AN EPIDERMAL FACTOR WHICH STIMULATES THE SYNTHESIS OF COLLAGENASE IN FIBROBLASTS IN A RECONSTITUTED DERMAL MODEL

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ABSTRACT

A dermal model was prepared by embedding rat dermal fibroblasts in three-dimensional lattices of hydrated collagen fibrils. A factor (or factors) which stimulates collagenolysis of the dermal model was found in the epidermal-cell-conditioned medium (ECCM). The factor was secreted from epidermal cells into culture media at a time point between 6 and 7 days of culture. The gel filtration chromatography indicates that the molecular weight of the factor is around 48 K. Collagenolysis was not stimulated when collagen gels were incubated in a mixture of conditioned media from cultures of epidermal cells and of fibroblasts in the dermal model, indicating that the factor is not a latent collagenase and is not an activator of the latent enzyme. That the factor is a stimulator of collagenase synthesis in fibroblasts was shown in experiments with cycloheximide and cortisol, where these chemicals completely abolished the stimulator activity in ECCM. Unlike fibroblasts in collagen gels, monolayered fibroblasts on plastic dishes were not sensitive to the factor, suggesting that the cell shape is essential for fibroblasts to respond to the factor. Several lines of evidence indicate that the epidermal factor is a unique stimulator of collagenase biosynthesis in fibroblasts which has not been reported hitherto.

Collagenase is one of the principal enzymes in the metabolism of connective tissues in both physiological and pathological processes. Many regulators of activity and production of collagenase have been reported (28): specific inhibitors such as tissue inhibitor of metallo-

proteinase (TIMP) (38, 55), steroid hormones as the suppressor (3, 11) and several stimulators such as prostaglandins (15), cytokines (27, 30) and lymphokines (13, 16, 37, 54). These regulators of collagenase have been usually found in tissues or cells cultured on plastic which is a completely artificial substrate.

Elsdale and Bard (17) tried to elaborate a method where dermal fibroblasts can be cultured in an environment similar to the *in vivo* condition. Thus, these authors developed a dermal model where fibroblasts are populated in three-dimensional lattices of hydrated collagen fibrils. The dermal model has been well characterized morphologically and biochemi-

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cally (20–22, 24, 35, 47, 48, 58–60). This model was further progressed in a skin equivalent by Bell and his associate (4), where the dermal model is overlaid with epidermal cells. In this skin model, epidermal cells adhere to the dermal model, form a multilayered epidermal sheet, and differentiate (5).

When the skin model was prepared with rat cells, we noticed an unexpectedly high level of collagenolytic activity, which led to a complete dissolution of the model (58). This activity was due to collagenase and was successfully suppressed by adding cortisol to the culture medium. In the present study, regulation of the collagenase activity in the skin model is further investigated. A new and unique stimulator of collagenase synthesis in fibroblasts was found in the culture media of epidermal cells. Some of its biological and biochemical characteristics are described in this report.

MATERIALS AND METHODS

Chemicals, Reagents and Culture Materials

Trypsin (EC 3.4.21.4) was obtained from Difco (Detroit); dispase (EC 3.4.24.4) from Godo Shusei (Tokyo); cortisol and molecular weight markers (bovine serum albumin, chymotrypsinogen A, myoglobin, ribonuclease A and cyanocobalamin) from Sigma Chemical (St. Louis, MO); Percoll and resins of Sephadex G-100 from Pharmacia Fine Chemicals (Uppsala); fetal calf serum (FCS) from Nippon Biotest (Tokyo); Dulbecco's modified Eagle's medium (DMEM) from GIBCO (Grand Island, NY); Hepes (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) from Dojin Chemical Institute (Kumamoto); antibiotics (penicillin and streptomycin) from Meiji Seika Kaisha (Tokyo); a specific inhibitor of collagenase from the bovine dental pulp (33), a gift from Dr T. Hayakawa; Cellgen (0.5% type I collagen solution) from Koken (Tokyo); Coster 12-well tissue culture clusters from Data Packing (Cambridge, MA); 24-well multiplates from Terumo (Tokyo); ultrafiltration membranes and Centriprep-10 from Grace Company (Lexington); and all other chemicals were of reagent grade, purchased from Wako Pure Chemical Industries (Tokyo) or Nacalai Tesque (Kyoto).

Animals

Newborn Fisher rats (F344, specific pathogen-free, 2–3 days after birth) were purchased from Nippon Biosup Center (Tokyo).

Cell Culture

Newborn rat skin was separated into epidermis and dermis with forceps after dispase digestion according to Kitano and Okada (34). Epidermal cells were isolated from epidermis with trypsin digestion (50), and were further purified by Percoll density gradient centrifugation (39). No fibroblasts were noticed at least up to 10 days when the preparation of epidermal cells thus obtained was cultured. Fibroblasts were collected as cells migrating from dermal explants and were subcultured (57). Both cells were stored in liquid nitrogen until use. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air. DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM NaHCO₃, 20 mM Hepes and 10% FCS was used as culture medium for epidermal cells and fibroblasts.

Preparation of ECCM (Epidermal-Cell-conditioned Medium)

Epidermal cells were plated at 1.5×10^6 per well (diameter 22 mm) with 2 ml of medium and were allowed to attach for 24 h. Then the culture medium was replaced by fresh medium (2 ml) and the cells were further cultured for the days indicated in each experiment. Culture media were collected and centrifuged at 1,500 g for 15 min. The supernatant was stored at –20°C and was used as ECCM.

Preparation of Dermal Models

Dermal models were prepared as described previously (21, 58). Fibroblasts were suspended at 2.5×10^5 cells/ml in the culture medium containing 0.2% collagen. A solution of 500 µl of the cell suspension was poured into wells (diameter 15 mm) and was allowed to form collagen fibrils at 37°C. After 24 h, the models were overlaid with 0.5 ml of fresh culture medium or ECCM and incubated for the indi-

cated period. Dermal-model-conditioned-medium (DMCM) was prepared by incubating the dermal models for 4 days at 37°C. Media were obtained by centrifuging models at 17,000 g for 10 min and used as DMCM in the experiment shown in Fig. 3.

Gel Filtration of ECCM

All manipulations were carried out at 4°C. ECCM was concentrated about 25-fold by an Amicon ultrafiltration cell with a YM-10 Diaflo membrane. Four milliliters of concentrated ECCM was applied onto a 1.6 × 90 cm Sephadex G-100 column equilibrated with an elution buffer containing 0.15 M NaCl and 10 mM Tris-HCl, pH 7.4. The column had been calibrated with molecular weight markers: bovine serum albumin (67 K), chymotrypsinogen A (25 K), myoglobin (17 K), ribonuclease A (13.7 K), and cyanocobalamin (1.3 K). For the assay of collagenase-stimulating activity, each fraction was concentrated and was exchanged with DMEM by using a Centriprep-10.

Assay for Collagenolytic Activity of Dermal Models

Dermal models were centrifuged at 17,000 g for 10 min. The supernatants were hydrolyzed in 6 N HCl at 110°C for 24 h. Concentrations of hydroxyproline in the hydrolysate were determined by the method of Bergman and Loxley (6). The collagenolytic activity of dermal models was quantitated by the amount of hydroxyproline in the supernatants. This represents the collagenase activity, because no hydroxyproline was detected when the dermal model was incubated in the presence of a specific collagenase inhibitor purified from the bovine dental pulp. ECCM itself showed collagenolytic activity, which was less than 7% of that of the dermal model in most cases. The collagenase activity of the dermal model was obtained by subtracting the activity of ECCM when the model was incubated with ECCM.

RESULTS

Effect of ECCM on Collagenase Activity of Dermal Models

The skin model showed a high activity of col-

lagenolysis. This collagenolysis resulted from degradation of collagen fibrils by collagenase produced by fibroblasts and/or by epidermal cells, because the presence of a collagenase-specific inhibitor (1 unit/ml) or cycloheximide (100 ng/ml) completely inhibited the collagenolysis. Previous study has also shown that the skin model expresses collagenase activity which is higher than that obtained by arithmetical summation of activities of the epidermal cells and dermal model (58). This synergetic activity of collagenase in the skin model suggests the presence of epidermis-dermis interactions for the expression of collagenase of each of both epidermal cells and fibroblasts. To biochemically clarify an aspect of this interaction, ECCM was prepared and was placed onto the dermal model. ECCM was obtained by culturing epidermal cells for 6 days in the presence or absence of FCS. ECCM markedly stimulated the collagenase activity of dermal models: about 6-fold stimulation as compared to unconditioned fresh medium was observed (Fig. 1). The presence of FCS during the culture of epidermal cells did not affect the extent of stimulation.

Fig. 2 depicts a time course of response by fibroblasts in dermal models to ECCM. Fibroblasts started to respond to ECCM at day 3 of culture and were activated enough at day 4. In experiments described below, the collagenase activity of fibroblasts was measured at day 4 of culture with ECCM.

The Presence of A Stimulator in ECCM for Collagenase Production by Fibroblasts

The results shown in Fig. 1 and Fig. 2 suggest four possibilities as to the activation of collagenase in the dermal model. First, the latent collagenase (procollagenase) produced by fibroblasts is activated by some protease in ECCM. Second, procollagenase in ECCM is activated by proteolytic enzymes from fibroblasts. Third, the first and second possibilities occur simultaneously. Fourth, ECCM contains a stimulator which activates the synthesis of collagenase in fibroblasts in the dermal model.

To see which of the four possibilities is likely, DMCM was prepared and mixed with ECCM. The mixture was assayed for the collagenolytic activity (Fig. 3). ECCM alone

Fig. 1 Activation of dermal collagenase by epidermal-cell-conditioned medium (ECCM). ECCM was prepared in the presence (10%) or absence of fetal calf serum (FCS) and was overlaid onto the dermal model. ECCM which had been prepared in the absence of FCS was supplemented with FCS at a final concentration of 10% and was then overlaid on the dermal model. Collagenase activity was determined at day 4 of culture as detailed in the text, and expressed as μg of hydroxyproline from the dermal model in a 15-mm well. The values are averages of three determinations. SE was within 15% of the mean. a, ECCM with FCS; b, ECCM without FCS; c, unconditioned fresh medium

Fig. 2 Responsiveness of fibroblasts to ECCM. Dermal models were prepared and left 24 h before exposure to ECCM, at zero day. Each value represents a single determination. $\square$ — $\square$, without ECCM; $\blacktriangle$ — $\blacktriangle$, with ECCM

Fig. 3 Collagenase activity of ECCM and DMCM. ECCM was incubated alone, with DMCM, or with the dermal model for 4 days and the collagenase activity was then determined. Each column represents the average of two determinations shown by closed circles. a, ECCM alone; b, DMCM alone; c, ECCM with DMCM; d, ECCM with the dermal model

showed some activity, while DMCM alone had no activity. No stimulation of collagenolysis was observed in the mixture of ECCM and DMCM as compared to that of ECCM alone or that of the dermal model with ECCM where about 7.5-fold stimulation was obtained. This result supports the fourth possibility.

The presence of an epidermal stimulator for the synthesis of collagenase by the dermal model was further supported by the experiment, where dermal models were incubated with ECCM in the presence of cortisol (200 ng/ml) or cycloheximide (3 $\mu\text{g}/\text{ml}$). Cortisol is known to suppress the synthesis of collagenase in fibroblasts (3, 11). More than 95% of protein synthesis in fibroblasts was inhibited by this concentration of cycloheximide. These two chemicals almost (in case of cortisol) or completely (in case of cycloheximide) abolished the stimulator activity of ECCM (Fig. 4).

The relationship between the concentration of the stimulator in ECCM and the extent of stimulation is shown in Fig. 5. When diluted

Fig. 4 Effects of cortisol or cycloheximide on the activity of ECCM. The dermal model was incubated with ECCM in the presence of cortisol (200 ng/ml) or cycloheximide (3 µg/ml) for 4 days, and was assayed for collagenase activity. Each column represents the average of two determinations shown by closed circles. a, ECCM in the presence of cortisol; b, ECCM in the presence of cycloheximide; c, ECCM alone; d, fresh medium

to 80% of original ECCM, ECCM showed as low as 50% stimulation of the full strength. No stimulation was observed at more than 50% dilution.

Epidermal cells were found to release this stimulator of collagenase production by fibroblasts at a time point between 5th and 7th day of culture but not at days earlier than this (Fig. 6). Gross appearance of epidermal cells during culture is presented in Fig. 7. Epidermal cells actively releasing the stimulation factor are larger in size and show diffused boundaries of cells in colonies.

Some Characterization of the Stimulation Factor

The factor did not lose its activity on repeated freezing-thawing (no loss up to at least 7 times operations). The factor was very heat-unstable; complete inactivation occurred at 100°C for 10 min.

The factor did not pass ultrafiltration membranes with a cut molecular weight of 10 K. The molecular weight of the factor was

Fig. 5 Response of fibroblasts to diluted ECCM. ECCM was diluted to 80% (c), 50% (b), and 20% (a) of original strength (d) and was assayed for the collagenase stimulation activity. e, unconditioned fresh medium. Each column indicates the average of two determinations shown by closed circles.

Fig. 6 Epidermal stimulator for the collagenase synthesis in fibroblasts. Epidermal cells were cultured for 24 h in DMEM-10% FCS and media were replaced with fresh serum-free DMEM. The cells were cultured for days indicated below. ECCM thus obtained was assayed for the collagenase stimulating activity. The values are the averages of three determinations. SE is within 13% of the average. a, ECCM obtained by culturing epidermal cells between day 1 and day 3; b, ECCM between day 3 and day 5; c, ECCM between day 5 and day 7; d, ECCM between day 1 and day 7; e, fresh medium

Fig. 7 Gross appearance of epidermal cells during culture. Epidermal cells were cultured in the presence or absence of FCS and were photographed at days indicated below. a through d, in the presence of FCS; e through g, in the absence of FCS; a, day 1; b and e, day 3; c and f, day 5; d and g, day 7. Bar, 50 μ m

estimated to be around 48 K by molecular sieve chromatography with Sephadex G-100 (Fig. 8).

Importance of Shape of Fibroblasts for Their Responsiveness to the Factor in ECCM

Fibroblasts in Fig. 9b are the cells that are actively producing collagenase in response to the factor in ECCM. They are more round in shape as compared to the cells in control culture (Fig. 9a). Importance of cell shape for fibroblasts to respond to the stimulation factor is further suggested by the following experiment. Fibroblasts were cultured on plastic surface as a conventional monolayer, exposed to ECCM, and collagenolytic activity of the culture media was assayed (Fig. 10). No stimulation of collagenase production was seen at all, in contrast to fibroblasts in the dermal model which were stimulated by ECCM. The shape of fibroblasts were compared between cells on the plastic and those within collagen gels (Fig. 11). The shape was quite different: flat and with less cytoplasmic protrusions on the plastic, whereas elongated and slim and with many protrusions in the gels.

DISCUSSION

Recently, methods of culture of mammalian functional cells have been greatly improved due to discovery of several essential growth factors (23), and elucidation of the important roles of extracellular matrices (ECM) for morphology and physiology of normal mammalian cells (49). Successful attempts to reconstitute organized architectures which morphologically and biochemically mimic the corresponding organ in the living body have been reported for the liver (44), blood capillaries (18) and vascular walls (53). Bell and his associates developed a skin equivalent by seeding epidermal cells onto a dermal equivalent where dermal fibroblasts had been populated in three-dimensional lattices of hydrated collagen fibrils (4, 5).

During studies on a skin equivalent, we noticed unexpectedly high activity of collagenase when a skin model was reconstituted with rat cells (58). It was inferred that there was an interaction between epidermal cells and fibroblasts for the expression of collagenase in the skin model. There has been reports which describe cell-cell interactions for the

Fig. 8 Gel filtration chromatography of the stimulation factor in ECCM. ECCM was pooled and concentrated about 25-fold, 4 ml of which was applied to a column of Sephadex G-100 (1.6×90 cm). Eluates were collected every 10 ml, concentrated and exchanged with DMEM by using a Centriprep-10, and assayed for the collagenase activity in fibroblasts in the dermal model. The elution positions of molecular weight markers are shown by arrows. BSA, bovine serum albumin (67 K); CTA, chymotrypsinogen A (25 K); RNase, ribonuclease A (13.7 K). —, absorbance at 280 nm; $\circ$ — $\circ$, collagenase activity in the dermal model

Fig. 9 Gross appearance of fibroblasts in collagen gels. Fibroblasts in the dermal model were exposed to ECCM for 4 days. The bar indicates $80 \mu\text{m}$. a, with unconditioned fresh medium; b, with ECCM

collagenase production since the study by Grillo and Gross (19) where they first demonstrated that collagenase production by one cell type can be greatly modified by the

presence of another cell type (56). From the viewpoint of reconstitution of the skin model, the high expression of collagenase raises a difficulty in developing structurally stable skin

Fig. 10 Unresponsiveness of fibroblasts cultured on the plastic to the factor in ECCM. Fibroblasts (9.2×10^4 cells) were cultured on the plastic (22 mm in diameter) or in collagen gels (0.5 ml in 22 mm-dish). They were exposed to ECCM for 4 days and collagenase activity of the media was determined. Each column represents the average of two determinations shown by closed circles. a, b, fibroblasts on the plastic; c, d, fibroblasts in the dermal model; a, c, cultured with unconditioned fresh medium; b, d, cultured with ECCM

equivalents. The present study was performed to know the reason for this high activity of collagenase production.

In this paper, we clearly demonstrate the presence of a stimulator for the synthesis of collagenase in fibroblasts. Up to now several factors have been reported which activate the collagenase production in fibroblasts, and are collectively called cytokines (46). Interleukin 1 (10, 36, 37, 43, 54), tumor necrosis factor (TNF) (12) and platelet-derived growth factor (2) increase collagenase production, whose molecular weights are 12 K–18 K (2, 41), 45 K (42) and 28 K–31 K (45), respectively. The molecular weight of the factor we found is 48 K. Interleukin I is produced mainly by monocytes and macrophages and also by other cells including keratinocytes (40). TNF are produced mainly by macrophages (7). These cytokines are effective on fibroblasts cultured on the plastic as a monolayer, whereas the factor reported in the present paper does not exert any stimulating action on the monolayered cells. It thus appears that the factor reported in the present study is different in its nature from these cytokines.

Corneal epithelial cells have been reported to produce cytokines with molecular weights of 20 K, 54 K and ≥ 90 K which stimulate collagenase production in stromal cells (29–31). The similar cytokines were found also in adult

Fig. 11 Shape of fibroblasts cultured for 4 days on the plastic (a) or in lattices of hydrated collagen fibrils (b). Bar, 50 μ m

and fetal epidermal cells of rabbit skin, whose apparent molecular weights were 20.5 K and 55 K (32). These are potent stimulators for collagenase production in dermal cells cultured on the plastic, suggesting that these cytokines are different in their nature from the stimulator found in the present study. Johnson-Wint (30) noticed that cytochalasin B significantly potentiates the enzyme production in connective tissue cells in response to epidermal cytokines. This indicates the importance of cell shape for the cytokines in cell interactions, which is also demonstrated in the present study.

The epidermal cell-derived factor is active on fibroblasts populated in three-dimensional lattices of collagen fibrils, where the cells differ in their shape from those cultured on the plastic. Cells in the lattice are slim and spindle-shaped, with prominent cytoplasmic protrusions. The shape of cells is closely associated with the arrangement of cytoskeletons. It is thus relevant to point out that drugs such as cytochalasin B, colchicine, and phorborester, which interfere with cytoskeletons, are known to induce collagenase in fibroblasts (8, 14, 25, 26). It appears that some specific arrangement of cytoskeletons is essential for cells to respond to the stimulator of collagenase production (9). Aggeler *et al.* (1) studied collagenase synthesis in rabbit synovial fibroblasts which had been treated with a variety of agents affecting cell morphology (12-*o*-tetradecanoylphorbol-13-acetate, cytochalasins B and D, trypsin, chymotrypsin, and trifluoperazine), and found a good correlation between the extent of cell-shape change and the extent of collagenase gene expression.

Unemori and Werb (52) investigated the collagenase production in rabbit synovial fibroblasts cultured on and in hydrated collagen gels and pointed out the importance of reorganization of polymerized actin for triggering the induction of procollagenase. It is possible that collagen fibrils induce some specific cell shape by interacting microfilaments via the cell membrane, and this particular cell shape allows the cell to respond to the factor and express collagenase genes. This postulation is further supported by the observation that collagen modulates cell shape and cytoskeletons of fibroblasts (51, 59).

Epidermal cells, which are cultured less

than 5 days and show normal appearance, do not produce the stimulator. The cells older than 7 days appear to be deteriorated and start to release the factor. It thus seems that the stimulator we found may be expressed in abnormal epidermal cells as a sort of emergency signal, but not expressed in intact epidermal cells. Though biochemical characterization of this stimulator is still under investigation, all available data indicate that the stimulator is a unique and new factor which belongs to cytokines.

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