Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue

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ABSTRACT **Many physiological and pathological processes involve tissue remodeling due in part to degradation of extracellular matrix. Several factors limit current approaches used for detection of matrix-degrading enzymes in tissues. Matrix metalloproteinases (MMPs), enzymes specialized in catabolism of extracellular matrix constituents, require processing from inactive zymogen precursors to gain enzymatic function. Presently available antibodies do not distinguish between precursor and proteolytically processed forms of MMPs. Also, ubiquitous tissue inhibitors of metalloproteinases (TIMPs) could prevent matrix degradation by MMPs even if the enzymes were in an active form. For these reasons immunocytochemistry does not provide information regarding the functional state of these enzymes. Biochemical studies of tissue extracts preclude localization and entail the possibility of artifactual activation of the enzymes consequent to tissue disruption. To obviate these problems, we have adapted substrate zymography to frozen tissue sections to assess net proteolytic activity in situ. We report here the details and the validation of this methodology. Initial experiments defined casein fluorescently labeled with resorufin as a useful substrate for detecting stromelysin, and fluoresceinated gelatin or autoradiographic emulsion as suitable for detecting gelatinolytic activity by this approach. Either TIMP-1 or the Zn chelator 1,10-phenanthroline reduced the zymographic activity in cryosections of atheroma from humans or rabbits. Inhibitors of serine proteases did not reduce the extent of substrate lysis substantially. In situ zymography preserves the fine morphological details of the tissue and can complement the study of enzyme expression by other microscopic techniques, such as immunocytochemistry. This approach may prove generally applicable for the detection of protease activity in tissue sections permitting exploration of the roles of these enzymes in pathobiology.—Galis, Z. S., Sukhova, G. K., Libby, P. Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue.** ***FASEB J.*** **9: 974–980; (1995)**

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VASCULAR CELLS PRODUCE IN VITRO (1–4) and in vivo (5, 6) matrix metalloproteinases (MMPs)[2] (7). Through the enzymatic digestion of various components of the extracellular matrix (ECM), MMPs probably participate in tissue remodeling during physiological and pathological processes. Two main mechanisms control MMP enzymatic activity (8). First, to attain function the secreted latent proenzyme forms of MMPs must undergo proteolytic activation. Second, ubiquitous endogenous tissue inhibitors of metalloproteinases (TIMPs) can interfere with MMP proteolytic activation and enzymatic activity. Temporal or spatial variation of active MMPs in relation to their inhibitors may regulate the local accumulation or resorption of ECM. For example, during the evolution of atherosclerotic lesions, processes that produce intimal thickening (such as vascular cell migration and proliferation) or leukocyte immigration, as well as compensatory enlargement of the arterial wall during atherogenesis, involve ECM degradation. At later stages in the plaque's natural history, focal erosion of the matrix in vulnerable areas, often in the shoulders of the fibrous cap, could favor plaque disruption (9–11). Ruptures of the plaque's fibrous cap can trigger thrombosis, which causes myocardial infarction or stroke.

We recently detected increased expression of immunoreactive MMPs in advanced human atherosclerotic lesions (5). However, available antibodies do not distinguish between the zymogen or activated forms of MMPs. A stoichiometric excess of the ubiquitous TIMPs would mitigate the effects of MMPs even if the immunoreactive enzymes were activated, hence the importance of assessing the net functional activity of MMPs in tissues. Limited availability of antibodies recognizing MMPs in various spe-

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[2]Abbreviations: ECM, extracellular matrix; PMSF, phenylmethysulfonyl fluoride; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; EDTA, ethylenediaminetetraacetic acid.

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cies also restricts the use of MMP immunodetection. Homogenization of tissue for assay of enzymatic activity precludes localization of the cell type exhibiting activity. The extraction procedure itself can artifactually activate enzymes or permit contact of enzymes and inhibitors localized in distinct compartments in the intact cells or tissues. In situ zymography, a method previously used for detection of enzymatic activity released by explants of developing amphibian tissue (12), allows preservation of the tissue structure while detecting enzymatic activity, and does not require species-specific reagents (13). We have recently applied this approach to demonstrate expression of matrix degrading activity in human atherosclerotic plaques (5). Here we describe details of the methodology and validation of this technique.

MATERIALS AND METHODS

Casein-resorufin and the fluorescein labeling kit were from Boehringer Mannheim (Indianapolis, Ind.). Brij 35, ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, and phenylmethysulfonyl fluoride (PMSF) were from Sigma Chemical Co., St. Louis, Mo. (1). Sheep polyclonal antibodies raised against human stromelysin (MMP-3) cross-reacting with rabbit MMP-3 (14) were generously provided by Constance Brinckerhoff (Darmouth Medical School, Hanover, N.H.). Anti-gelatinase A (also called 72 kDa gelatinase or MMP-2) monoclonal antibody was purchased from Molecular Oncology (Gaithersburg, Md.) and anti-human macrophage HAM-56 monoclonal antibody from Dako Corporation (Carpinteria, Calif.). Recombinant TIMP-1 was kindly provided by Michael Lark (Merck Research Laboratories, Rahway, N.J.).

Detection of enzymatic activity in sections of tissue: In situ zymography

Tissue specimens

Rabbit arterial tissue, in which experimental lesions were induced by a combination of hypercholesterolemic diet and balloon injury (15), were embedded and frozen in O.C.T. (Miles, Elkhart, Ind.) without fixation. Blocks of embedded tissue were stored at -70°C until sectioning. Fresh human arterial specimens obtained from endarterectomy or excess tissue from donor hearts were treated similarly.

Principle of in situ zymography

A specific enzymatic substrate detectable by microscopy is brought into close contact with cryostat sections of unfixed tissue. Lysis of the substrate demonstrates the presence of active enzymes and localizes sites of enzymatic activity in the tissue.

Substrates

Preliminary experiments with MMP substrates evaluated the use of various preparations based on unlabeled or fluorochrome-coupled casein or gelatin. We also tested two fluorogenic synthetic peptide MMP substrates (see below). These substrates permit detection of activity associated with stromelysins or gelatinases (5). We report here the evaluation of these various substrates for the microscopic detection of caseinolytic or gelatinolytic activity in tissue specimens. In addition, we report the use of a novel fluorescent substrate for an improved assay of in situ gelatinase activity.

Detection of enzymatic activity using fluorescent *substrates*

Substrates used were casein or gelatin coupled to fluorescent molecules. We tested casein-resorufin detectable by red fluorescence, casein-FITC (Sigma), and fluorescein-coupled gelatin emitting green fluorescence. We fluoresceinated the gelatin (Bio-Rad Laboratories, Melville, N.Y.) using a kit for fluorescent labeling (Boehringer) according to the manufacturer's instructions.

Protocol. Fluorescent substrate (1 mg/ml) was mixed (1:1) with 1% agarose melted in Tris-HCl (50 mM, pH 7.4) containing 10 mM Ca chloride and 0.05% Brij 35 (Tris buffer). The liquid mixture was spread on prewarmed glass slides by a maneuver similar to that used to produce blood smears. The film was allowed to gel at room temperature and was inspected with the microscope. We discarded slides with nonhomogeneous-appearing substrate layers. Frozen sections (6–10 μm) of unfixed tissue were cut and applied on top of the substrate film. A drop of the Tris buffer was added over each tissue section and a coverslip was placed on top. Slides, kept in a horizontal position, were incubated, light-protected, in humidified chambers at 37°C for various lengths of time (see below). Lysis of the substrate was assessed by examination under a fluorescent microscope.

Rationale. Enzymatic activity produces areas of substrate lysis shown in illuminated sections by black zones in the otherwise brightly fluorescent substrate films.

Controls. To establish the nature of the enzymatic activity, consecutive sections were layered on glass slides covered with substrates diluted in Tris buffer containing selective inhibitors of various proteolytic enzyme classes. This buffer can also be applied on top of sections. For this purpose we tested the effect of human recombinant TIMP-1, 1,10-phenanthroline, EDTA, or PMSF. Parallel incubations of specimens at 4°C can also be used for comparison.

Detection of gelatinolytic activity using photographic emulsion

Substrate. This variation used as substrate a gelatin-based photographic emulsion (NTB-2, Kodak, Rochester, N.Y.) intended for use in autoradiography.

Protocol. Frozen tissue sections were applied to uncoated glass slides and allowed to reach room temperature. Glass slides holding sections were dipped into a photographic emulsion (NTB-2, Kodak) that was diluted 1:2–1:3 with distilled water. Emulsion-covered slides were placed horizontally in humidified chambers and incubated for various lengths of time at 37°C.

At the end of incubation, the emulsion was allowed to dry and the slides were processed by photographic development using D19 developer (Kodak) according to the manufacturer's instructions. The specimens were examined under a microscope in transmitted light.

Rationale. After developing, the background of these specimens appears black due to exposure of emulsion to ambient light during incubation and processing. Lysis of gelatin in the emulsion produces transparent spots on the slides.

Optimization of conditions for in situ zymography

We determined the optimum time of incubation of the sections with the substrates by monitoring the progress of substrate lysis up to 14 days at 37°C.

Detection of enzymatic activity in tissue extracts

Fresh surgical specimens of human arterial tissues were extracted by using chilled glass homogenizers in either ice-cold 50 mM Tris-HCl, pH 7.3, containing 10 mM Ca chloride and 0.05% Brij 35 or in 10 mM Na phosphate, pH 7.2, containing 150 mM Na chloride, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, and 0.2% Na azide. Protein concentration in extracts was measured using the Bio-Rad (Bio-Rad Laborato-

ries) protein assay. MMP enzymatic activity in tissue extracts was measured by fluorometry using two different fluorescent substrates, casein-resorufin and a synthetic peptide M-1895 (16) (Bachem Biosciences Inc., Philadelphia, Pa.).

Immunocytochemistry

Serial frozen sections were used for identification of MMPs by immunocytochemistry, as previously described (5).

RESULTS

Enzymatic activity detected by in situ zymography in relation to localization of immunoreactive MMPs in arterial tissue

Normal rabbit arterial tissue did not stain for stromelysin nor did it exhibit caseinolytic activity by in situ zymography (Fig. 1, left panels). Iliac arteries of rabbits injured by balloon withdrawal and fed a hypercholesterolemic diet develop intimal lesions that contain a large number of foam cells (15), which stain for stromelysin by immunohistochemistry (Fig. 1). Areas of intense staining for stromelysin in the lesions corresponded to areas of casein digestion on serial sections (Fig. 1, right panels). Lysis of the casein overlay also occurred in regions of thrombosis (not shown). The zone of lysis usually extended into the lumen of injured vessels, suggesting that the caseinolytic activity diffused from the tissue during the incubation period. In serial sections, corresponding zones of lysis occurred in fluorescently labeled gelatin or in the gelatin-based photographic emulsion (not shown).

Specimens of human atheroma obtained at carotid endarterectomy (Fig. 2) displayed substrate digestion in areas described as being vulnerable (9–11). We previously found that these areas of atherosclerotic plaques contain immunoreactive stromelysin and produce lysis of casein (Fig. 2), as well as gelatin substrates. Proteolytic activity in these specimens is frequently associated with accumulations of inflammatory cells, identified by immunostaining (Fig. 2). Sections of normal human arterial tissue studied in parallel with plaques did not lyse the substrates (not shown).

Nature of enzymatic activity

We incubated serial sections of vascular tissues in parallel

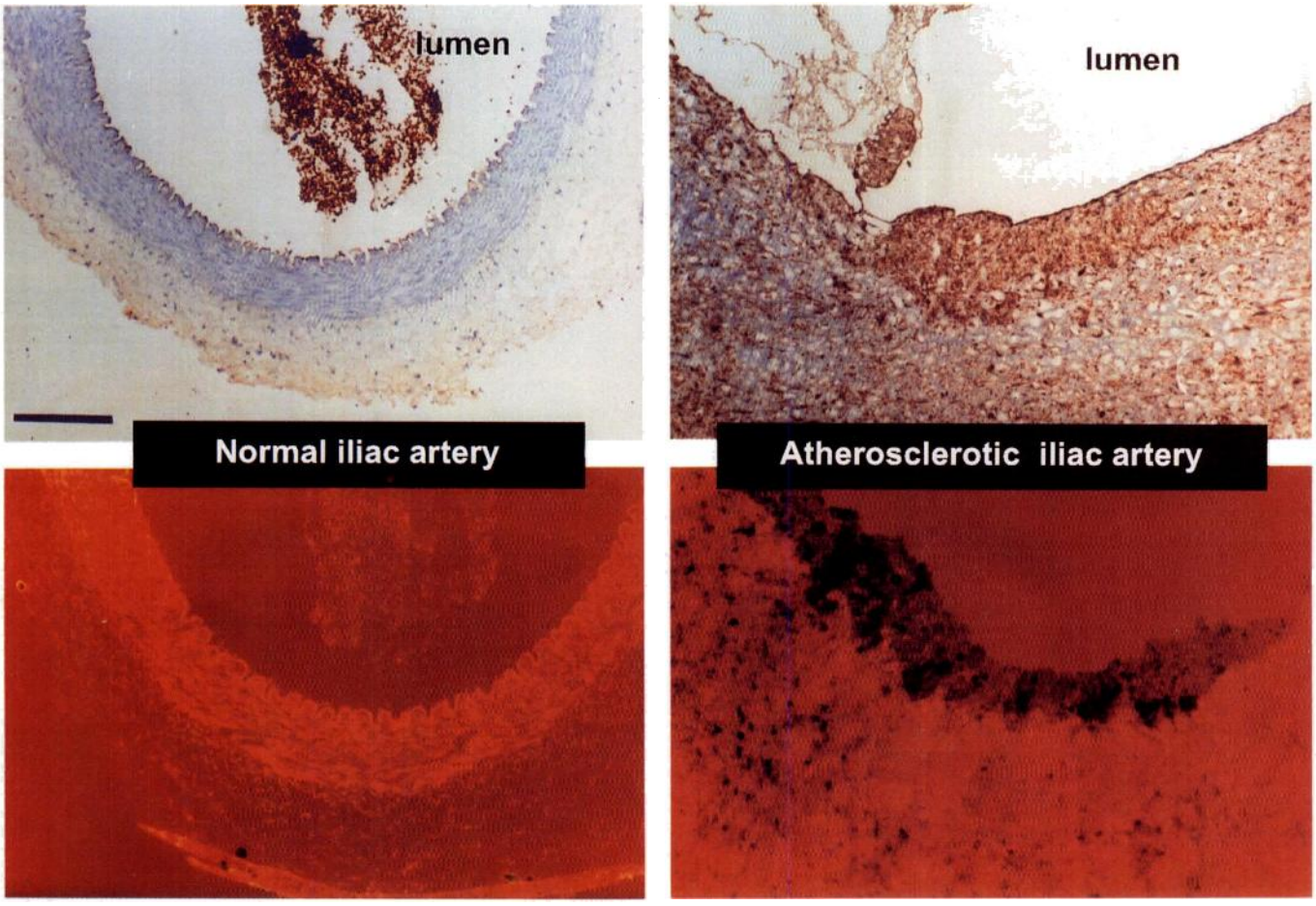

Figure 1. Rabbit arterial tissues studied by immunocytochemistry (upper panels) and by in situ zymography (lower panels). Normal or atherosclerotic iliac arteries were immunostained for stromelysin. Serial frozen sections were placed on glass slides coated with casein-resorufin and incubated at 37°C for 24 h. Sections from normal iliac arteries did not produce lysis of the fluorescent substrate (lower left). Atherosclerotic lesions displayed immunoreactive stromelysin shown by brown reaction product (upper right) and caseinolytic activity (lower right) shown by digestion of the fluorescent substrate both within the lesion and in a band surrounding the luminal surface. All panels show the specimens at the same magnification. Bar = 100 μm.

in the presence or absence of various protease inhibitors. A solution of recombinant TIMP-1 (290 μg/ml) layered over the specimens reduced the extent of casein lysis (Fig. 2). Phenanthroline, a small-molecule organic inhibitor of MMPs, abolished the lytic activity (not shown). Incubation of serial specimens in the presence of PMSF, an inhibitor of serine proteases, had little effect on casein lysis. Taken together, these findings indicate that most of the activity was due to the action of MMPs. However, serine proteases possibly present in our specimens may have also contributed to lysis of the casein substrate.

Choice of substrate

In preliminary experiments we tested as substrates for in situ zymography uncoupled proteins, including casein, from dry milk (13) or as a purified preparation (Sigma), as well as gelatin. These substrates did not yield sufficient contrast for detection by light microscope at the desired magnification. Subsequently, we tried to visualize zones of lysis in the substrate coating of the slides after using protein stains based on Coomassie brilliant blue or naphthol dyes. This approach stained both the substrate films and the tissue specimens, obscuring any possible differences.

In contrast to these initial efforts with unlabeled substrates, digestion of fluorescently tagged substrates produced high contrast and permitted following of the reaction in time by repeated observations. The red fluorescence of casein-resorufin produced better contrast than the green fluorescence of casein-FITC; it was not quenched by repeated observations of the specimens and remained visible for up to 2 wk. Because simple aqueous solutions of both casein and gelatin solutions are liquid at 37°C, we selected 1% agarose as a carrier for stabilizing the substrate film after testing several other possible candidates.

Optimum time and temperature conditions for in situ zymography

For atherosclerotic plaque specimens, visible lysis of substrate began after approximately 4–8 h of incubation at 37°C (Fig. 3). The reaction progressed more slowly at room

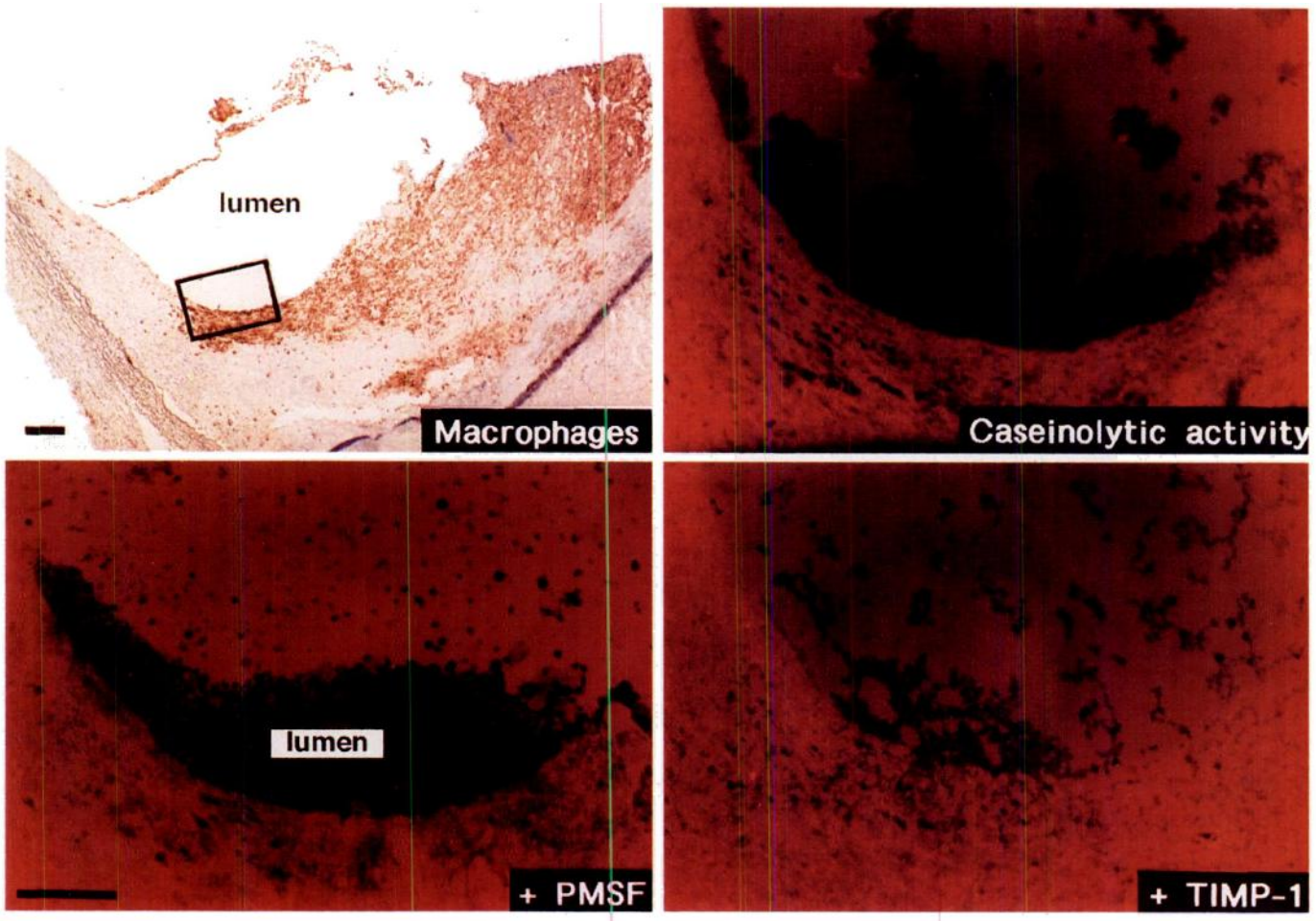

Figure 2. Effect of protease inhibitors on caseinolytic activity in advanced human atherosclerotic plaque detected by in situ zymography. Tissue obtained at endarterectomy contains abundant macrophages, identified by immunostaining with specific antibodies (upper left, bar = 100 μm). Serial sections were incubated in the absence or presence of protease inhibitors, and the area highlighted by the box is shown at higher magnification in the other three panels. Macrophage-rich areas consistently produce lysis of substrate underlying the lesion itself, but the lytic activity also diffuses away from tissue into the substrate surrounding the specimen. Inhibitors of MMPs, such as recombinant TIMP-1(290 μg/ml) or phenanthroline (not shown) reduce lysis substantially. The serine protease inhibitor, PMSF (10 mM), has less effect on digestion of the substrate. Bar = 100 μm.

temperature. Incubation of the slides at 4°C further reduced the lytic activity (not shown). The extent of substrate lysis increased with time (Fig. 3). This time and temperature dependence are as expected for an enzymatic reaction. We ultimately chose incubation of slides at 37°C for 24–72 h as optimum conditions, depending on the substrate and nature of the tissue under examination. A key practical point regarding this technique is the necessity of maintaining a high ambient relative humidity during incubations. The substrate films have a high surface area-to-volume ratio and desiccate easily, rendering interpretation difficult. Incubating the slides in a closed chamber surrounded with water-saturated paper towels suffices for this purpose.

Reproducibility of observations

Serial sections derived from 20 frozen blocks of either human or rabbit arterial tissue were analyzed in 3–5 independent experiments each. In both types of tissue, substrate lysis occurred consistently in the same areas on consecutive sections using either casein or both types of the substrates for gelatinases (not shown).

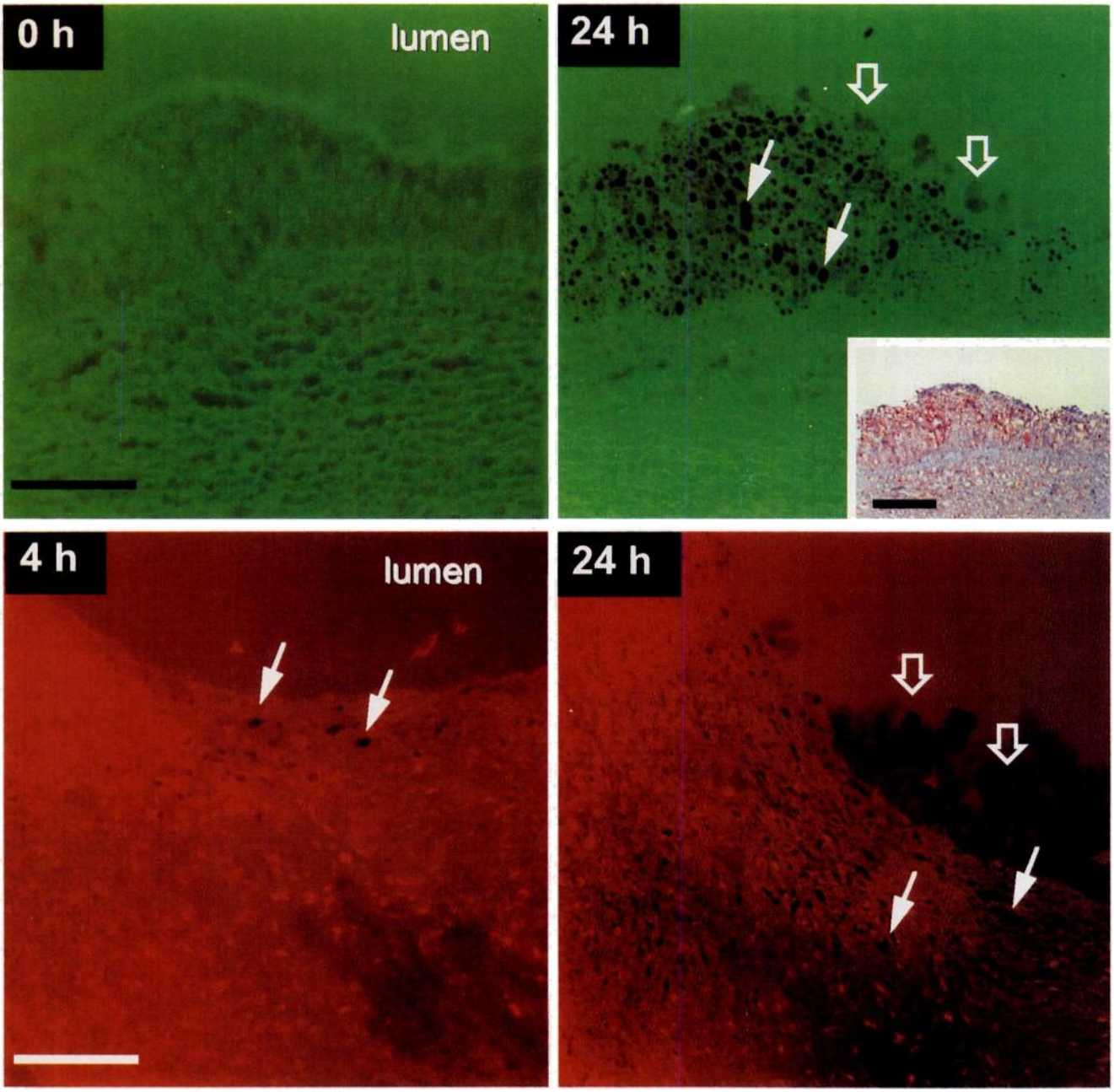

Figure 3. Substrate lysis increases with time. Upper panels: frozen sections of an atheromatous rabbit aorta examined immediately after application on a gelatin-fluorescein substrate film (0 h) and after incubation of specimen at 37°C for 24 h. Inset shows a serial section of the same specimen stained with antibodies against gelatinase A (the 72 kDa gelatinase). Lower panels: caseinolytic activity displayed by a section of human carotid endarterectomy specimen after 4 and 24 h of incubation. Thin, solid arrows point to examples of substrate digestion underneath the lesion, open arrows indicate some examples of diffusion of lytic activity into the substrate surrounding the innermost edge of lesion. Bars = 100 μm.

Detection of MMP activity in tissue extracts

The cleavage of fluorogenic substrates (casein-resorufin and peptide M-1895) by extracts of arterial tissue increased with time (data not shown). Extracts of normal or atherosclerotic tissue showed no substantial differences in their capacity to degrade the fluorescent substrates.

DISCUSSION

We describe here the details and validation of a histochemical method that enables the identification and microscopic localization of enzymatic activity within thin sections of unfixed frozen tissue specimens. We developed this method in relation to our interest in elucidating the role that MMPs may play in atherosclerosis. Assay of MMP enzymatic activity in tissue homogenates based on digestion of exogenous substrates remains experimentally challenging (17). Competition by abundant endogenous substrates and the presence of tissue inhibitors of these enzymes complicate the assay of MMPs in crude tissue homogenates. The approach described here, although qualitative, permits microscopic localization of proteinase activity. We identified several substrates suitable for the microscopic detection of caseinolytic or gelatinolytic activity. Of several different substrates tested, casein-resorufin (for stromelysin) or fluoresceinated gelatin (for gelatinases) produced the best results, as discussed earlier.

The initial protocol for localizing gelatinolytic activity using gelatin emulsion (5) proved more difficult to standardize. Slides covered with photographic emulsion cannot be coverslipped and prove very sensitive to variations in humidity during the incubation. Uneven thickness of the emulsion layer influences the quality of the results as well. In the case of fluorescent substrates, microscopic inspection allows elimination of slides that contain defects in the substrate coating before placing the tissue sections on the slides. Our later experiments using fluorescently labeled gelatin yielded more satisfactory results, comparable to those we obtained with commercially available fluorescent casein.

As previously reported, in situ zymography demonstrated that vulnerable areas of atheroma contain matrix-degrading activity decreased by inhibitors of MMPs. This finding suggested that the balance of matrix metabolism may favor matrix degradation in the shoulder regions of advanced plaques, areas known to be prone to rupture (18). Assay of tissue extracts of whole atherosclerotic lesions could easily obscure localized excess of active MMPs over inhibitors. Indeed, our attempts to demonstrate excess overall MMP activity by fluorimetric assay of tissue extracts showed no consistent difference between normal arteries and atheromatous plaques. In solution, the bulk of enzymes and inhibitors from the whole tissue sample may well overwhelm local excess of activated proteinases over inhibitors in the focal zones of lysis revealed by the in situ zymographic technique.

In situ zymography demonstrates enzymatic activity in tissue and indicates its cytological location. Serial sections can be processed for identification of cells (exemplified in Fig. 2) or enzyme protein (Fig. 1 and Fig. 3) by immunocytochemistry, as well as detection of messenger RNA expression by in situ hybridization in areas displaying substrate lysis. As in the case of similar techniques applied to tissue explants, we detected diffusion of the lytic activity from tissue sections, leading to digestion of substrate adjacent to the atherosclerotic lesion itself. Lysis of substrate outside the specimens may reflect lack of competition with endogenous extracellular matrix and absence of inhibitors.

The technique illustrated here applies directly to microscopic detection of enzymes with caseinolytic or gelatinolytic activity. Neoplasia or inflammatory diseases may also involve locally increased matrix degradation (19, 20). The substrates described in this study may prove useful for analysis of such nonvascular specimens as well. Because casein is a general protease substrate, it can be used to detect a variety of proteolytic enzymes. The specificity of digestion in this case can be assessed by comparing the effects of particular inhibitors. The approach described here is also easily adaptable to detection of proteinases with other specificities by substituting the substrate in the specimen overlays, in view of the increased commercial availability of several fluorescently labeled substrates. Such modifications should enable extension of the in situ zymographic approach reported here to the study of a variety of issues related to modulation of enzymatic activity, e.g., organ and tissue development and blood coagulation. Beyond the application to vascular biology established here, this method may help elucidate pathophysiologic mechanisms, identify potential therapeutic targets, or test the local efficacy of compounds used to inhibit MMPs or other proteinases in a variety of tissues.

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ERRATUM

The letter by Darren R. Flower [*FASEB J.* (1995) The up-and-down β-barrel proteins: three of a kind, **9**, 566] contained a typographical error in the penultimate sentence of the first paragraph. That sentence should now read: Beyond an obvious similarity of function—all three families bind small, water-insoluble molecules—this triumverate of protein families is characterized by a similar overall folding pattern: a cup or calyx-shaped antiparallel β-barrel, with a repeated +1 topology, possessed of an internal ligand binding site. This is 8-stranded and continuously hydrogen-bonded for the lipocalins and avidins but 10-stranded and discontinuous for the FABPs.

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