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# Cellular Source of Collagenase and TIMP-1 in Carrageenin-Induced Granuloma

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We have examined the occurrence and cellular localization of interstitial collagenase and TIMP-1 mRNAs in a model of granuloma induced by carrageenin in guinea pigs. Granulomas were studied at 4, 7, 10, and 14 days after carrageenin injury using a combined protocol for *in situ* hybridization and immunofluorescence. Antivimentin monoclonal antibody was used to identify fibroblasts. Avidin-FITC and Texas red horse antimouse IgG were employed for detection of probes and antibody, respectively. Our results showed that during the extracellular matrix deposit phase (4 and 7 days), interstitial collagenase and TIMP-1 mRNAs were expressed only by fibroblasts as demonstrated by the colocalization of mRNA and vimentin. By contrast, during the initiation of the resorptive phase (10 and 14 days), fibroblasts and vimentin-negative cells, probably macrophages, expressed collagenase and TIMP-1. This study suggests that fibroblasts are the cell type expressing interstitial collagenase and TIMP-1 mRNA during all phases of the evolution of carrageenin granuloma and that macrophages, by contrast, express the mRNA for the enzyme and the inhibitor exclusively in the degradative phase. 

## INTRODUCTION

Regulation of collagen metabolism plays a crucial role in maintaining structural and functional properties of the tissue, and disturbances in collagen turnover may provoke a spectrum of pathological changes. After any injury, there is usually an inflammatory response which may be followed by the partial or complete restitution of tissues, by a progressive destructive erosion of supporting structures, or by the progression to fibrosis (Woessner, 1991; Krane *et al.*, 1990; Pardo and Selman, 1991). The mechanisms involved in the heterogeneous type of response are related to the regulation of extracellular matrix macromolecules. Interstitial collagenase (MMP-1), together with other members of the matrix metalloproteinase family, as well as the family of tissue inhibitors of metalloproteinases (TIMP), has been implicated in these dynamic processes, playing an active role in tissue remodeling.

The model of carrageenin-induced granuloma in guinea pigs constitutes a useful tool for analyzing the mechanisms involved in connective tissue regulation after inflammation (Pérez-Tamayo, 1970). This model is characterized by an initial inflammatory and fibroproliferative response with active accumulation of connective tissue (deposit phase) followed by a progressive and complete healing of the granuloma (resorptive phase). Interstitial collagenase, usually associated with collagen fibers, has been demonstrated by immunofluorescence during all stages of the granuloma, although the enzyme cell source could not be determined (Pardo *et al.*, 1983). Collagenase and TIMP are produced *in vitro* under several experimental conditions by a variety of cell types, but studies concerning the *in vivo* expression in normal and pathological processes are scant.



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The aim of this study was to determine the cellular source of interstitial collagenase and TIMP-1 during the progression of the carrageenin-induced granuloma.

## MATERIALS AND METHODS

### *Experimental Model*

Carrageenin granulomas were prepared in guinea pigs as described previously (Figueras and Pardo, 1986). Groups of three animals were sacrificed at 4, 7, 10, and 14 days after the injection of carrageenin (Viscarin 402 Marine Colloids, Springfield, NJ).

### *Probe Labeling*

The human collagenase cDNA (clone pCllase 1) containing pSP64 vector and TIMP cDNA containing pTZ vector were obtained from the American Type Culture Collection. Two micrograms of probe (collagenase or TIMP) were labeled by nick translation in the presence of bio-11-dUTP. The reaction was finished when probe fragments achieved a size of about 200 bp as monitored by electrophoresis in 1% agarose gel. The probe was purified through a G-50 sephadex column and stored at −20°C until its use.

### *In Situ Hybridization and Detection*

Several fresh tissue samples of each granuloma were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline solution (PBS), pH 7.4, for 1 hr at room temperature. Tissues were rinsed in 0.2 *M* glycine in PBS (three times for 10 min each). The fixed tissues were embedded in paraffin and sections for *in situ* hybridization were cut at 4 μm and mounted on slides previously coated with 0.1% gelatin and 0.01% $CrK(SO_4)$ (Pardue, 1990). Tissue sections were deparaffinized in xylene, dehydrated through graded ethanols, and rehydrated in PBS. Cells were then permeabilized with 0.2% Triton X-100 plus 0.5% normal goat serum for 5 min at 4°C.

A combined protocol for *in situ* hybridization followed by immunofluorescence was performed essentially as described (Jiménez-García and Spector, 1993). Briefly, dried dewaxed slides were equilibrated in 2× SSC for 10 min and then hybridized at 42°C overnight in a mixture containing 100 ng of probe per slide. Avidin–FITC (Vector) was used for detection. After detection and rinsing in 4× SSC for 30 min, 0.1% Triton X-100 in 4× SSC for 10 min, 4× SSC for 10 min three times, and PBS, immunofluorescence was performed. Antivimentin monoclonal antibody (Dako) was used diluted 1:5 in PBS. Texas red horse anti-mouse IgG (Vector) was used for detection of antibody. Cells were then counterstained with 4′,6-diamidino-2-phenylindole-2HCl (DAPI) at a concentration of 1 mg/ml. Slides were mounted in 90% glycerol, 10% PBS, plus 4% (w/v) *n*-propyl gallate. Cells were examined with a Nikon FXA epifluorescence microscope equipped with a 60× 1.4 NA objective. As controls for nonspecific hybridization sections in each experiment were hybridized with labeled pSP64 or pTz (plasmids without the insert).

Tissue sections obtained at 4, 7, 10, and 14 days were also stained with hematoxylin–eosin and Masson's thricrome for conventional light microscopy.

*Cell Culture*

A human normal lung fibroblast cell line, CCD-25 Lu, and a guinea pig lung fibroblast cell line, CCL-158, were obtained from the American Type Culture Collection. The cells were cultured under standard conditions, in 25-$cm^2$ Falcon T-flasks, using F-12K medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). Guinea pig fibroblasts at early confluence were stimulated with 10 ng/ml of 4B-phorbol 12-myristate 13-acetate (PMA) in F-12K serum-free medium and incubated for 24 hr. Human and guinea pig lung fibroblasts were collected for RNA extraction.

*Northern Blot Analysis*

Total cellular RNA was isolated by the acid guanidinium thiocyanate/phenol chloroform extraction method (Chomczynski and Sacchi, 1987). Total RNA (10 μg/lane) was fractionated on a 1% agarose gel containing 0.66 *M* formaldehyde. Ribosomal RNA was visualized with ethidium bromide and the fractionated RNA was transferred onto Nytran transfer membranes (Schleicher & Schuell Keene, NH) by capillary blotting overnight. The membranes were air-dried and then baked at 80°C for 2 hr. The membranes were prehybridized at 42°C for 16 hr in 5× SSC, 50% formamide, 5× Denhardt's solution, and 0.5% SDS, containing 100 μg/ml of denatured salmon sperm DNA. Hybridization was carried out at 42°C for 16 hr in hybridization buffer containing 50% dextran sulfate plus heat-denatured $^{32}$P-labeled probe. The membranes were washed in 2× SSC, 0.1% SDS at room temperature twice for 15 min, followed by 0.1× SSC, 0.1% SDS at 42°C for 30 min and 0.1× SSC, 0.1% SDS at 55°C for 15 min. After drying, the membranes were exposed to Kodak X-AR film at −70°C with an intensifying screen. The cDNA probes were radiolabeled with [$^{32}$P]dCTP to specific activity of 200 × $10^6$ dpm/μg using a multiprime DNA labeling kit (Dupont NEP-103).

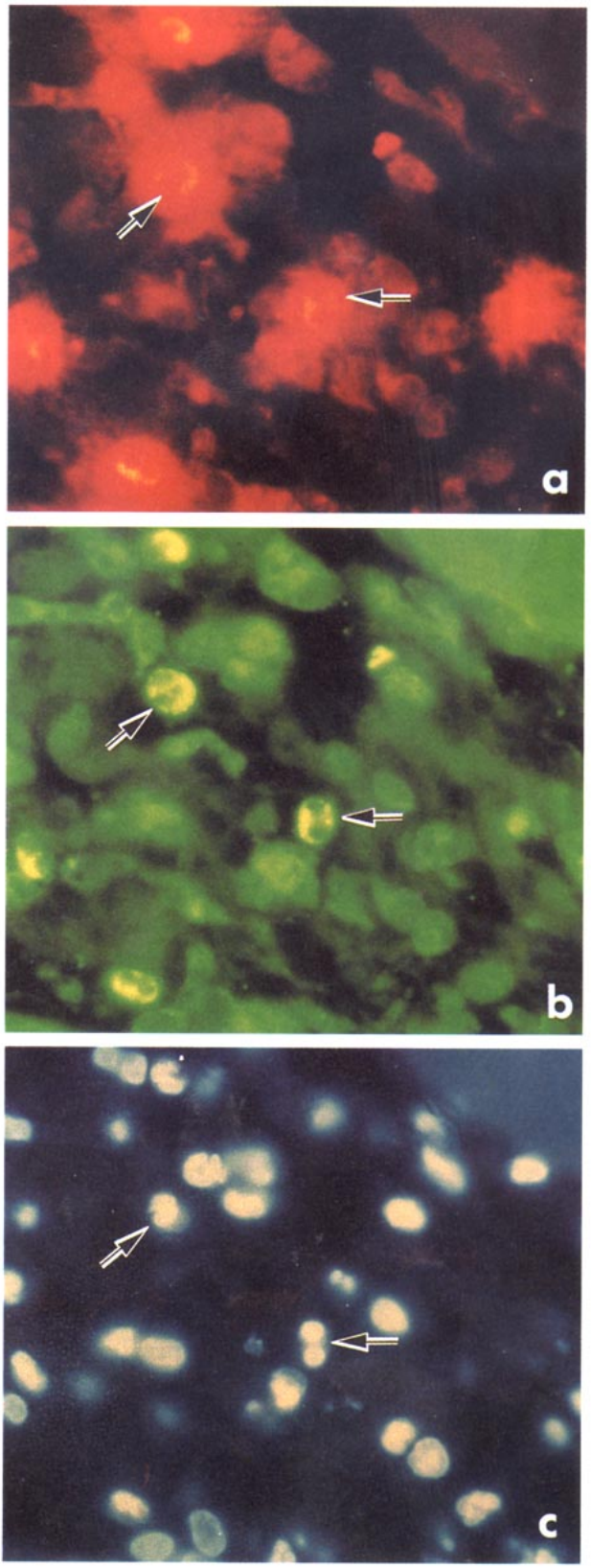
## RESULTS

A triple-labeling protocol which includes a combined *in situ* hybridization technique to detect mRNA followed by immunolocalization to visualize vimentin and counterstaining with DAPI was used. With this triple labeling we were able to observe interstitial collagenase and TIMP-1 mRNA, to distinguish fibroblasts from other cells, and to detect DNA. In addition, Nomarski's differential interference contrast microscopy was used to visualize the morphology of the tissue. Therefore, the protocol is useful for studying the spatial relationships among DNA, proteins, and mRNA.

Figure 1 exemplifies the colocalization of collagenase mRNA and vimentin in the same cells observed after 4 days carrageenin injury. In this early stage of granuloma evolution, only vimentin-positive cells were expressing the message.

Figure 2 illustrates the localization of collagenase mRNA in the different stages of the granuloma progression. At 4 and 7 days after carrageenin injury, the RNA coding for interstitial collagenase (Fig. 2c) always colocalized with vimentin im-

FIG. 1. Triple fluorescence labeled for vimentin, collagenase mRNA, and DNA in 4-day carrageenin granuloma. Arrows indicate vimentin localization by a secondary antibody coupled to Texas red (a), collagenase mRNA detection by avidin–FITC complex (b), and DNA distribution by DAPI staining (c). The cytoplasmic staining in (a) and (b) corresponds to the presence of two cells as confirmed by DAPI staining. 63×.


a
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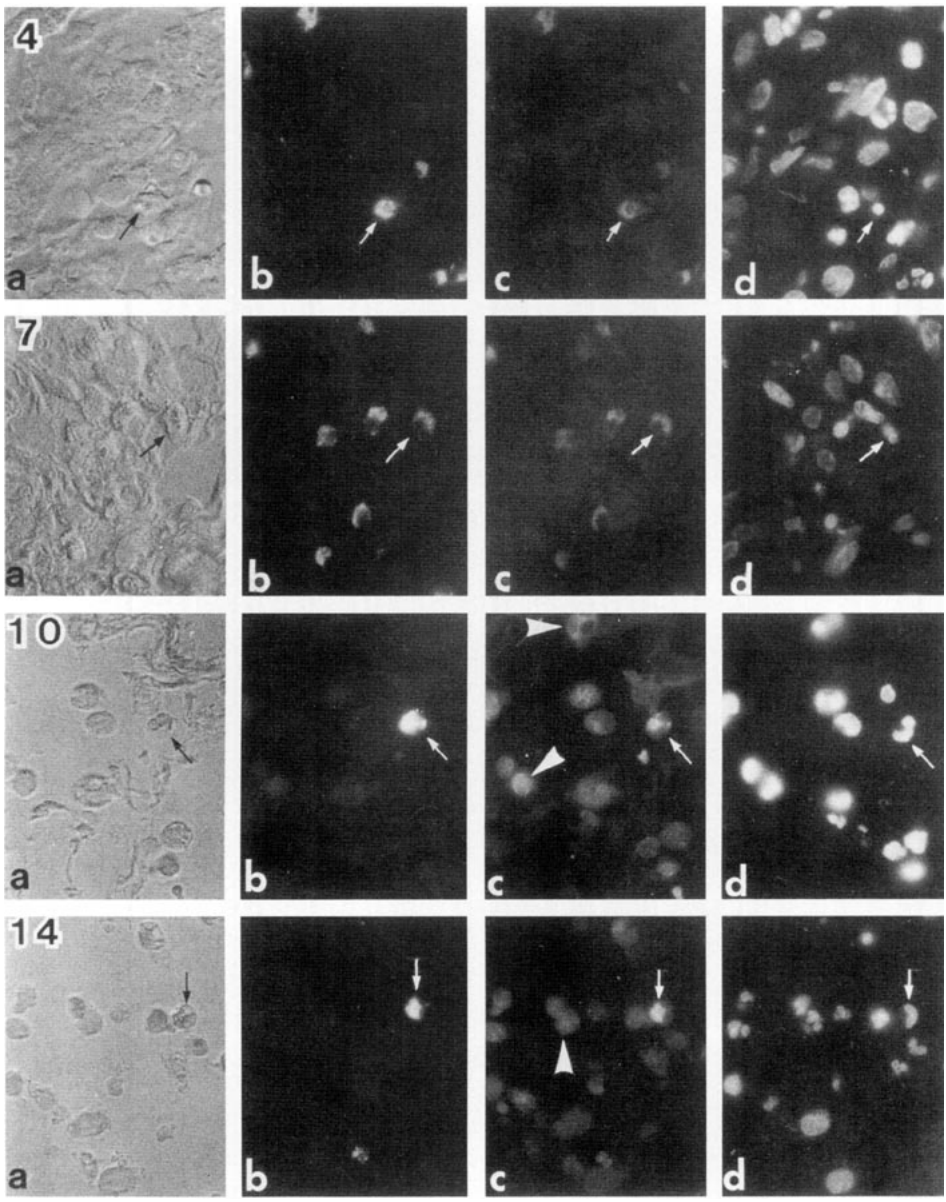


FIG. 2. Localization of collagenase mRNA in carrageenin granuloma by fluorescence *in situ* hybridization. Numbers indicate days after carrageenin injection. Panel (a) illustrates Nomarsky illumination of the same field shown in panels (b–d). Panel (c) exhibits collagenase mRNA distribution, panel (b) immunofluorescence to detect vimentin, and panel (d) DAPI staining for DNA. Arrows indicate vimentin-positive cells (b) that display the message for collagenase in the cytoplasm (c). Arrowheads illustrate vimentin-negative cells that express the specific mRNA 50×.

munofluorescence in fibroblasts (Fig. 2b). At 10 and 14 days, however, in addition to fibroblasts, some vimentin-negative cells were also stained for collagenase mRNA. Usually more positive cells were detected at 10 days. As observed by DAPI staining (Fig. 2d), the collagenase label was concentrated in the cytoplasm and excluded from the nuclei.

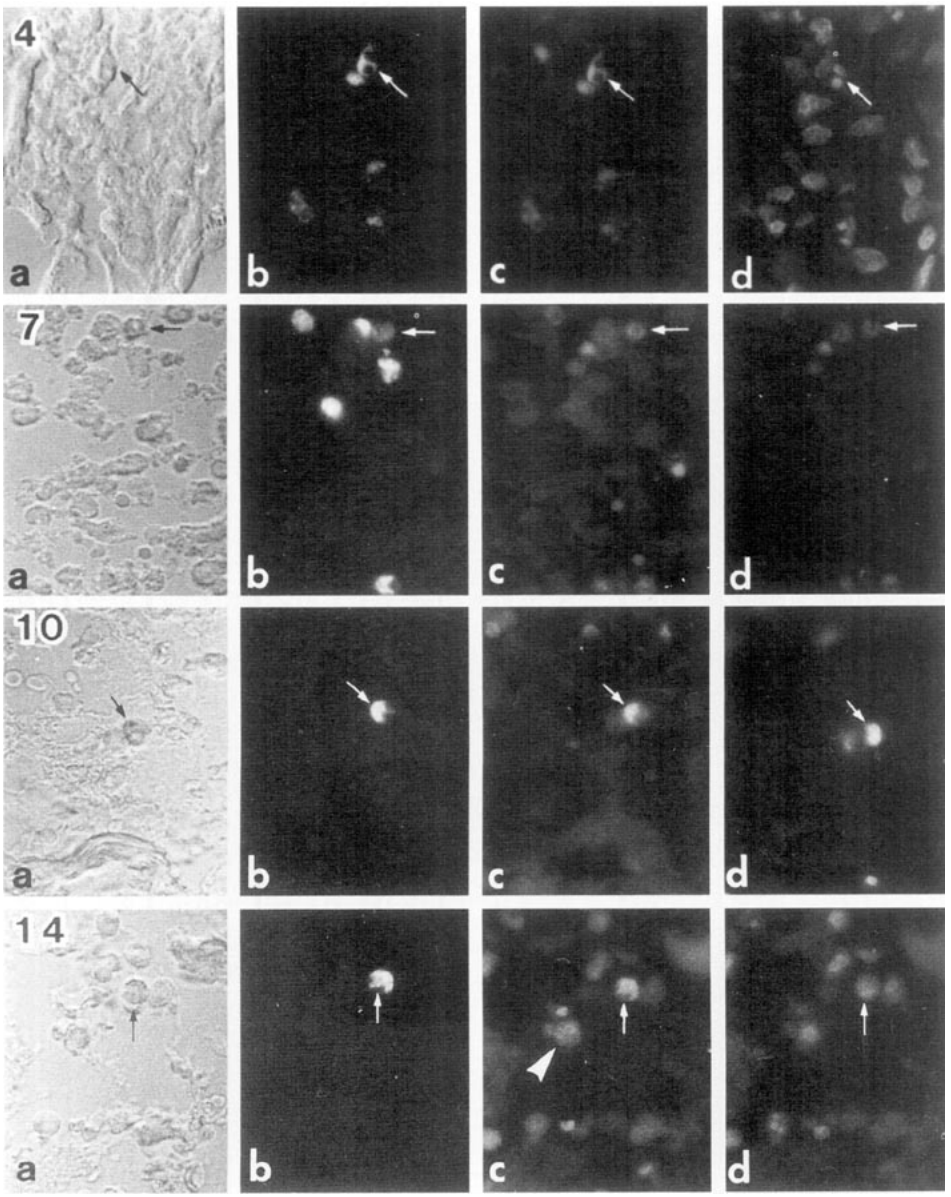


FIG. 3. Localization of TIMP-1 mRNA during the evolution of carrageenin granuloma by fluorescence *in situ* hybridization. Numbers indicate days after carrageenin injury. (a) Normarsky illumination of the same field shown in (b–d). (c) TIMP-1 mRNA expression. (b) Immunofluorescence for vimentin. (d) DAPI staining for DNA. Arrows indicate vimentin-positive cells (b) that display the message for TIMP in the cytoplasm (c). Arrowheads indicate vimentin-negative cells that express the specific mRNA. 50×.

As for the localization of TIMP-1 mRNA (Fig. 3), the staining pattern was similar to that of collagenase mRNA during all phases of the granuloma formation. At 4 and 7 days the signal was only present in fibroblasts. At 10 and 14 days, several vimentin-negative cells expressed the inhibitor. Nevertheless, in contrast to that observed with collagenase, TIMP-1 mRNA was present in more cells at 14 days than at 10 days.

Figure 4 illustrates negative controls in which biotinylated vectors without insert was used. To verify collagenase probe specificity, total RNA was isolated from human lung fibroblasts and guinea pig lung fibroblasts stimulated with PMA. In Fig. 5 the 2.5-kb transcript for human mRNA collagenase and a similar transcript for guinea pig collagenase mRNA can be observed. No other transcripts that could be related to different members of the metalloproteinase family were observed.

## DISCUSSION

The subcutaneous injection of carrageenin in guinea pigs induces a predominantly mononuclear inflammatory response, followed by fibroblasts proliferation and active deposit of extracellular matrix. Approximately 2 weeks after initial injury, connective tissue of the granuloma is under resorption, and by 3 weeks there is a complete healing of the tissue (Williams, 1957; Pardo and Pérez-Tamayo, 1974). Although it was logical to assume that during the degradative phase collagenase should be present, we previously showed that immunoreactive collagenase was universally present on the extracellular structures at both the collagen-deposition stage and the collagen-resorption stage (Pardo *et al.*, 1983). Regarding functional activity, we have demonstrated that collagen accumulation occurs in the presence of low collagenolytic activity and high levels of free TIMP, whereas a rise in collagenolytic activity with a decrease in free TIMP is observed during resorptive granuloma (Figueras and Pardo, 1986; Pardo *et al.*, 1992). In the present study, we confirmed by *in situ* hybridization that the expression of collagenase mRNA paralleled that of enzyme synthesis, and that TIMP-1 expression is also maintained during all of the progression of the granuloma.

The primary aim of this study, however, was to identify the cellular types involved in the expression of both the enzyme and the inhibitor at the different stages of the granuloma evolution. Three major cell types are present in carrag-

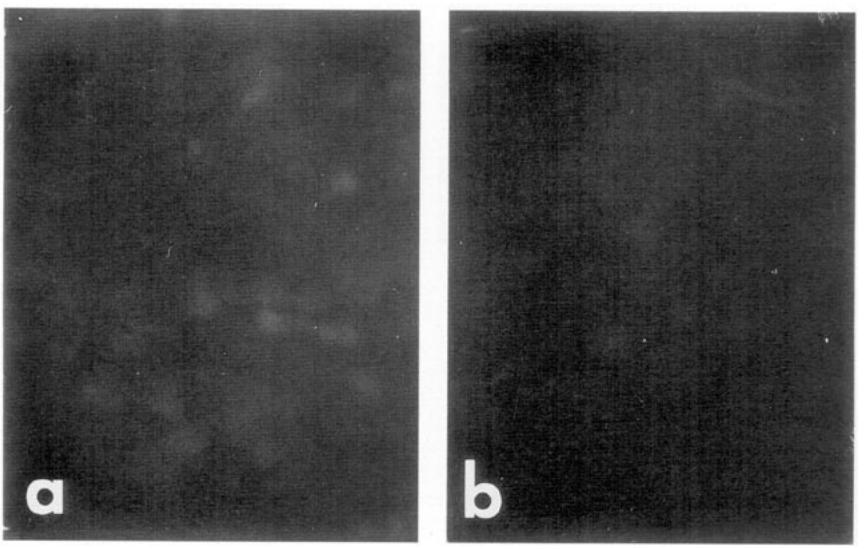


FIG. 4. *In situ* hybridization of a 10-day carrageenin granuloma using biotinylated vectors without insert, followed by detection with avidin–FITC. No signal was observed. (a) pSP64, vector for collagenase cDNA. (b) pTZ, vector for TIMP insert. 63×.

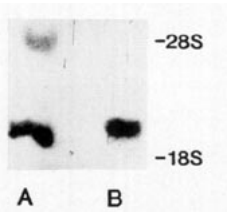


FIG. 5. Northern blot analysis for collagenase mRNA. Total RNA was prepared from guinea pig fibroblasts stimulated with PMA (A) and human fibroblasts (B). Each track was loaded with 10 μg of total RNA. Collagenase transcript appears above 18s indication.

eenin granuloma, namely, polymorphonuclear leukocytes, macrophages, and fibroblasts. Neutrophils, usually in small numbers, disappear before the onset of involution, and mononuclear phagocytes are the dominant inflammatory cells present in the early stages. The number of fibroblasts increase progressively with time and during the degradative phase they are the predominanting cellular element, always coexisting with macrophages (Pérez-Tamayo, 1970). Interestingly, in this study we found by *in situ* hybridization that during the deposit phase, when collagen is actively being deposited, only fibroblasts expressed interstitial collagenase and TIMP-1 mRNA. In all of the fields examined, exclusively vimentin-positive cells exhibited the presence of the specific messengers in spite of the fact that as it has been reported (Williams, 1957; Perez-Tamayo, 1970) that numerous macrophages are intimately mixed with the fibroblasts. This finding is similar to that reported early repair in other tissues. In this context, Girard *et al.* (1993) have illustrated that only resident stromal fibroblasts synthesized collagenase in the repairing corneal stroma, within 1 day after injury. Likewise, fibroblasts have also been demonstrated as the collagenase-synthesizing cells in healing skin as well as in a pathological skin model (Porras-Reyes *et al.*, 1991; Hembry and Erlich, 1986).

By contrast, at 10 and 14 days of granuloma progression, when the deposit phase has achieved its maximum level and massive degradation of extracellular matrix is taking place, vimentin-negative cells identified as macrophage-like cells, also expressed the messengers for both the enzyme and the inhibitor. There exists a flood of information reporting the production of collagenase and TIMP *in vitro* by stimulated macrophages (Werb and Gordon, 1975; Wahl and Winter, 1984; Welgus *et al.*, 1985; López-Escalera and Pardo, 1987), and recently the expression of the enzyme by macrophages has been shown in the rheumatoid synovial pannus *in vivo* (McCachren, 1991).

The mechanisms explaining why macrophages do not produce collagenase and TIMP in the early days of carrageenin granuloma are unknown. One possible explanation for this relatively late expression by macrophages might be related to mononuclear phagocyte differentiation. It can be assumed that granuloma macrophages arrive after carrageenin injury as monocytes and differentiate in the tissue with time. In fact, in this experimental model it has been documented that the ultrastructural aspect of macrophage cytoplasm varies with time in a rather characteristic pattern. In the early stages there are many different types of cytoplasmic inclusions, some with membranous structures, and in the later stages, by contrast, the macrophage cytoplasm depicted many small and irregular vacuoles, mostly empty or with granular material (Pérez-Tamayo, 1970). It is known that differentiation of monocytic cells in tissues involves together with morphological changes, several biochemical modifications, including a transition in the profile of

proteinases synthesized. For example, it has been shown that differentiation of U937 monocytic cells is accompanied by a markedly enhanced production of both interstitial collagenase and TIMP (Shapiro *et al.*, 1993a).

Another possible physiological explanation for the delayed macrophage commitment in collagen degradation could be related to macrophage stimulation by changes in the microenvironment. It has been proposed that modifications in the amount and composition of extracellular matrix may influence macrophage behavior. In this sense, Shapiro *et al.* (1993b) have found that exposure of human alveolar macrophages to type I and type III collagens stimulated the expression of interstitial collagenase. Concerning TIMP synthesis, they observed that although it was also increased, the magnitude of stimulation was relatively small compared to collagenase.

We can speculate that in the carrageenin model both mechanisms, monocyte differentiation and dynamic changes in the microenvironment reflected by accumulation of extracellular matrix, are responsible for the delayed collagenase and TIMP production by macrophages. Therefore, although mononuclear phagocytic cells are present almost immediately after injury, extracellular matrix deposit and progressive cell differentiation stimulate the late expression of both interstitial collagenase and TIMP-1 mRNA.

In summary, this study supports the notion that fibroblasts are the cell type expressing interstitial collagenase and TIMP-1 mRNA during all phases of the evolution of carrageenin granuloma. Macrophages, by contrast, express the mRNA for the enzyme and the inhibitor exclusively in the degradative phase.

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