IMMUNOCYTOCHEMICAL LOCALIZATION OF COLLAGENASE IN HUMAN SKIN AND FIBROBLASTS IN MONOLAYER CULTURE*

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ABSTRACT

The development of a functionally monospecific antiserum to human skin collagenase has permitted the precise localization of this enzyme using fluorescence microscopy in tissue sections of human skin and in fibroblasts from primary cell culture. In human skin, collagenase is primarily localized to the upper or papillary dermis. Collagen fibers show pronounced staining suggesting that much of the enzyme present in skin is extracellular and perhaps bound to its collagen substrate. Cytoplasmic staining was also seen in fibroblast-like cells scattered in the upper dermis. Specific fluorescent staining was virtually absent in the mid- and lower dermis and in the epidermis.

Fibroblasts obtained from human skin explants displayed a granular and frequently reticulated staining pattern primarily in the perinuclear region when examined either by immunofluorescent or immunoperoxidase techniques. By radioimmunoassay the culture medium contains material immunologically identical to skin collagenase. These findings suggest that the fibroblast is the major cell responsible for collagenase production.

Specific neutral collagenases have been isolated from organ cultures of a number of human and animal tissues [1] and recent studies indicate that this group of enzymes is actively involved in the remodeling of collagen in vivo. For example, collagenase has been detected in extracts of normal human skin [2], in rheumatoid synovial fluid [3], and in involuting rat uterus [4]. Despite the apparent importance of collagenases in collagen metabolism, the cell type responsible for the production of the enzyme has not been established. In order to obtain an accurate assessment of the role of collagenase in the in vivo degradation of collagen in normal and pathologic states, it is essential to identify the cell of origin of the enzyme as well as the localization of collagenase in the extracellular elements of the tissue.

A major deterrent to the cellular localization of collagenase production has been the inability to maintain cell cultures in serum-free medium, a condition necessary for the enzymatic detection of collagenase, since these enzymes are inhibited by whole serum [5]. The availability of a functionally monospecific antiserum to human skin collagenase [6] suggested that an immunologic approach might be useful in establishing the cytotopic origin of this enzyme in the intact organism and in cell cultures of human skin fibroblasts. Immunologic detection of collagenase is more sensitive than the enzymatic assay [7] and the enzyme can be measured in tissue extracts in the presence of serum and perhaps other tissue inhibitors of collagenase activity [2, 5].

This report describes the cellular localization of human skin collagenase by immunohistochemical techniques in normal human skin under conditions where enzymatic activity cannot be detected. In addition, immunologic localization of collagenase has been accomplished in fibroblast cultures at a time when immunoreactive collagenase can be demonstrated in the culture medium in which the fibroblasts have been grown.

METHODS

Preparation of antiserum. Functionally monospecific antiserum to human skin collagenase (anti-HSC) was prepared in rabbits as previously described [6] and was taken to 40% saturation with ammonium sulfate at 0°C and pH 7.0 to obtain the gamma globulin fraction. This preparation was dissolved in 0.05 M Tris·HCl (pH 7.5) with 0.15 M NaCl (Tris·NaCl buffer), dialyzed against the same buffer, and adjusted to a protein concentration of 20 mg/ml.

Nonimmune rabbit gamma globulin was obtained from rabbits prior to immunization. Rabbit antibovine serum albumin and goat antirabbit IgG were purchased commercially (Gateway Immunochemicals). These sera were subjected to an identical 40% ammonium sulfate fractionation prior to use and their protein concentrations were adjusted to 20 mg/ml in Tris-NaCl buffer.

Preparation of fluorescein-conjugated goat antirabbit IgG. Goat antirabbit IgG was conjugated to fluorescein isothiocyanate (FITC) as described by Nairn [8]. The reaction mixture contained 2 ml of 0.2 M NaHPO, buffer (pH 9.5), 100 mg of the gamma globulin fraction of goat antirabbit IgG, and 1.25 mg of FITC (Isomer, I, Sigma Chemical Co.). The reaction mixture was stirred for 30 min at 25°C, after which the FITC-labeled goat antirabbit IgG was separated from the free FITC by gel filtration on a column (2.0 × 25 cm) of Sephadex G-25 equilibrated with 0.01 M phosphate buffer (pH 7.2). The conjugated antiserum was applied to a column (1.2 × 30 cm) of DEAE cellulose (Whatman DE-32) equilibrated in the same buffer. Elution was accomplished with a linear gradient to 1 M NaCl [9, 10]. The FITC-labeled an-
tiserum obtained by this procedure had an F/P molar ratio of 3.8 and a protein concentration of 4.9 mg/ml. Following dialysis into Tris-NaCl buffer, the samples were divided into 1 ml aliquots and lyophilized. They were subsequently stored at -20°C and protected from light until used. The conjugated antisera retained precipitin activity against rabbit IgG by Ouchterlony double diffusion [11].

Preparation of peroxidase-antibody conjugates. Goat antirabbit IgG was conjugated to horseradish peroxidase (Type IV, Sigma Chemical Co.) using glutaraldehyde as the bifunctional cross-linking reagent [12]. A typical reaction mixture contained 50 mg of horseradish peroxidase and 20 mg of goat antirabbit IgG in phosphate buffer (pH 6.9) to which was added 0.2 ml of 1% glutaraldehyde in dropwise fashion with constant stirring. After allowing the reaction to proceed for 6 hr at room temperature, the mixture was placed on a column (1.6 x 70 cm) of Sephadex G-200 equilibrated with Tris-NaCl buffer. Eluent fractions were monitored at an absorbance of 403 nm and peroxidase activity was assessed with guaiacol reagent and H_2O_2. Conjugated active fractions were pooled, dialyzed, and lyophilized for storage at -20°C.

Tissue sources. For localization of collagenase in intact human skin, 3-mm punch biopsies from healthy adult volunteers were obtained under 1% lidocaine local anesthetic. Specimens were quick-frozen and cut into 8-μ thick sections on a cryostat at -25°C.

Primary human fibroblast cultures were initiated from two 3-mm skin punch biopsies which were minced finely with a razor blade and placed in 20 ml of Dulbecco's modified Eagle's medium containing 2 mg/ml of bacterial collagenase (Clostridium histolyticum, Worthington Biochemical), 5 mg/ml of trypsin, 200 units/ml of penicillin and 200 μg/ml of streptomycin. The suspension was incubated at 37°C for 45 min with gentle stirring. Cells and tissue were sedimented by low-speed centrifugation at room temperature, the supernatant fractions were discarded, and the procedure repeated if the tissue was not sufficiently fragmented. After incubation the tissue was suspended in Dulbecco's modified Eagle's medium-HG + glutamine containing 15% fetal calf serum, 200 units/ml of penicillin and 200 μg/ml of streptomycin and placed in disposable sterile plastic culture flasks (Falcom Plastics) or glass slide-bottom flasks (Lab Tech). Cultures were maintained at 37°C in an air/CO_2 (95:5 v/v) incubator and the medium changed every 3 days. Subcultures were handled in a similar fashion.

To ensure that the majority of cells present in the monolayer cultures were primarily fibroblasts and not macrophages, which have recently been shown to contain collagenase [13], the phagocytic index was determined [14]. Confluent cultures were incubated for 24 hr in fresh medium containing polylysine latex particles 0.81 μ in diameter (Difco). The cells were washed and the number of particles ingested per cell were counted using phase contrast microscopy. In no instance was the average number of particles ingested greater than one, suggesting that there was little contamination of the fibroblast cultures with macrophages.

Staining procedures. After sectioning, tissue specimens were stained by the indirect immunofluorescent technique. The sections were incubated with a 1:30 dilution of anti-HSC gamma globulin (20 mg/ml) for 30 min at room temperature. The slides were gently washed 3-6 times in Tris-NaCl buffer, after which they were incubated with a 1:20 dilution of FITC-labeled goat antirabbit IgG for 30 min at room temperature and again washed in Tris-NaCl buffer. Tissue sections were mounted in buffered glycerol (pH 8.0) and stored, protected from light, at 4°C until viewed. Fibroblasts adhering to the glass slide bottoms of cell culture flasks were stained in an identical manner except that following removal of the culture medium the slides were rinsed with buffer and placed in 95% ethanol fixative for 30 sec. Specimens were examined with a Leitz Orthoplan fluorescence microscope equipped with a Ploem vertical illuminator (activation filter BG-12, barrier filters K-510 and K-530). The light source was a high-pressure mercury vapor 200-watt lamp.

Peroxidase staining was accomplished using rabbit anti-HSC gamma globulin in the same concentration as with the fluorescence technique and goat antirabbit IgG conjugated to horseradish peroxidase as the second layer. The peroxidase-goat antirabbit IgG was incubated on the tissue sections for 30 min at room temperature and then rinsed with Tris-NaCl buffer. The sections were subsequently incubated for 30 min in 0.05 M Tris-HCl (pH 7.6) containing 0.3 mg/ml diaminobenzidine and 0.001% H_2O_2 and examined by standard light microscopy.

Controls. Control specimens were stained in an identical fashion except that gamma globulin fractions of nonimmune rabbit serum or of rabbit antitoxine serum albumin were used as the first layer in place of anti-HSC gamma globulin. To further define the specificity of the staining, anti-HSC gamma globulin was absorbed with an excess of enzymatically active human skin collagenase. Precipitates were removed by centrifugation prior to incubation of the absorbed antiserum with the tissue specimens. Collagenase for these experiments was prepared as previously described [15] and its activity assessed on native reconstituted 14C-labeled collagen gels [16]. Protein was determined according to Lowry et al [17].

Radioimmunoassay for human skin collagenase. Culture medium was harvested at the termination of cell cultures and examined for immunoreactive collagenase by radioimmunoassay [7]. Prior to use in the assay, medium was concentrated by a 0-70% ammonium sulfate fractionation and reconstituted in a small volume of Tris-NaCl buffer. After dialysis, serial doubling dilutions of this material were examined for extent of cross-reactivity with purified human skin collagenase in standard inhibition curves.

RESULTS

Whole tissue sections. Normal human skin, stained by indirect immunofluorescence using anti-HSC gamma globulin as the first layer and FITC-labeled goat antirabbit IgG as the second layer is shown in Figure 1A. Collagenase is localized primarily to the upper or papillary portion of the dermis. There is specific green fluorescence not only of cellular elements but also diffuse staining of the collagen fibers, suggesting that much of the enzyme present in vivo is extracellular and perhaps bound to its collagen substrate. Epidermal staining is absent, and, although brightly refractile nonfluorescent fibers are present in the mid- and lower dermis, specific fluorescence appears to be limited primarily to the papillary dermis with minimal enzyme localized in the deeper dermal layers. Controls (Fig. 1B) in which nonimmune rabbit gamma globulin or rabbit antitoxine serum albumin was substituted for anti-HSC gamma globulin show no staining either of the cellular elements or of the collagen. Only refractile fibers
are present in the mid- and lower dermis. Direct staining of the sections with FITC-labeled goat antirabbit IgG was also nonreactive.

**Fibroblast cultures.** Fibroblasts obtained from human skin explants and allowed to reach near confluency were examined in a fashion identical to sections of human skin. As shown in Figure 2A and B, the fibroblasts display a granular and frequently reticulated staining pattern in the perinuclear region consistent with the organization of the endoplasmic reticulum. At this level of resolution it is not possible to determine whether the granular staining pattern indicates that collagenase is present within cytoplasmic vacuoles. Diffuse staining is occasionally present in some of the long cell processes. In cells undergoing mitosis, fluorescence staining is homogeneous and of greater intensity (Fig. 2C). Whether this merely reflects greater cytoplasmic density or represents greater synthesis of the enzyme by cells during mitosis is unclear at present. Staining of the fibroblasts is not evident when either nonimmune rabbit gamma globulin or rabbit antiovine serum albumin is used in the place of specific anti-HSC gamma globulin as the first antibody.

To confirm these observations, fibroblasts were stained with horseradish peroxidase coupled to goat antirabbit IgG. Indirect immunoperoxidase staining also reveals a granular perinuclear staining pattern in fibroblasts virtually identical to that obtained with fluorescent techniques (Fig. 3). The nonimmune rabbit gamma globulin control was again negative. The use of the immunoperoxidase technique should permit more precise enzyme localization at the resolution of the electron microscope.

Further specificity of the fibroblast staining was ascertained by reacting the cells with anti-HSC gamma globulin which had previously been absorbed with an excess of human skin collagenase. Pretreatment of the antiserum in this fashion completely abolished all specific staining of the cells.

**Radioimmunoassay of culture medium.** In addition to demonstrating collagenase immunocytochemically, fibroblasts are capable of elaborating collagenase into the culture medium as assessed by radioimmunoassay. Figure 4 shows that the standard curve obtained with serial doubling dilutions of culture medium is identical in its cross-reactivity with the standard curve of purified human skin collag enase. Identity of the immunoreactive material with pure human skin collagenase by this sensitive technique lends further support to the
concept that the fibroblast is the cellular source of collagenase.

**DISCUSSION**

Immunocytochemical techniques used in this study have permitted the precise localization of human skin collagenase in whole sections of human skin. Previous in vitro studies utilizing explants of human skin maintained on a short-term basis in serum-free medium [18] suggested that the papillary dermis was responsible for collagenase production. In addition, under certain circumstances [18, 19], the epidermis also appeared to be capable of elaborating the enzyme. The present study shows that under normal in vivo conditions, the upper, or papillary, dermis is the major site of collagenase production. Using indirect immunofluorescent staining, specific fluorescence was observed in fibroblast-like cells and on collagen fibers principally located in the upper dermis. Whether the epidermis is indeed capable of elaborating the enzyme under conditions such as wound healing [18] is currently under investigation.
Our previous studies have indicated that collagenase can be detected immunologically in tissue extracts of human skin [2, 19] and rheumatoid synovium [20] and that following chromatographic separation from serum inhibitors of collagenase, at least part of this immunoreactive material is also enzymatically active. Immunofluorescent staining patterns indicate that much of the enzyme in vivo is bound to its collagen substrate extracellularly. This suggests that the enzyme may be synthesized continuously and that at least partial control of collagenase activity occurs at an extracellular level. Whether the extracellular control of enzyme activity is accomplished by serum or perhaps other tissue collagenase inhibitors and/or by a zymogen form of the enzyme is unknown. Thus far, our studies have failed to disclose a procollagenase for human skin [20], although the existence of a zymogen for tadpole [22, 23] and mouse bone collagenases [24, 25] has been suggested.

Further localization of the cellular source of collagenase has been accomplished with cell cultures of human skin fibroblasts. The reticular staining seen in the cytoplasm of these cells is consistent with the organization of the endoplasmic reticulum. However, the granular appearance of both the immunofluorescent and peroxidase staining material in the fibroblasts may indicate that the enzyme is present, at least in part, in storage granules. Studies on the subcellular localization of the enzyme at the electron microscopic level will be needed before it can be determined whether human collagenase in skin fibroblasts can be demonstrated in lysosomes.
Although immunoreactive collagenase is detectable in fibroblast culture medium, it has not yet been possible to detect enzyme activity directly in the medium. This is understandable since the culture medium is rich in serum, a potent inhibitor of human skin collagenase [5]. The possibility that the enzyme may be complexed to other as yet unidentified inhibitors or is present as an inactive precursor has not been ruled out by these studies. Nevertheless, the fact that the immunoreactive material is identical with pure, active human skin collagenase in the radioimmunoassay provides strong evidence that fibroblasts are capable of elaborating collagenase into the culture medium.

It is of interest that in cell cultures most, if not all, of the fibroblasts show positive staining for collagenase (Figs. 2, 3). Since numerous studies [26] have shown that fibroblasts in culture produce collagen, and hydroxyproline-containing peptides are present in the cell cultures used in this study (unpublished observations), it is possible that the same cell elaborates both collagen and collagenase. The use of double labeling fluorescent antibody techniques should provide a basis for future experiments on this aspect of collagen metabolism.

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REFERENCES

10. The TH. Feltkamp TWE: Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. Immunology 18:875-881, 1970