EXPRESSION OF METALLOPROTEINASES (MMP-1, MMP-2, AND MMP-9) AND THEIR INHIBITORS (TIMP-1 AND TIMP-2) IN SCHISTOSOMAL PORTAL FIBROSIS

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Abstract. Focal extracellular matrix degradation morphologically identified in human portal pipestem fibrosis due to Schistosoma mansoni did not express immunohistochemical reactivity for metalloproteinases (MMP-1, MMP-2, and MMP-9) and their inhibitors (TIMP-1 and TIMP-2). However, when active schistosomal periovular granulomas were present, a strong reactivity for MMP-1, MMP-2, TIMP-1, and TIMP-2 was observed. No reactivity was ever observed for MMP-9. However, the positive pattern of immunohistochemical expression was not seen in old fibrotic periovular granulomas, which were sometimes situated in other areas of the same microscopic section. Positive staining for MMPs and TIMPs was observed at the same time in hepatocytes and within the apical portion of bile duct epithelium. These findings are consistent with the concept that matrix degradation in recent and old fibroses, in addition to differing at the ultrastructural level, also differs in immunohistochemical expression of metalloproteinases and their inhibitors.

Extracellular matrix degradation occurs in several physiologic and pathologic conditions. In human portal fibrosis due to schistosomiasis, resorption of fibrous tissue may be quite impressive, since a large amount of hepatic fibrosis can disappear a few years following curative chemotherapy.1 Metalloproteinases (MMPs) are key enzymes in the metabolism of collagen and in the physiopathology of fibrosis. The distinguishing properties of MMPs include putative zinc binding at the catalytic site, secretion in a zymogen form, activation by organomercurials, and regulation by tissue inhibitors of metalloproteinases (TIMPs), creating a balance between matrix degradation and synthesis.3

Histologic and ultrastructural changes associated with degradation of fibrosis after curative treatment have been described in humans4 and in experimental animals infected with Schistosoma mansoni.5–7 Degradation of early fibrosis in hepatic periovular granulomas formed during an 8–12-week mouse infection starts a few days after treatment and is almost complete after 2–4 months.5,8 However, in granulomas formed during prolonged experimental infections and in human pipestem portal fibrosis, partial or total resorption of fibrosis takes a prolonged time, usually more than four months in the mouse5 and 2–3 years in humans with schistosomal hepatosplenic disease.1,2 Interestingly, ultrastructural findings concerning matrix degradation have been observed to differ in recent and old fibrosis.9 Based on such ultrastructural observations, there seems to be two different types of extracellular matrix degradation. One is acute, which is commonly seen in the experimental models available,10–12 The other is chronic, which has a prolonged course and a peculiar ultrastructural pattern. The present work examined the activities of metalloproteinases and their inhibitors in fibrous tissues showing morphologic evidence of chronic matrix degradation. Liver biopsy materials from cases of advanced schistosomiasis due to S. mansoni, in which previous light and electron microscopic investigations had demonstrated the presence of characteristic ultrastructural changes of focal, chronic, extracellular matrix degradation,4 were subjected to immunohistochemical methods for detecting metalloproteinases (MMP-1 MMP-2, and MMP-9) and their inhibitors (TIMP-1 and TIMP-2).

MATERIALS AND METHODS

This study was reviewed and approved by Review Board of the Centro de Pesquisas Goncalo Moniz (FIOCRUZ) (Bahia, Brazil). Informed consent was obtained from all patients. Liver biopsy specimens received at the Laboratory of Pathology, Gonçalo Moniz Research Center, FIOCRUZ as routine material from a public hospital (Roberto Santos Hospital, Salvador, Bahia, Brazil) were used. This material represented surgical biopsies performed in 14 patients during splenectomy for treatment of portal hypertension (esophageal and gastric hemorrhages and hypersplenism). All patients were diagnosed on clinical, parasitologic, and histologic grounds to have schistosomal portal (pipestem) fibrosis that was apparently not associated with other liver diseases. Unequivocal evidence of anti-schistosome treatment was obtained for three patients. Controls consisted of liver sections from paraffin blocks that were obtained from surgical biopsies of two unrelated patients that were histologically within normal limits.

Preparation of tissues. The tissues were immediately fixed in phosphate-buffered 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. Formalin-fixed tissues were processed using standard methods of paraffin embedding and staining with hematoxylin and eosin.

Immunohistochemical studies. Immunohistochemical staining was performed on formalin-fixed paraffin sections using the avidin-biotin immunoperoxidase technique. Briefly, unstained sections mounted on polylysine-coated slides were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Nonspecific binding was blocked with 1.5% normal horse serum. Avidin and biotin binding sites contained in the samples were blocked using a commercial avidin-biotin blocking kit (Vector Laboratories Inc., Burlingame, CA). Sections were then incubated for 30 min at room temperature with the following mouse anti-human monoclonal antibodies diluted in phosphate-buffered saline (PBS) containing normal horse serum: anti-MMP-1 (2.5 μg/ml), anti-MMP-2 (5 μg/ml), anti-MMP-9 (5 μg/ml), anti-TIMP-1 (7.5 μg/ml), and anti-TIMP-2 (7.5 μg/ml) (all from Oncogene Science, Cambridge, MA). The tissue sections were washed in ice-cold saline and incubated with a

9
secondary biotinylated anti-mouse IgG. Endogenous peroxidase activity was blocked using 0.3% H2O2 in horseradish peroxidase (Vector Laboratories, Inc.). Peroxidase activity was visualized using diaminobenzidine (Vector Laboratories, Inc.). This technique uses unlabeled primary antibody, biotinylated secondary antibody, and a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. In accordance with the recommendations of the manufacturer, the avidin-biotin complex reagent contains avidin and biotinylated horseradish peroxidase reagents that were specifically prepared to form ideal complexes for immunoperoxidase staining. The slides were then rinsed in water and lightly counterstained with hematoxylin. Before the blocking procedure, the samples incubated with anti-MMP-2 and anti-MMP-9 were preincubated with 0.1% trypsin in PBS for 12 min at 37°C, as suggested by the manufacturer.

**Immunohistochemical control procedures.** Negative control immunohistochemical procedures included omission of the primary antibody from the described staining protocol and its replacement with PBS plus normal horse serum.

To rule out the possibility that no artifacts were distorting our findings, two antibodies unrelated to the MMP family or their inhibitors were used. For this control, the primary antibodies were replaced with anti-cathepsin B (5 µg/ml) or anti-cathepsin D (5 µg/ml) (Oncogene Science).

**RESULTS**

Fibrous tissue in the expanded portal spaces was negative throughout for MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 activities. A search for focal staining in areas of collagenous rarefaction and/or fragmentation within the fibrous tissue of the portal spaces was thoroughly performed, but the results were always negative. Fibroblasts and macrophages within or near the focal areas of diminished collagen density did not show reactivity. Strong expression of reactivity for metalloproteinases and their inhibitors occurred in the cytoplasm of cells and also in the interstitium of active perivascular granulomas (Figure 1). The positivity for MMPs was occasionally observed in elongated cells around the schistosome eggs, without ground staining. The more active the inflammatory reaction within the granuloma, the more accentuated the staining for MMPs and TIMPs. When multinucleated giant cells were present, they exhibited a diffuse cytoplasmic staining (Figure 2). In older granulomas, with dense and concentric fibrosis around remnants of egg shell, no metalloproteinase activity was observed, even in giant cells in the proximity of the egg shell or engulfing remnants of it (Figure 3). When antibodies against MMP-2, TIMP-1, and TIMP-2 were used, a similar pattern of staining was obtained. However, it was always less intense than when antibodies against MMP-1 were used.

Strong positive staining was regularly observed in the cytoplasm of hepatocytes and in the biliary epithelium. There was an increase in density around the perinuclear area in the former and positivity was stronger at the apical portion of the cells (Figure 4) in the latter. The best positive staining was obtained for MMP-1, which is an interstitial collagenase. Intense staining was regularly seen in the one or two rows of hepatocytes bordering the portal spaces or adjacent to the fibrous septa. Signs of increased matrix degradation (fragmentation of collagen fibers and/or foci of rarefaction) were not apparent in the vicinity of the limiting plate of hepatocytes.

Consistent negative results (lack of staining) were observed in the sections treated with anti-MMP-9, or to the unrelated antibodies against cathepsin B and cathepsin D, as well as when the primary antibody was omitted (controls).

**DISCUSSION**

Conceptually, fibrosis results from an imbalance in the equilibrium of the normal processes of synthesis and degradation of extracellular matrix components. The extent to which alterations in matrix proteolysis play a role in the development and regression of fibrosis is poorly understood.13

The cause and mechanisms by which focal matrix degradation occurs in old scars are not yet known. Our immunohistochemical study did not reveal focal activity for metalloproteinases or TIMP in the expanded fibrous tissue of the portal spaces of advanced human schistosomiasis, although light and electron microscopic evidence of multifocal collagen resorption similar to that reported in a previous work was present.4,9

Takahashi and others demonstrated that there is a parallel increase in collagen and collagenase synthesis in the liver during early murine infection with S. mansoni.14 Collagenase activity was demonstrated immunocytochemically in perivascular granulomas formed in the liver of infected mice by Biempica and others.15 Both active and inactive collagenases are secreted simultaneously with the activity of collagen synthesis during the formation of perivascular granuloma in mouse liver.16

Our present findings demonstrated that even during prolonged infection, when an active granulomatous reaction occurs, the presence of both metalloproteinases (MMP-1 and MMP-2) as well as their inhibitors (TIMP-1 and TIMP-2) can be demonstrated by immunocytochemistry. However, in the dense fibrous tissue, presumably of cicatricial origin, and where the granulomas occurred, the immunocytochemical methods used did not show the presence of the factors usually associated with extracellular matrix degradation. Even in older perivascular granulomas, when the morphologic signs of inflammatory activity disappeared and the granulomas became fibrotic, the reactivity for metalloproteinases and their inhibitors was absent. However, this does not indicate that the chronic focal degradative collagen changes seen by light and electron microscopy4,9 do not depend on the classical factors known to participate in matrix degradation. Perhaps biochemical or molecular methods, and/or immunoelectron microscopy will be necessary to rule out such a possibility. However, the present findings seem to indicate that chronic matrix degradation also shows differential features when acute and chronic matrix degradation are investigated by immunohistochemical techniques.

Three other points deserve consideration. One relates to accentuation of the staining for metalloproteinases and TIMPs at the hepatocytes bordering the portal fibrous tissue. Although focal areas of rarefaction can be found at the limits of parenchyma and stroma, we did not obtain clear evidence that this is a peculiar site of matrix degradation. However,
FIGURES 1 and 2. 1, (top) periovular granuloma in schistosomiasis exhibiting strong positivity for metalloproteinase-1 (MMP-1) (brown staining). The center is occupied by a *Schistosoma mansoni* egg, showing the dark blue staining of the miracidium structures (immunohistochemical method for MMP-1, original magnification $\times 400$). 2, (bottom) a loose-structured periovular granuloma showing positive staining for MMP-1 in the cytoplasm of fibroblasts, chronic inflammatory cells, and the interstitium. A multinucleated giant cell seen at the lower left is also strongly stained (immunohistochemical method for MMP-1, original magnification $\times 250$).
Figures 3 and 4. 3, (top) an old fibrotic granuloma centered by a giant cell engulfing remnants of the egg shell appears negative for metalloproteinase-1 (MMP-1) reactivity. However, the hepatocytes are positively stained, especially those arranged as a limiting plate around the fibrous tissue (immunohistochemical method for MMP-1, original magnification × 200). 4, (bottom) fibrotic portal space with the portal vessels being represented by dilated, thin-walled venules. The bile ducts show a brown staining of the cytoplasm of the epithelial cells, indicating the presence of MMP-1. The dense fibrous tissue discloses small focal areas of rarefaction but is negative for MMP-1 reactivity (immunohistochemical method for MMP-1, original magnification ×200).
the differential distribution of metalloproteinases and their inhibitors in hepatocytes was consistent enough to deserve further investigation.

The second point concerns staining of the biliary epithelium. Bile duct hyperplasia can sometimes be a prominent finding in experimental schistosomiasis. Since new biliary canals supposedly induce the formation of new supportive stroma, biliary hyperplasia, frequently found in experimental murine pipestem fibrosis, may contribute to increase portal fibrosis. This change has been linked to excessive proline produced by the worms, which can by itself stimulate fibrosis. Although bile duct hyperplasia was not a special feature of the cases of human schistosomal portal fibrosis considered, it may be a contributory factor not only in fibrogenesis but also in fibrolysis, as the present findings suggest.

The third point refers to anti-MMP-9. Since this reagent had been previously tested, its negativity in the present material reflects special selectivity for metalloproteinases during matrix degradation and serves as a good control for the results obtained with the other MMP types.

Taken together, our findings are consistent with the concept that matrix degradation in recent and old fibroses, besides differing at the ultrastructural level, also differ in their immunohistochemical expression of metalloproteinases and their inhibitors.

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