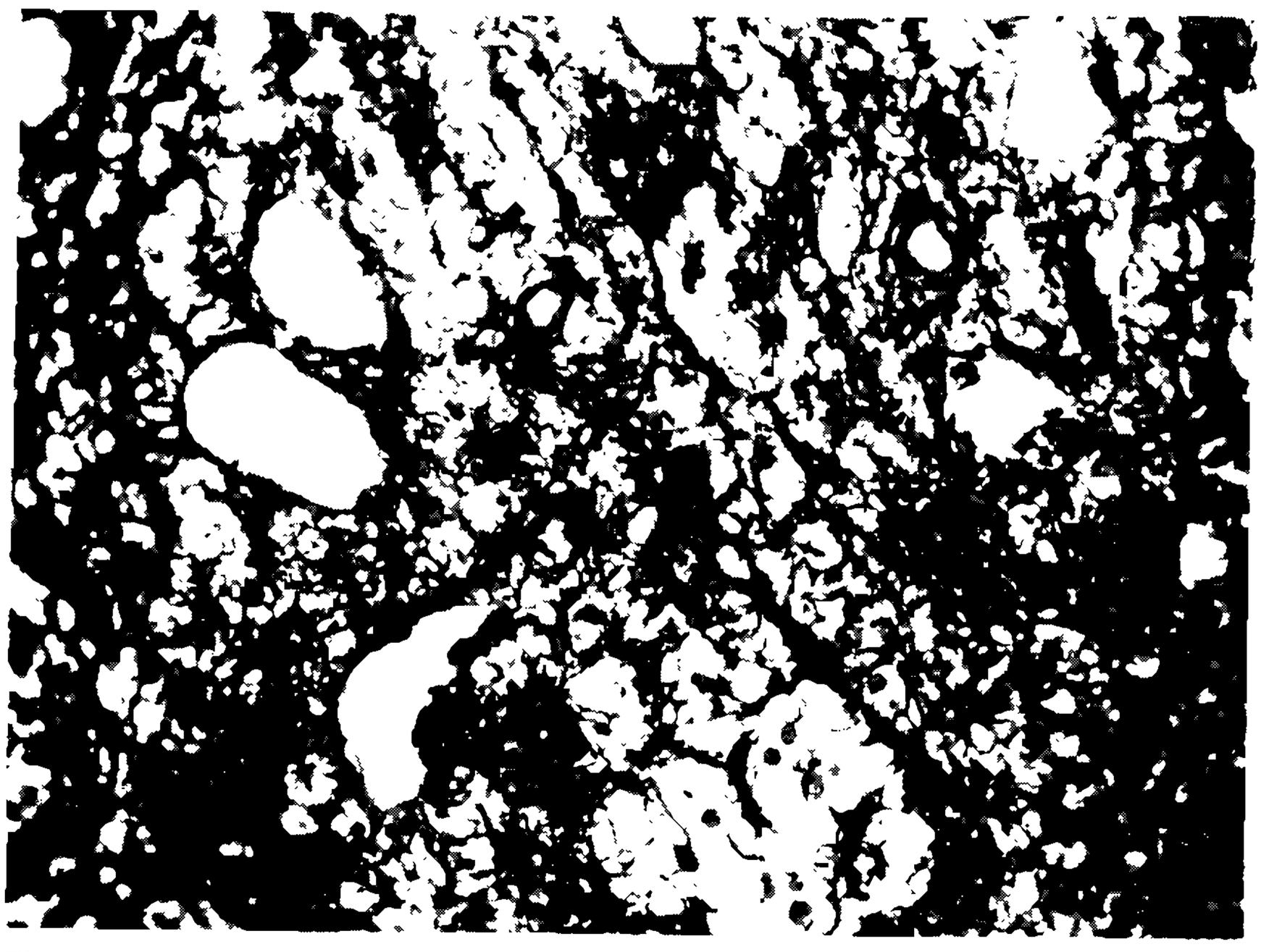

Fig. 11: collagen fibers presenting a reticulated pattern, with areas of variable densities, in the portal space with pipe stem fibrosis. Picrosiriusred method. 400 X.

Therefore, it seemed that evidence of collagen degradation in human pipe-stem fibrosis has always been under our noses, but we failed to see them, probably because we lacked a proper conceptual foundation.

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