Enhanced Collagenase Production by Fibroblasts Derived from Human Basal Cell Carcinomas

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ABSTRACT

Fibroblasts derived from human basal cell carcinomas demonstrated an increased capacity to synthesize and secrete collagenase. Although the levels of collagenase were up to 8-fold greater than those of normal control cell lines, this phenotypic trait was not permanent and was expressed only for a few passages following primary explantation. The basal cell carcinoma fibroblast collagenase was secreted as a procollagenase. The kinetics of activation and the catalytic efficiency of the basal cell carcinoma fibroblast enzyme were equal to control collagenase, indicating that increased activity was due to increased synthesis of enzyme protein. Increased synthesis of collagenase was not due either to altered cell growth or to an overall increase in protein synthesis. Furthermore, synthesis of another major protein, collagen, was not enhanced. The data suggest that the tumors may have stimulated adjacent fibroblasts to produce more collagenase which is of importance in tumor invasion.

INTRODUCTION

BCC’s4 typically produce locally destructive, ulcerated lesions of the skin and deeper tissues. Since the mechanisms by which neoplasms destroy tissue locally are of pathogenetic importance in tumor invasion, these tumors should be an ideal prototype for examining this process.

Previous studies (5, 14, 36) have shown an increase in collagenase in tissue extracts and organ cultures of BCC, suggesting that the elevated levels of this enzyme might be important in the resorption of connective tissue near the tumors. Of further interest was the immunocytochemical observation that HSC was localized in vivo to the connective tissue stroma adjacent to tumor islands (5). These findings raised the question of whether the HSC protein might have been synthesized by fibroblasts in the area of the tumor rather than by the BCC itself.

In a broader sense, these and other studies done both with human (10, 11, 20, 34) and animal (7, 15, 23, 27, 30, 35) cells and tissues suggest that epithelial-stromal interactions may be of fundamental importance in tumor invasion. The present investigation was designed to assess (a) whether fibroblasts derived from BCC display altered in vitro properties, either in the expression of major gene products or in growth patterns, (b) whether any such phenotypic aberration is stable in culture and persists through many cell passages, and (c) the specificity of any altered characteristics observed in BCC-derived fibroblasts.

MATERIALS AND METHODS

Cell Cultures. Control and BCC-derived fibroblasts were processed in the same manner. Briefly, a portion of tissue measuring approximately 3 cu mm was removed from each of 9 BCC which had been therapeutically removed. In each case, the sampled tissue was taken from a nodular, nonulcerated area of the tumor. This tissue was minced finely and explanted in a 25-sq cm culture flask (Falcon Plastics, Cockeysville, Md.) in Dulbecco’s modified Eagle’s medium:high glucose plus glutamine (Microbiological Associates, Walkersville, Md.) with 0.03 M n-2-hydroxyethylpiperazine-N’-ethanesulfonic acid buffer (pH 7.6), 20% fetal calf serum, 200 units of penicillin, and 200 µg of streptomycin per ml at 37° to allow outgrowth of the fibroblasts. Histological examination of the explanted tissue specimens from different patients revealed no overall difference in the quantity of tumor or stromal tissue in each primary culture. However, variable amounts of tumor tissue and connective tissue elements were present within each individual explant. When the primary fibroblast cultures were confluent, they were sequentially passed in a 1:3 to 1:4 ratio for growth area. At the third or fourth passage, one-half of the cells from each line were frozen for later use. The remainder of the cultures was utilized directly.

Quantitation of Collagenase. In experiments to determine the concentration of IHSC in the culture medium, fibroblasts were grown to early confluence, a density at which collagenase accumulation has been shown to be greatest in normal cell lines (1). Serum-containing medium was then removed, the cells were washed 4 times with Hanks’ balanced salt solution, and the cultures were maintained for 24 hr in the same pH-adjusted (9) medium used for cell growth except that the fetal calf serum was omitted. This serum-free medium was then dialyzed against 4000 volumes of distilled water, lyophilized, and stored at -80° until assayed.

In studies to quantitate collagenase activity, fibroblasts were grown in 75-sq cm culture flasks (Falcon Plastics). At confluence, the cells were maintained for 24 hr in serum-free medium, after which the medium was made 10 µm with respect to CaCl2 (25) and assayed for collagenase activity and immunoreactive enzyme protein.

Human skin procollagenase was activated proteolytically with trypsin as described previously (6). For each enzyme preparation, a range of trypsin concentrations (0.1 to 2.0 µg trypsin per 50 µl enzyme sample) was used to ensure that maximal collagenase activity was measured. After preincubation with trypsin for 10 min at 25°, at least a 5-fold molar
TRYPsin activity. Each mixture was then assayed for collagenase activity at 37°C in 0.05 M Tris-HCl (pH 7.5) in the presence of 10 mM CaCl₂ using native reconstituted [*¹⁴C]glycine-labeled collagen fibrils containing approximately 3000 cpmp per substrate gel (19).

Immunoreactive HSC was measured by a slight modification of the double antibody radioimmunoassay previously reported (4). The pro-collagenase used as the unlabeled standard and for iodination in the radioimmunoassay was purified to homogeneity from cell culture medium as described by Stricklin et al. (28). This same enzyme preparation was used to produce specific antiserum to the enzyme (29). Standard radioimmunoassay curves were performed in duplicate in disposable plastic microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) that contained a 1:2500 dilution of the γ globulin fraction of antiserum to HSC, [*¹¹I]-labeled HSC (approximately 20,000 cpm/tube), and 0 to 100 ng of electrophoretically homogeneous HSC in a total volume of 250 μl. After incubation for 24 hr at 4°C, goat anti-rabbit IgG was added in excess, and the resulting precipitates were isolated, washed, and counted in a single-channel γ scintillation spectrometer. Unknowns, consisting of the various collagenase preparations, were reconstituted in a small volume of 0.05 M Tris:0.15 M NaCl buffer and assayed for enzyme protein in an identical fashion using 100-μl portions of serial doubling dilutions of the enzyme preparations. Controls in which nonimmune rabbit γ globulin was substituted for specific antiserum were routinely included.

Protein Synthesis Studies. General protein synthesis was measured in control and BCC-derived cells. For these experiments, the cells were grown to confluence, washed, and incubated with medium containing [*¹⁴C]proline (5 μCi/ml). After 24 hr at 37°C, the cultures were harvested, and total protein synthesis was determined as the amount of [*¹⁴C]proline incorporated into 10% trichloroacetic acid-insoluble material in the medium and cells.

Collagen Biosynthesis. For studies on collagen biosynthesis, the cells were incubated with [*¹⁴C]proline under conditions which we have previously shown to be optimal for collagen biosynthesis (32). The cells were labeled on Day 1 or 2 after the cultures had reached visual confluence since the rate of collagen biosynthesis, measured as hydroxyproline formation, is maximal at this stage of growth. Ascorbic acid (50 μg/ml) was added to the culture medium 4 hr prior to labeling. The labeling was initiated by placing the cultures in 7 ml of culture medium containing 20% dialyzed fetal calf serum, ascorbic acid (50 μg/ml), and β-aminopropionitrile fumarate (20 μg/ml). The radioactive proline (3 μCi/ml) was added, and the cultures were incubated for 20 hr by slow shaking in humidified tissue culture incubators at 37°C. At the end of the labeling period, the medium was removed, and the cell layer was rinsed with 3 ml of medium and combined with the incubation medium. Protease inhibitors were added to given final concentrations of 20 mM disodium EDTA, 10 mM N-ethylmaleimide, and 1 μM phenylmethylsulfonyl fluoride. Aliquots of the media were dialyzed against running tap water for assay of [*¹⁴C]hydroxyproline (see below). Part of the medium was precipitated with ammonium sulfate (114 mg/ml) (20% of saturation) for DEAE-cellulose chromatography.

The cell layer was rinsed 3 times with 10 ml of Hanks' balanced salt solution, and the cells were scraped using a rubber policeman into 5 ml of 0.4 M NaCl:0.1 M Tris-HCl (pH 7.5) containing the above-mentioned protease inhibitors. The cells were sonicated at 60 Hz for 30 sec. Aliquots of the cell lysates were dialyzed against running tap water for assay of [*¹⁴C]hydroxyproline as well as for assay of DNA and total cell protein.

To measure the synthesis of [*¹⁴C]hydroxyproline, the dialyzed medium and cell samples were hydrolyzed in 6 M HCl at 110°C for 24 hr. The [*¹⁴C]hydroxyproline in the hydrolysate was then separated from [*¹⁴C]proline on a 10 × 0.75-cm column of Beckman type W-2 polystyrene resin eluted with 0.02 M sodium citrate buffer (pH 3.24). Fractions of 1.0 ml were collected, and 0.4-ml aliquots were counted using a liquid scintillation spectrometer. Alternatively, in some experiments, the [*¹⁴C]hydroxyproline was assayed by a specific radiochemical method (16).

For DEAE-cellulose chromatography, the [*¹⁴C]proteins recovered from the medium by ammonium sulfate precipitation were dissolved in starting buffer consisting of 2 mM urea and 1 mM disodium EDTA in 0.025 M Tris-HCl (pH 7.5) and dialyzed against the same buffer. The samples were then chromatographed on DEAE-cellulose using a linear gradient from 0 to 0.22 M NaCl in 0.025 M Tris-HCl buffer (pH 7.5) as described elsewhere (26, 31).

Other Assays. The growth kinetics of control and BCC-derived cells were determined by seeding cultures from a single large pool at low density. Duplicate cultures were fed serum-containing medium every 2 days for the length of the experiment. Duplicate cultures were harvested daily for cell counts. Protein (18) and DNA (8) were determined using established methods.

RESULTS

A total of 9 different histologically proven BCC specimens, each from a different patient, were used. The clinical characteristics of the tumors from the individual patients are detailed in Table 1. Ten separate experiments were performed to determine IHSC levels in the fibroblast culture medium of these lines. As shown in Table 2, taken as a group irrespective of the passage level, the IHSC content of the 9 BCC-derived fibroblast lines was increased about 1.6-fold when based on DNA content of the cultures (p < 0.05) and increased about 1.5-fold when based on medium protein (p < 0.025).

Although the BCC-derived cultures as a group had significantly elevated levels of IHSC, the range of values was indicative of a substantial degree of heterogeneity within each cell line. The one factor which appeared to have a clear effect on the capacity of these cells to produce collagenase was the

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WUT 7537</td>
<td>M</td>
<td>65</td>
<td>2-cm nodule, thigh</td>
</tr>
<tr>
<td>WUT 7548</td>
<td>M</td>
<td>83</td>
<td>1-cm nodule, nose</td>
</tr>
<tr>
<td>WUT 7551</td>
<td>F</td>
<td>30</td>
<td>1.5-cm plaque, shoulder</td>
</tr>
<tr>
<td>WUT 7554</td>
<td>M</td>
<td>52</td>
<td>4-cm nodule, forehead</td>
</tr>
<tr>
<td>WUT 7571</td>
<td>M</td>
<td>82</td>
<td>3-cm nodule, neck</td>
</tr>
<tr>
<td>WUT 76115</td>
<td>M</td>
<td>67</td>
<td>1-cm nodule, nose</td>
</tr>
<tr>
<td>WUT 76116</td>
<td>M</td>
<td>70</td>
<td>1-cm nodule, cheek</td>
</tr>
<tr>
<td>WUT 76121</td>
<td>M</td>
<td>60</td>
<td>2-cm nodule, forehead</td>
</tr>
<tr>
<td>WUT 77220</td>
<td>F</td>
<td>79</td>
<td>2-cm nodule, cheek</td>
</tr>
</tbody>
</table>

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passage level. When the IHSC content of the BCC-derived cell lines was determined at each passage level (Chart 1), all 8 cell lines examined at passage 6 or earlier had significant increases in IHSC ($p < 0.005$). Some cell lines had levels ranging as high as 8-fold greater in individual experiments. In contrast, no differences were observed between BCC-derived fibroblasts and controls after the sixth passage ($p$, not significant). Furthermore, there were no differences within the control lines themselves between early and late passage (Chart 1).

These findings are more clearly illustrated by examining representative individual lines. As depicted in Chart 2A, in the fourth passage, the BCC-derived line WUT 7537 had greater than a 2-fold increase in IHSC based on medium protein and an approximate 3-fold increase based on DNA. This level persisted through the sixth passage but showed consistent reversion to control levels of IHSC thereafter. A similar trend was observed in WUT 76121 (Chart 2B) where at passage 5, there was a 3- to 4-fold increase in IHSC which decreased significantly with each serial passage. It is important to emphasize that not every BCC-derived cell line displayed the capacity to synthesize and secrete elevated amounts of IHSC through the sixth passage. As shown in the 2 cell lines depicted in Chart 2, C and D, increased IHSC was only observed in the fourth passage, the earliest level examined. These data indicate that the absolute numerical level of passage is not relevant but rather that the phenotypic characteristic could be observed only for a few passages after explantation and then was lost.

In separate experiments, we sought to define whether storage of the cells had any effect on the expression of this trait. In these studies, cells which had been stored frozen in the fourth or fifth serial passage were compared with one series of cells subcultured directly from the primary. As illustrated by the 2 lines in Table 3, BCC-derived fibroblasts stored frozen retained the capacity to synthesize and secrete increased amounts of IHSC but, like the cells subcultivated directly from the primary, reverted to control levels of enzyme within 2 to 3 additional serial passages.

In addition to determining immunoreactive enzyme protein, collagenase activity was quantitated in the BCC-derived cell lines. As shown in Chart 3, the low-passage BCC-derived fibroblast cultures displayed increased enzyme activity. In the absence of trypsin activation, all of the collagenase was found in latent form. Both the control and BCC-derived collagenases showed similar activation by trypsin and were maximally activated with 0.5 to 2.0 $\mu$g of trypsin per 50 $\mu$l of medium, depending upon the quantity of enzyme in the sample. For example, in Chart 3, the control line (WUN 76130) required 0.5 $\mu$g of trypsin for complete activation, while the tumor-derived line (WUT 7537) required 1.0 $\mu$g of trypsin for maximum activity.

Table 4 depicts a comparison between collagenase activity and immunoreactive protein in 4 representative BCC-derived cell lines. In each of 3 different experiments, collagenase activity in the BCC-derived cultures was increased over that in control lines in direct proportion to the increase in immunoreactive material. This finding was further emphasized by examining the catalytic efficiency (activity per unit immunoreactive protein) of the BCC-derived collagenases (Table 4). In each case, the catalytic efficiency ranged from 76 to 117% of the control enzyme preparations in the same experiment, indicating...
Collagenase in BCC

Effect of freeze-storage on collagenase production by BCC fibroblasts

Table 3

<table>
<thead>
<tr>
<th>Culture</th>
<th>Conditions*</th>
<th>Passage</th>
<th>Immunoreactive collagenase (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WUT 7537</td>
<td>Subcultured directly from primary</td>
<td>4</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>Stored frozen in Passage 5; thawed and subcultured through the eighth passage</td>
<td>5</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>216</td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>94</td>
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<td></td>
<td></td>
<td>8</td>
<td>94</td>
</tr>
<tr>
<td>WUT 76121</td>
<td>Subcultured directly from primary</td>
<td>6</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>Stored frozen in Passage 5; thawed and subcultured through the eighth passage</td>
<td>5</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>80</td>
</tr>
</tbody>
</table>

* In each cell line, the IHSC content of the frozen cells was compared to the IHSC content at low passage (sixth passage) of one set of cultures subcultivated directly from the primary. The IHSC content of these cultures was determined as ng/mg protein and expressed as the percentage of control.

In addition, since several studies with fibroblasts derived from other types of epithelial tumors have implied the existence of altered growth properties (10, 11, 20, 34), the growth kinetics of the BCC-derived fibroblasts were determined. As shown in Table 5, the 6 BCC fibroblast lines examined showed no alteration in mean population doubling times. The cells for all of these studies were used in the third through sixth serial passages, although no significant differences in the growth rates were observed within any given line through the tenth passage. Thus, the increased concentration of IHSC in the BCC fibroblast cultures could not be attributed to altered growth kinetics.

To characterize further the properties of the BCC-derived cells, their capacity to synthesize and secrete procollagen, another major gene product, was examined. Under the incubation conditions used, the synthesis of radioactive hydroxyproline in relation to DNA or total cell protein, was taken as an index of collagen formation. Incubation of low-passage (sixth...
passage) fibroblast cultures demonstrated that the rate of collagen synthesis in the BCC cell lines was equal to that of the control cell lines (Table 6). Examination of the secreted [14C]-hydroxyproline in the cell fraction demonstrated that after a 20-hr labeling period more than 80% of the newly synthesized collagen was found in the extracellular space in both cell types.

The capacity of these cells to synthesize genetically distinct types of procollagens, types I and III, was also assessed. To estimate the relative synthesis of the 2 procollagens, [14C]-procollagen in the medium was partially purified by ammonium sulfate precipitation and types I and III procollagen molecules were then isolated by DEAE-cellulose chromatography. The elution patterns of [14C]-labeled protein were qualitatively similar in samples obtained both from BCC and control fibroblasts (Chart 4). In all chromatograms, 2 major peaks of radioactivity, Peaks A and B, were observed. On the basis of their [14C]-hydroxyproline content, susceptibility to digestion by bacterial collagenase, α-chain composition as estimated from carboxymethyl-cellulose chromatography after limited pepsin proteolysis, and cyanogen bromide peptide mapping, Peaks A and B have been shown to contain types I and III procollagen respectively (17, 32). However, since Peak B occasionally contains noncollagenous, [14C]proline-containing proteins, the isolated types I and III procollagens were quantitated by determining the [14C]hydroxyproline in each peak (Chart 4A). The distribution of radioactivity between types I and III procollagen was relatively constant with a mean ratio of [14C]hydroxyproline in types I and III procollagen in BCC cell cultures of 6.5:1 compared to a mean value of 7.2:1 (range, 5.5 to 9.1:1) for the control cell lines.

**DISCUSSION**

The present study indicates that fibroblasts derived from explants of human BCC express, as a phenotypic trait in culture, an enhanced capacity to synthesize and secrete collagen. The increased production of collagenase appears to be a temporary rather than a permanent characteristic, since each cell line reverted to control enzyme levels within a few passages.

Like the enzyme produced by normal human skin fibroblasts (28, 29), the BCC-derived collagenase was synthesized as a proenzyme. Furthermore, its catalytic efficiency was unaltered when compared to control preparations. Thus, the collagenase obtained from these cells appears to be identical to that made by control cells. These findings stand in contrast to those in fibroblast cultures of patients with the hereditary disorder, recessive dystrophic epidermolysis bullosa, in which enhanced in vitro synthesis of a collagenase of diminished catalytic efficiency has been shown to be a stable and, therefore, presumably genetically determined trait (2, 3). Also, since the capacity for enhanced production of the enzyme was lost in the BCC-derived cells with passage, the trait is not analogous to the increased levels of plasminogen activator which have been observed in association with cellular transformation (21, 33) and represent a stable trait in transformed cells.

Although the mean increase in collagenase synthesis by the tumor-associated fibroblasts is relatively small when compared to the several hundredfold stimulation induced by activators in other non-tumor-derived systems (13), the increase in the BCC-derived fibroblast cultures appears to be a selective trait at least as shown by the failure to find any overall increase in protein synthesis. In addition, despite the approximate 2-fold overall increase in enzyme production, there was no alteration in the synthesis of collagen, a protein which constitutes the major extracellular gene product of skin fibroblasts.

The present studies do not establish the precise source of the fibroblasts which synthesize collagenase, although our previous immunohistological examinations of similar tumors suggest that the stromal elements adjacent to the tumor islands are the most likely source (5). Initially using immunological quantitation of in vivo enzyme protein, we observed an approximate 2-fold increase in IHSC in crude tumor extracts (5). Furthermore, by taking multiple samples from a single large tumor, we noted that the periphery of the tumor contained significantly greater amounts of IHSC in vivo as determined by

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**Table 6**

<table>
<thead>
<tr>
<th>Culture</th>
<th>No.</th>
<th>(14C)Procollagen synthesis</th>
<th>(14C)Procollagen synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dpm/μg DNA</td>
<td>dpm/mg protein</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>266 ± 45</td>
<td>11,150 ± 2835</td>
</tr>
<tr>
<td>BCC</td>
<td>9</td>
<td>266 ± 45</td>
<td>11,150 ± 2835</td>
</tr>
</tbody>
</table>

* Number of different cell lines examined.
* Mean ± S.E. dpm [14C]hydroxyproline incorporated per μg DNA or per mg cell protein.
* p, not significant compared to the control cell lines.
radioimmunoassay, suggesting in vivo stimulation of connective tissue elements by the tumor (5). These findings, in addition to the immunofluorescent studies in which IHSC was localized to the stroma surrounding the BCC (5), suggested that connective tissue cells might be the source of the enzyme rather than the tumor itself. The present cell culture studies thus appear to extend and support the concept that fibroblasts represent the major in vivo source of the enzyme in BCC.

Evidence obtained from a variety of investigations in humans (10, 11, 20, 34) and animals (30) suggests that epithelial-stromal interactions may be quite important in tumor pathogenesis. Although our in vitro system imposes certain limitations which prohibit a direct correlation with in vivo tissue destruction, it seems possible that the BCC may have elicited enhanced collagenase synthesis by the surrounding fibroblasts. Any mechanism whereby in vivo modulation of collagenase synthesis might occur is as yet undefined, but can be postulated to occur (a) through elaboration of a factor acting directly to increase collagenase synthesis, (b) through gene amplification (24), or (c) through a decrease in some normal suppressor mechanism for collagenase synthesis. However, factors such as proenzyme activation, substrate binding, and inhibition by specific tissue inhibitors all interact in vivo and cannot be adequately evaluated in cell culture. Indeed, it is possible that other enzymes produced by the tumor, such as plasminogen activators (12, 22), may be important in a sequence leading to activation of the latent collagenase synthesized by the fibroblasts. This biological amplification system would then result in enhanced tissue destruction and facilitate local tumor invasion.

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REFERENCES

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