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REPORTS

QUANTITATION AND IMMUNOCYTOCHEMICAL LOCALIZATION OF HUMAN SKIN COLLAGENASE IN BASAL CELL CARCINOMA

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Human skin collagenase was quantitated by radioimmunoassay in 21 basal cell carcinomas. Immunoreactive collagenase protein was found to be approximately 2-fold greater in extracts of these tumors than in extracts of normal skin, suggesting that this enzyme may be important in the pathogenesis of soft tissue destruction in vivo. To further define the role of collagenase in such destruction, immunofluorescent staining with specific antiserum to human skin collagenase was used to localize collagenase in the basal cell carcinomas. The enzyme was found only in the stromal elements surrounding the tumor islands. No staining of the epithelial components of the basal cell carcinomas was found. These findings suggest that the normal connective tissue elements may have been stimulated to produce an increased amount of collagenase and emphasize the importance of epithelial-stromal interaction in soft tissue invasiveness.

Basal cell carcinomas are tumors of epithelial origin which typically produce local invasion but rarely metastasize. Since the invasive properties of a variety of malignant tumors may depend in part upon proteases, as suggested by the correlation of increased proteolytic activity with both in vivo and in vitro malignancy [1, 2], basal cell carcinomas should be an ideal prototypic tumor in which to examine the mechanisms by which tumors produce local destruction and invasion. Collagen constitutes the major structural protein of skin, and one model for the pathogenesis of cutaneous tumor invasion suggests a role for collagenase, the enzyme required for the initiation of collagen breakdown [3, 4]. Both in vitro [5] and in vivo [6] studies have implicated collagenase in the invasive properties of basal cell carcinomas. However, neither the tissue responsible for the production of this enzyme nor the levels of collagenase protein in these tumors have been established.

The difficulties in accurately quantitating collagenase activity in vivo [3] have led to our use of immunologic techniques to measure collagenase in extracts of both normal [7, 8] and diseased [9, 10] tissues. This study was designed to determine whether the tumor cells of basal cell carcinoma are capable of producing collagenase and, if so, whether increased levels of the enzyme are present within an individual tumor. This was accomplished by using a specific radioimmunoassay for collagenase [8] to quantitate levels of the enzyme in basal cell carcinomas. In addition, immunofluorescent staining, employing specific antiserum to human skin collagenase (HSC), was used to localize collagenase in the tumors.

MATERIALS AND METHODS

Tissue Extracts of Basal Cell Carcinomas

A total of 21 different, histologically confirmed, basal cell carcinoma specimens were examined in this study. For determination of skin collagenase levels, two 3-mm punch biopsies were taken from within the clinically defined borders of the tumors immediately following excision. Both tissue samples were blotted on filter paper and one was used for determination of dry weight after drying for 48 hr at 130°C in an Abderhalden pistol. The second specimen, which contained both tumor cells and stromal elements, was minced finely and homgenized at 0° using 3 separate 1.0-ml aliquots of 0.05 M Tris-HCl (pH 7.5) containing 0.15 M NaCl (Tris-NaCl buffer), as described previously [8, 10]. Following brief ultrasonication of the tissue, the supernatant fractions from these 3 sequential extractions were pooled, dialyzed, and lyophilized. The lyophilized extracts were reconstituted in Tris-NaCl buffer and diluted for use in the radioimmunoassay. In addition, an aliquot of the undiluted material was examined for collagenase activity by incubation with [^{14}C]collagen fibrils (vide infra).

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Abbreviations:
HSC: human skin collagenase
iHSC: immunoreactive human skin collagenase
PBS: phosphate-buffered saline

Radioimmunoassay for Human Skin Collagenase

The radioimmunoassay for HSC was performed using a slight modification of the double antibody technique detailed previously [8]. HSC was prepared from tissue culture medium as described by Stricklin et al [11] and iodination of the pure enzyme was carried out by the lactoperoxidase method [12]. Functionally monospecific antiserum to HSC was prepared in rabbits as previously described [13]. Goat antirabbit IgG was obtained commercially (Gateway Immunochemicals).

Immunofluorescent Staining Procedures

Indirect immunofluorescent staining, using functionally monospecific antiserum to HSC as the first antibody [14] and fluorescein-labeled goat antirabbit IgG as the second antibody, was employed to localize collagenase in tissue sections of the basal cell carcinomas. Fluorescein isothiocyanate-conjugated goat antirabbit IgG (Fluorescein:protein molar ratio = 4:1) was the generous gift of Dr. Boyd K. Hartman of Washington University School of Medicine [15, 16].

To prepare the tissues for immunofluorescent staining, a portion of each tumor was fixed in 10% formalin in phosphate-buffered saline (PBS) (pH 7.4) for 48 hr at 4°C. The tissues were then placed in 20% buffered sucrose for 24 hr at 4°C [16] after which 4-μ sections were made with a cryostat. The use of buffered 10% formalin in fixation specifically prevents the loss of soluble proteins from tissues, as described in detail by Hartman [16]. Although fresh-frozen tissue yielded identical results, fixed tissue gave better resolution. The cut sections were taken up on slides previously coated with 30% egg albumin in buffered glycerol and stored at −80°C until stained [16].

For staining, sections were brought to room temperature and incubated for 15 min at 25°C with rabbit antiserum to HSC diluted 1/60 with PBS (pH 7.4) containing 0.3% Triton X-100. After washing the sections twice with PBS containing 2% Triton X-100, fluorescein-labeled goat antirabbit IgG diluted 1/80 in PBS with 0.3% Triton X-100 was added for 15 min at 25°C. The tissues were again washed with buffered 2% Triton X-100 and mounted in buffered glycerol (0.5 M carbonate, pH 8.6) [16]. In each case, control sections were obtained by substituting normal rabbit serum for rabbit anti-HSC antiserum as the first antibody.

The specificity of the staining was further defined by blocking experiments. In these studies, the diluted antiserum to HSC was preincubated with 50 ng pure HSC [11] for 30 min at 25°C. The mixture was then centrifuged to remove any precipitate and the resulting absorbed antiserum was used as the first antibody. The remainder of the staining procedure was identical to that previously described (vide supra).

The stained sections were examined with a Leitz Orthoplan fluorescence microscope equipped with a Ploem vertical illuminator with a mercury vapor lamp as the light source. The activating filter was a KP490 and the barrier filter was a K530 [16]. Photomicrographs were taken using a Leitz SLR system and high-speed Ektachrome film (Kodak ASA 160).

Other Assays

Collagenase activity was measured by the release of soluble [^{14}C]glycine-labeled peptides from native, reconstituted collagen fibrils, containing 4000 cpm per substrate gel [17] after incubation with 100 μl of active HSC obtained from organ culture as a control or undiluted tumor extract for 18 hr at 37°C. Protein was determined [18] using crystalline bovine serum albumin as a standard. Double diffusion in agar was performed according to Ouchterlony [19] using antiserum to HSC for comparison of tumor extracts of basal cell carcinomas with pure HSC obtained from tissue culture [11]. Statistical analysis was performed using Student's *t*-test.

RESULTS

Studies performed to evaluate the degree of immunologic similarity between active HSC obtained from tissue culture medium and the immunoreactive material found in tumor extracts are shown in Figure 1. Using Ouchterlony analysis there was a reaction of immunologic identity between the purified active enzyme and a representative basal cell carcinoma extract. Further evidence of the close similarity of immunoreactive collagenase (iHSC) from the tumor extracts to enzymatically active collagenase can be seen in the standard immunodisplacement curve of the radioimmunoassay (Fig. 2). The slopes of the curves obtained using serial doubling dilutions of 3 different basal cell carcinoma extracts were parallel with the standard curve of the purified active enzyme. Thus, both the gel diffusion analysis and the immunoassay confirmed that collagenases from in vitro and in vivo sources were antigenically closely related and that the radioimmunoassay could be used to quantitate the enzyme protein in tumor extracts.

Immunoreactive HSC (iHSC) was quantitated in 21 different basal cell carcinomas. As shown in the Table, the mean concentration of enzyme protein in the tumors, expressed as ng/mg tissue dry weight, was approximately 2-fold greater than that of the controls ($p < 0.02$). The range of values for the controls was 4.7–46.5 ng iHSC/mg dry weight, while that of the basal cell carcinoma group was 12.4–165.2 ng iHSC/mg dry weight. Although not included in the statistical analysis of the data, it is noteworthy that one patient had a tissue level of iHSC that was approximately 35 times the mean value (981.8 ng/mg dry weight).

The tumor extracts were also examined for collagenase activity using lysis of [^{14}C]collagen gels [17]. No significant collagenase activity could be found in any of 8 different basal cell carcinomas examined.

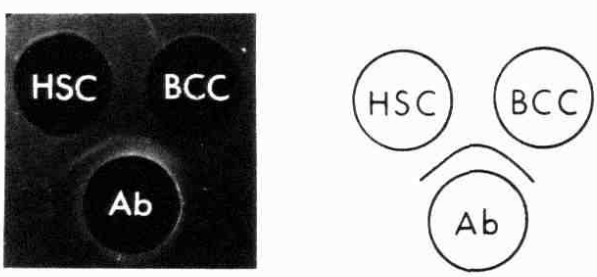

FIG. 1. Comparison of basal cell carcinoma extract with pure human skin collagenase by immunodiffusion. *HSC*, electrophoretically homogenous human skin collagenase; *BCC*, extract of a representative basal cell carcinoma; *Ab*, functionally monospecific antiserum to human skin collagenase.

To determine whether the elevated levels of iHSC in basal cell carcinomas (Tab.) were the result of increased synthesis and secretion of the enzyme by the tumor cells themselves and/or by the cells within the stromal tissue surrounding the tumor, immunofluorescent staining was employed to localize the source of the enzyme. Figure 3 depicts the staining pattern typically seen in these tumors with specific antiserum to HSC. In a low-power view of one such tumor (Fig. 3*A*), no specific staining of the basaloid cells was seen, whereas the surrounding connective tissue showed staining for collagenase. Under high power (Fig. 3*B*), the epithelial cells of the basal cell carcinoma showed no fluorescence, and specific staining was localized to the stromal components coursing throughout the tumor (Fig. 3*B*, *arrow*). In some cases, the staining appeared to surround or outline epithelial cells, but in no case was staining of the tumor cells seen. The collagenase appeared to be primarily extracellular and bound to the collagen; however, the degree of resolution of the method did not permit definitive cellular localization within the connective tissue components. Figure 3*C* depicts the identical staining pattern as seen in a second tumor.

The specificity of these findings was confirmed

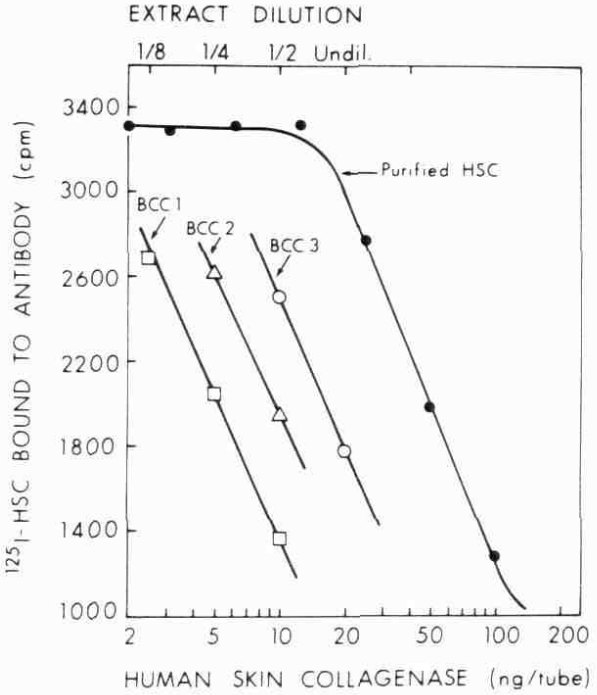

FIG. 2. Standard immunoassay curve for human skin collagenase. Aliquots of 100 μl of either pure HSC containing 0–100 ng protein or representative crude tissue extracts from 3 different basal cell carcinomas (*BCC*) were reacted in the radioimmunoassay as described in *Materials and Methods*. In control tubes, to which an equivalent amount of nonimmune rabbit gamma globulin was added, 5% of the counts was precipitated.

TABLE. *Collagenase content of basal cell carcinomas*

Patients	Number	Immunoreactive human skin collagenase (ng/mg dry weight)[a]
Control	23	27.9 ± 2.4
Basal cell carcinoma	20	50.8 ± 8.0[b]

[a] Expressed as mean ± SE.

[b] $P < 0.02$ with respect to the control value.

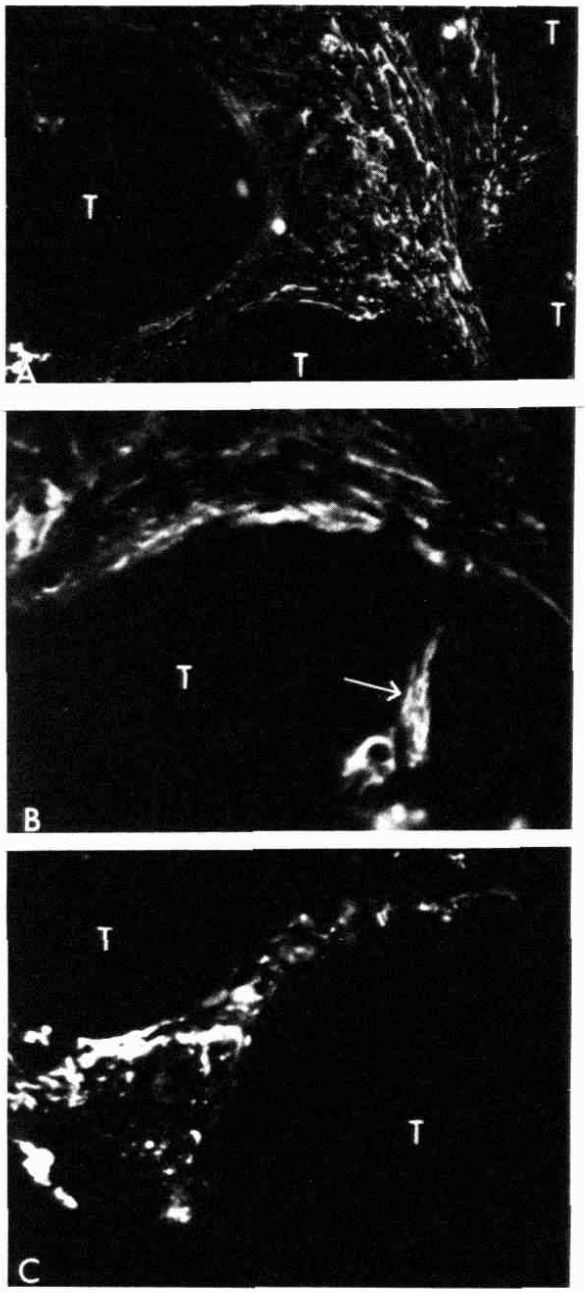

FIG. 3. Immunofluorescent localization of HSC in basal cell carcinomas. Sections were stained with rabbit anti-HSC antiserum followed by FITC-labeled goat antirabbit IgG as described in *Materials and Methods*. *A*: Low-power view of a basal cell carcinoma. Note the intense staining of stromal elements and failure of tumor islands (*T*) to stain (× 163). *B*: High-power view of the same tumor seen in (*A*). Note the staining of connective tissue components (*arrow*) in the center of a tumor island (*T*) (× 312). *C*: Fluorescent localization of HSC in a second basal cell carcinoma (× 163). After absorption of the anti-HSC antiserum with 50 ng pure HSC as noted in *Materials and Methods* this fluorescence disappeared.

in two ways. First, the substitution of normal rabbit serum for anticollagenase antiserum as the first antibody resulted in total loss of fluorescence (not shown). Second, in blocking experiments in which specific anticollagenase antiserum was previously absorbed with 50 ng of electrophoretically pure HSC, all specific fluorescence was absolished (not shown). Thus, the immunocytochemical studies demonstrate that the primary localization of collagenase in vivo is in the stromal tissue rather than in the epithelial component of the tumor.

This localization of collagenase suggested that the surrounding connective tissue elements might have been stimulated to produce increased amounts of collagenase. Thus, collagenase production was assessed by performing multiple biopsies on a large (3 cm in diameter) basal cell carcinoma to compare the iHSC content of the center of the tumor to that of the advancing margin of the tumor, where its interaction with the connective tissue might be expected to be the greatest. The collagenase content at the center of the tumor was 45.1 ng iHSC/mg dry weight, while that of the margin of the lesion was 113.3 ng iHSC/mg dry weight, approximately 2.5 times greater.

DISCUSSION

The invasion of malignant tumors almost certainly involves the degradation of collagen in the surrounding extracellular matrix. In the present study we have been able to demonstrate that the mean tissue levels of iHSC are significantly increased in biopsies of basal cell carcinomas when compared to normal control skin (Tab). This finding supports the postulate that collagenase may play a role in the destructive properties of these tumors. Immunologic techniques were required to measure the enzyme, since, unlike Yamanishi et al [6], we were unsuccessful in extracting collagenase activity directly from the tumors despite the elevation in immunoreactive collagenase protein. It should be emphasized that, although it has previously been shown that under some conditions enzymatically active forms of collagenase can be detected in tissue extracts [7, 9, 20–23], the methods employed are laborious and the estimates of collagenase activity are not quantitative. Thus, such an approach is of little value in accurately measuring the in vivo levels of collagenase in disease states. In view of the identical immunologic properties between pure HSC and the iHSC obtained from basal cell carcinoma extracts, the use of a specific radioimmunoassay has proved to be a practical approach to this problem [8, 10].

Although the reason for our inability to detect enzyme activity in vivo is at present unclear, it is possible that this may be related to the presence in the crude tissue extracts of serum inhibitors [7, 24, 25] or other tissue inhibitors [26, 27] of collagenase. In addition, collagenase has been shown to be synthesized in human skin fibroblasts as a proenzyme [11, 26] and, thus, may exist in the extracts primarily in an inactive form. It is possible that trypsin activation of the proenzyme in the tissue extracts will in the future provide a more reliable method for detecting collagenase activity in vivo.

The immunocytochemical techniques used in this study indicate that in vivo collagenase is found almost exclusively in the stromal elements around the islands of tumor rather than in the tumor cells per se (Fig. 3), as has been suggested by others [6, 28]. Thus, in any given specimen the level of iHSC appears to be a reflection of the relative amount of connective tissue in the sample. Since collagenase could, in no instance, be localized to the tumor cells themselves, it seems unlikely that they are the source of the enzyme. Nevertheless, it is possible that the tumor cells might synthesize and secrete an enzyme which is then bound to the adjacent stroma as recently demonstrated with V_2 ascites-cell carcinoma in rabbits [29]. Even though the limits of resolution of immunofluorescent staining do not permit more precise localization of enzyme synthesis, perhaps if effective methods can be developed to completely separate the tumor cells from stroma, the site of production can be definitively determined.

It should be noted, however, that the inability to detect collagenase in an epidermal-derived cell is in agreement with previous in vitro [30,31] and in vivo [14] studies in human skin which indicate that under normal conditions collagenase production is localized to the dermis. This pattern of localization of collagenase to the extracellular collagen substrate has also been observed in animal tissues (32, 33]. In addition, previous studies have established that the human skin fibroblast is the primary source of collagenase under physiologic conditions [14, 26, 34]. Thus, since the tumor cells do not appear to be the source of the enzyme, a model for local tissue destruction in which normal connective tissue elements, such as fibroblasts, have been stimulated to produce more collagenase would be consistent with the current concepts of collagenase synthesis.

It is of interest that electron microscopic studies of basal cell carcinoma demonstrate the loss of collagen adjacent to the tumor [28, 35], a morphologic finding that would also seem to correlate well with the immunofluorescent localization of collagenase. Since the tumor cells display several features which have been associated with transformed cells [35], it is possible that they may produce an as yet unidentified diffusable factor capable of stimulating collagenase production by adjacent fibroblasts. In this regard, it is of interest that our preliminary studies (unpublished) of two epidermoid carcinomas of the skin revealed a similar pattern of localization of collagenase to the adjacent connective tissue.

In a general sense, these studies emphasize the importance of epithelial-stromal interactions in tumor pathogenesis. Several investigations indicate that fibroblasts isolated from connective tissue subjacent to epithelial tumors display altered growth properties and agglutinability [36–39]. In

addition, during experimental carcinogenesis in the skin of animals, changes in dermal collagen precede the onset of epidermal neoplasia [40]. It, thus, seems likely that the alteration in collagenase content observed in patients with basal cell carcinoma may represent yet another aspect of this interaction.

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