Electron Microscopic Observations of Collagenolytic Activity of Basal Cell Epithelioma of the Skin in Vivo and in Vitro

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SUMMARY

Electron microscopic examination of the stromal collagen surrounding the tumor mass of basal cell epitheliomas of the skin revealed severe degenerative changes that led to disappearance of the normal pattern of the dermal collagen. In most cases, a complete disappearance of the dermal collagen took place and resulted in the formation of a band-like clear space. Electron microscope examination showed this space to be filled with ill-defined, amorphous substances.

Basal laminae delimiting the tumor islands were also abnormal. They were swollen in some areas, thinned out in others, and often interrupted or absent. Segment-long-spacing collagen crystallites reconstituted from tropocollagen treated with basal cell epithelioma homogenate (crude enzyme) were often cleaved at the β2 locus, namely, 75% from the A end and 25% from the B end of the whole molecule. It is postulated that the clear space surrounding basal cell epithelioma parenchyma is produced by the degradation of dermal collagen, initially by specific collagenase and perhaps subsequently by other proteases.

INTRODUCTION

In our previous communication (37) it was demonstrated that homogenate prepared from BCE4 of the skin (crude enzyme) released radioactivity from reconstituted acid-soluble collagen prepared from the skin of rats and guinea pigs which were given i.p. injections of proline-14C and glycine-14C. Caseinolytic activity of the crude enzyme was minimal, and the collagenolytic activity was not inhibited by trypsin inhibitor (soybean) (37). It was inhibited by known collagenase inhibitors such as EDTA, cysteine, and normal human serum (37). Kinetic studies demonstrated a linear correlation between collagenolytic activity and crude enzyme concentration or the length of incubation time (37). Disc electrophoresis of a crude enzyme-tropocollagen mixture showed degradation products of tropocollagen, such as αA, βA, and other bands (37). Physical properties of acid-soluble tropocollagen were also changed when tropocollagen was incubated with the crude enzyme. There was a drop in the denaturation temperature midpoint (Tm) of about 5° below that of native tropocollagen at pH 4 and a loss of specific viscosity by 30 to 60% with no significant decrease in negative optical rotation (37). The present electron microscopic studies were undertaken to examine the fine structure of the stromal collagen surrounding the tumor parenchyma in vivo and to characterize the fine structural effect of BCE collagenase on tropocollagen in vitro by means of ATP reconstitution of BCE-treated tropocollagen.

As expected, the stromal collagen immediately surrounding the tumor islands and that enclosed within the parenchyma were either absent or exhibited severe degenerative changes. Basal laminae delimiting the tumor islands showed a number of abnormalities. Studies of SLS crystallites which were reconstituted from BCE-treated tropocollagen showed that BCE collagenase cleaves the collagen molecule into 2 major fragments (TCα5, TCβ5).

MATERIALS AND METHODS

Tissue Preparation. Specimens of BCE were obtained either by curettage or excision under 1% procaine anesthesia. All tumors were located either on the face or the scalp and were diagnosed histologically except for a few clinically typical ones. Normal skin from the lower extremities was used for control studies. Parts of several specimens of BCE's and normal skins were immediately cut into 1-cu mm tissue blocks and fixed in 5% glutaraldehyde for 3 to 5 hr in 0.2 M cacodylate buffer, pH 7.4. After an overnight rinse in the same buffer, tissue blocks were postfixed with 1% osmic acid in the same buffer for 3 hr. Dehydration was carried out with 50% ethanol for 10 to 15 min, and then the tissue blocks were stained with 1% uranyl acetate in 50% ethanol for 15 min. Dehydration was continued with 60% through absolute ethanol and propylene oxide. All specimens were embedded in Araldite. Thin sections, 400 to 600 Å, were cut in a Porter-Blum MT-2 ultramicrotome and stained first with 15% uranyl acetate in 50% methanol and then with lead citrate (32). Stained sections were observed with Hitachi HU-11C and HU-12 electron microscopes at accelerating voltages of 100 and 125 kV.

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4The abbreviations used are: BCE, basal cell epithelioma of the skin; SLS, segment-long-spacing.
Preparation of Tumor Homogenate. Curedt materials were used as such. Excised specimens were separated from the s.c. fat and necrotic material. Since each tumor was small, 10 specimens were mixed to prepare about 4 ml of homogenate. The pooled specimens were minced and homogenized in an ice bath with a ground-glass homogenizer in 4 ml of 0.05 M Tris buffer, pH 7.6, containing 0.001 M CaCl2. Fractions 2A and 2B of acid-soluble tropocollagen were prepared from calf skin by the method of Rubin et al. (34) and were used in the following experiments. Data on amino acid analysis of Fraction 2B is presented in Table 1.

Preparation of Reaction Mixture. The reaction mixture consisted of (a) 4 to 5 mg of Fraction 2A or 2B of acid-soluble calf skin tropocollagen in 3 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.04 M CaCl2, and (b) 1.0 ml of the whole-tumor homogenate prepared as described above. For determination of the purity of the substrate collagen, amino acid analysis of the fraction was done with a Beckman Model 120C analyzer and the standard 4-hr analysis. Hydrolysis was accomplished by adding 2 ml of glass-redistilled, constant boiling HCl, and 1 μl of phenol (saturated with water) to 1 to 2 mg of dry, lyophilized collagen samples in an ampul. The solution was flushed with N2 several times before the ampul was sealed under vacuum. The sealed ampul was placed in a temperature-regulated oven at 105° for a hydrolysis time of 20 to 100 hr. The results are presented in Table 1.

Determination of Collagenolytic Activity of the Homogenate. As an index of collagenolytic activity of the homogenate, the reduction of viscosity was measured at 27°, a temperature well below that of collagen denaturation. An Ostwald viscometer was used, with flow time for water of 75 to 90 sec. At various times of incubation, samples were taken out and centrifuged in the cold. The viscosity of each supernatant was measured at 27°. The maximum loss of specific viscosity was 60% of the original value after 24 hr incubation.

Table 1

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Citrate-soluble</th>
<th>Tropocollagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylysine</td>
<td>7.0</td>
<td>27</td>
</tr>
<tr>
<td>Lysine</td>
<td>27</td>
<td>4.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.5</td>
<td>45</td>
</tr>
<tr>
<td>Arginine</td>
<td>51</td>
<td>17</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>94</td>
<td>45</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>45</td>
<td>33</td>
</tr>
<tr>
<td>Thioreline</td>
<td>17</td>
<td>76</td>
</tr>
<tr>
<td>Serine</td>
<td>33</td>
<td>120</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>76</td>
<td>335</td>
</tr>
<tr>
<td>Proline</td>
<td>120</td>
<td>113</td>
</tr>
<tr>
<td>Glycine</td>
<td>335</td>
<td>22</td>
</tr>
<tr>
<td>Alanine</td>
<td>113</td>
<td>4.4</td>
</tr>
<tr>
<td>Valine</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>Methionine</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.5</td>
<td>13</td>
</tr>
</tbody>
</table>

Expressed as residues per 1000 residues. Corrected for threonine and serine hydrolysis destruction but not for methionine oxidation products.

RESULTS

Light Microscopy. The dermal collagen was absent in many areas around the tumor island; thus a clear zone of several μm was seen between the parenchyma and normal dermal collagen (Fig. 1). Empty spaces were also seen within the parenchyma, forming cyst-like clear areas of various sizes (Fig. 1).

Electron Microscopy of BCE. The parenchyma was either surrounded with basal lamina (Fig. 2) or exposed directly to the stroma (Fig. 3). The basal laminae, which were present more often than not, were often swollen, ill defined, irregular in width, interrupted, or smudged (Fig. 4). Normal collagen fibrils with regular banding pattern were practically absent from the clear space except for a sporadic remnant of a small collagen island composed of thin fibrils (Fig. 3). These spaces...
were either completely empty or sparsely filled with ill-defined, amorphous substances (Fig. 5). When the basal laminae were absent, the basal cytoplasm of peripherally located tumor cells bulged out in pseudopod-like processes (Fig. 3). Some peripheral tumor cells, which lacked the support of basal lamina, were often seen dropping into the clear space. In the stroma, fibroblasts, plasma cells, and mast cells were slightly increased but not the dermal macrophages as compared with their normal counterparts in the normal dermis. Polymorphonuclear leukocytes were absent unless tumors were ulcerated and grossly infected.

**Fine Structure of Control SLS.** SLS crystallites that were reconstructed by ATP from calf skin collagen showed morphology and a banding pattern identical to those described by Hodge and Schmitt (22). In the following, the terminology and designation of bandings of Hodge and Schmitt (22) will be used. Thus, along molecular length or longitudinal dimension, from the A- or NH2-terminal towards the B- or COOH-terminal end (Fig. 6) the following dense bands were recognized: δ3, δ3, δ2, βj, δ1. When the concentration of tropocollagen was high or ATP was added quickly, several molecules were chained in sequence tail to head or B-A junctions. Slight overlapping of molecules might not occur. Extremely wide or thin SLS were rare.

**SLS of BCE-treated Tropocollagen.** Calf skin tropocollagen treated with BCE crude enzyme yielded many fragments of 25% SLS. When matched with the control SLS or intact SLS in the same preparation, it was obvious that the molecule was severed at βj and thus the fragment represented TCβj5 (Fig. 6). When ATP was added to BCE-treated tropocollagen which had been incubated at 15°C for 100 hr, many fragments of 75% SLS were observed (Fig. 6). When compared with the control SLS or intact SLS in the same preparation, these fragments could be identified with TCβj5 (Fig. 6). When TCβj5 and TCβj5 were put together, a full-length molecule could be reconstituted (Fig. 6). No other types of segments, such as TCβj2, TCβj7 (21), were observed. Etching, erosion, tapering, and obscuring of the banding pattern occurred particularly in TCβj5 fragments (Fig. 6). Polymers with B-A junction and centrosymmetrical dimers, either A-A or B-B, were frequently encountered. Extremely wide, ribbon-like SLS, either intact or segmented, and extremely thin SLS, usually intact, were also seen.

**DISCUSSION**

One mechanism by which BCE spreads was thought to be the collagenolysis of the dermal collagen (19). Pinto et al. (31) also observed, at the light microscope level, the disappearance of normal collagen fibrils and basement membrane surrounding the particular area of the epidermis where experimentally induced BCE was emerging. It is understandable that the basal lamina, which contains collagen in addition to glycoproteins (25), is also susceptible to a certain extent to the action of probably the same collagenolytic enzyme or enzymes. In the present study, ultrastructural evidence *in vivo* and *in vitro* was presented in detail. There is no way to characterize the amorphous material filling the clear zone surrounding the tumor islands by fine structural analysis. It is, however, reasonable to speculate that this material represents an end product of collagenase action upon the dermal collagen from which water-soluble components were extracted during fixation and dehydration. It is also possible that proteolytic enzymes other than collagenase acted upon the collagen molecules, particularly after the initial segmentation by the specific collagenase or collagenases. Caseinolytic activities as expressed in trypsin equivalence were, however, not significantly higher in several BCE than those of the normal skin controls (37). The caseinolytic activity of the pooled specimens used in this study was not measured. Unlike most of the tissue collagenases thus far demonstrated (1–3, 5, 9, 10, 12–17, 23, 24, 33, 36), the amount produced by BCE, or alternatively, the amount present in free, active form in BCE, is large enough to be assayed directly without resorting to cumulative techniques such as tissue culture. In this regard, BCE collagenase falls into a minority group of collagenases which can be extracted directly from cells and tissues; examples are those from human leukocytes (27, 28) and crab hepatopancreas (8), respectively. Other investigators demonstrated collagenases *in vivo* in the human skin (17), in the extract of involuting rat uterus (18), and in the tadpole tailfin (35). BCE collagenase and the collagenase from normal human skin are identical insofar as the pH optimum, effects of inhibitors, and linearity of action on a collagen substrate with respect to time and enzyme concentration (9) are concerned. Visualization of the collagenolytic action on the tropocollagen molecules by means of observing SLS with the electron microscope revealed that the action of BCE collagenase is very similar to the most commonly observed type of tissue collagenases (9, 16, 20, 24), i.e., the scission of the molecule at βj into 75% and 25% segments. It has been generally agreed that only a class of proteases called collagenase can produce this type of molecular segmentation. As it is observed in the action of other tissue collagenases, the BCE collagenase induced dimer formation, with or without a slight overlapping. Trypsin is also known to produce such dimers (4). Erosion or stepwise chewing of the SLS molecule was previously shown in Pronase-treated tropocollagen (4). The formation of a large number of thin SLS might have been due to the variation of preparative techniques, concentration of tropocollagen, etc., and probably not due to endopeptidase which cleaved telopeptides. Although we need not consider contaminations due to an admixture of tissue culture media, bacterial protease, etc., in our system, our preparation was only a crude enzyme and, therefore, it is possible that tissue proteases were responsible for the production of a variety of abnormal SLS's such as eroded segments, which could not be attributed to pure collagenase action.

The present investigation does not allow us to attribute the source of the enzyme either to the tumor cells or to the stromal elements, since the whole tumor mass was homogenized as such. In our previous study, it was demonstrated that the optimal pH for BCE collagenase is near the neutral point (7 to 8.5) (37) and that the enzymatic activity is inhibited more than 90% by normal human serum in
a dilution of 1:10 (37). Lysosomal acid hydrodrolases such as may be contained in macrophages are capable of degrading collagen (11, 21), but they do so at acid pH. Collagenase from human polymorphonuclear leukocytes is not inhibited by normal human serum at a dilution of 1:10 (27, 28). Histopathologically (29, 30) as well as by the electron microscopic examination, macrophages and leukocytes are not the major stromal elements. Fibroblasts, which might produce collagenase (6, 26), and mast cells were increased in number in the stroma as compared with their population in the normal dermis, but they were still not the major cellular component of our material. This circumstantial evidence suggests that the source of BCE collagenase may well be the tumor cell itself. Physicochemical properties of our BCE crude enzyme, i.e., pH optimum and mode of scission of tropocollagen molecules, resemble most closely those of the human skin collagenase as demonstrated with tissue culture methods by Eisen et al. (9). It seems that the same enzyme present in the normal skin has increased quantitatively in BCE.

Since BCE the major component was proliferating tumor "basal cells" of the epidermis, it is reasonable to assume that the tumor basal cell is the source of the increased enzyme activity and not the stromal components. Also, the limitation of collagen degradation to the immediate vicinity of each tumor island indicates that the tumor cells produce the enzyme. This aspect, however, should be investigated more carefully by separating the parenchyma from the stroma, since previous investigators reported that the skin collagenase is present mainly in the papillary dermis (6).

It is now reasonable to postulate that the clear spaces that surround BCE parenchyma are the product of collagen degradation, initially by collagenase and subsequently by other proteases. The importance of such collagenase activity in relation to the invasiveness of BCE is obvious; BCE seldom metastasizes but destroys bones and cartilages locally. It may be further postulated that the milieu provided by the degradative products of collagen is indeed essential for the growth and survival of BCE.

REFERENCES

32. Reynolds, E. S. The Use of Lead Citrate at High pH as an

Fig. 1. Tumor islands are surrounded by empty spaces of various sizes. Beyond such spaces, the dermal collagen appears normal. Empty spaces are formed within some islands (*). X 100.

Fig. 2. Overall view of BCE. In these areas, the basal laminae (arrows) of various thicknesses appear to surround the periphery of each tumor island without interruption. Collagenous stroma between tumor islands (*), however, is mostly absent. X 3,000.

Fig. 3. Basal lamina (solid arrow) and half-desmosomes (hollow arrow) are present in the left portion (arrow) but absent in the middle to right portion of the tumor-stroma interface. A few cytoplasmic processes bud in the areas where the basal lamina is absent (*). A small island of collagen (C) and a fibroblast (F) are present, but the rest of the stroma is completely dissolved. X 15,000.

Fig. 4. Basal lamina is swollen (between arrows) and diffused out toward the stroma (S), in which no intact collagen remains. X 87,500.

Fig. 5. Basal lamina (arrow) still exists as an ill-defined, smudged band of irregular width. Stromal collagen is completely degenerated and amorphous substances (A) fill the space. X 31,100.

Fig. 6. A comparison of cleaved molecules with normal SLS. Juxtaposition of large (middle) and small (bottom) fragments of SLS to a normal full-length SLS reveals that they are TC2 (middle) and TC3 (bottom), respectively, and the locus of cleavage is at β1. Prominent dense bands such as δ1, δ2, δ3, and δ4 are marked for reference. 120,000 X 2.5.
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