Keratinocytes Cultured under Hyperthermal Conditions Secrete Factor(s) which Can Modulate Dermal Fibroblast Proliferation and Extracellular Matrix Production

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We have studied how keratinocytes cultured under hyperthermal conditions modulate skin fibroblast growth potential and their biosynthetic phenotypes in vitro. When keratinocytes were cultured at 30, 34, 37 or 39°C, the conditioned medium of the keratinocytes cultured at 39°C showed a greater inhibitory activity for fibroblast proliferation and greater synthetic activities of collagen and glycosaminoglycans than those incubated at 30, 34, or 37°C. Transforming growth factor (TGF-β1) production in skin fibroblasts was also stimulated by the keratinocyte conditioned medium cultured at 39°C. The stimulating activity of collagen and glycosaminoglycan syntheses by keratinocyte conditioned medium may be explained at least partly by enhanced TGF-β1 production. The results indicate that keratinocytes cultured at a higher temperature (39°C) may secrete factor(s) which modulate both fibroblast growth and matrix synthesis. This may provide evidence that under hyperthermal conditions epidermis can influence the functions of skin fibroblasts. Key words: hyperthermia; collagen cell proliferation; glycosaminoglycans.

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At physiological condition, the temperature of the epidermal layer, which faces the atmosphere and lacks circulating blood flow, is lower (25-30°C) than that of the dermis. Epidermal cells in culture have been shown to have optimal cell growth at 27-34°C (1), temperatures lower than those used in conventional cell culture systems (37°C). This implies that epidermal cell functions may differ depending on the culture temperatures.

Skin fibroblasts are thought to be responsible for the accumulation of extracellular matrices during wound healing. In the past, most wound care has consisted of providing an optimal environment for the wound. A good environment for cell growth and movement is created by warming or debridng the wound (2), controlling infection, and using pharmacological agents or different dressings (3). Hyperthermal therapy has been shown to improve a number of skin diseases, such as psoriasis (4), fungal infections (5, 6) and malignant skin tumors (7), and to improve delayed wound healing, possibly by increasing the blood flow of the dermal tissue, which results in the activation of skin fibroblast functions (8). However, it is also possible that some factors derived from epidermal cells may be induced by higher temperatures and modulate the proliferation and extracellular matrix production of dermal fibroblasts.

In the present report, we have attempted to study whether the conditioned media of keratinocytes cultured at different temperatures possess differential modulatory effects on mitogenic and biosynthetic phenotypes of dermal fibroblasts.

MATERIAL AND METHODS

Materials
Normal human keratinocytes (NHKs), originated from foreskin, were purchased from Sanko Junyaku (Tokyo, Japan). Highly purified bacterial collagenses isolated from Clostridium histolyticum came from Advanced Biofactures. Radioactive materials, [2-3H]proline (2.2 Tbq/mmol) and [6-3H]glucosamine hydrochloride (1.2 Tbq/mmol) came from Amerham.

Cell culture
NHKs were plated at a density of 2 x 10⁴/35 mm diameter-type I collagen-coated dish (collaborative Biomedical Products, Bedford, MA) and cultured for 3 days at 37°C in a medium containing insulin, epidermal growth factor (EGF), hydrocortisone and bovine pituitary extract (BPE) (designated as KGM) according to the supplier's protocols. Cultures were continued for 48 h at the temperatures of 30, 34, 37 and 39°C. At the termination of the cultures, the cell number was counted after trypsin (1:250) (Gibco) treatment, using a hemocytometer.

Normal human fibroblasts were established by a routine explant method. Fibroblasts were plated at a density of 1 x 10⁴/35 mm dish and grown for 3 days at 37°C in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS); incubation temperatures were then switched to 30, 34, 37 or 39°C. Cultures were continued for 48 h. At the end of the cultures, the cell number was counted as described above.

To study the effects of the conditioned medium of keratinocytes on fibroblast proliferation, NHKs were cultured for 3 days at 37°C in a medium containing insulin, EGF, hydrocortisone and BPE. The medium was replaced with serum-free DMEM to eliminate the influences of the factors contained in the keratinocyte medium, and incubation temperatures were shifted to 30, 34, 37 or 39°C. Cultures were continued for 48 h. At the termination of the incubation, the medium was harvested and stored at −20°C until ready for use. Fibroblast culture on day 3 after incubation received the conditioned medium at 37°C for 48 h, and the cell number was then counted.

In one experiment, the conditioned medium harvested from keratinocytes was heat-denatured at 100°C for 2 min prior to the treatment of fibroblasts.

Metabolic labeling and determination of collagen synthesis in skin fibroblasts
Fibroblast cultures were treated with keratinocyte-conditioned medium for 2 days and labeled with [2-3H]proline (20 µCi/ml) in the presence of ascorbic acid (30 µg/ml) during the last 24 h of the incubation. The medium was then collected and mixed with protease inhibitor cocktails, to yield 1 nM each of N-ethylmaleimide, EDTA, (pH 7.0) and phenylmethylsulfonyl fluoride. The cells were harvested with 9.25% trypsin, and the cell number was determined with a hemocytometer. After centrifugation cells were combined with the medium and stored at −20°C until ready for analysis. The amount of radioactivity incorporated into collagen was determined using

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purified bacterial collagenase (9), as previously described (10). Synthesis of collagen was expressed as collagenase-sensitive cpm/cell.

Synthesis of glycosaminoglycans in cultured skin fibroblasts

Fibroblasts were treated for 48 h with keratinocyte culture media and labeled with [6-3H]glucosamine (25 μCi/ml) for the last 24 h of the treatment. Cultured medium and cell pellet following trypsin treatment were combined together with carrier glycosaminoglycan mixtures, consisting of hyaluronic acid, dermatan sulfate, chondroitin 4- and 6-sulfate and heparan sulfate, and were then lyophilized. Isolated glycosaminoglycans were subjected to two-dimensional electrophoresis on cellulose-acetate membranes, using 0.1 M pyridine-0.45 M formic acid, pH 3.5, at 1 mA/cm2 for 1.5 h in the first dimension and 0.1 M barium carbonate, pH 8.1, at 1 mA/cm2 for 5 h in the second. The radioactivities incorporated in total glycosaminoglycan or in each component were determined with a liquid scintillation spectrometer (Hisachi 124) (11–13).

Determination of transforming growth factor (TGF)-β1 in cultured skin fibroblasts

TGF-β1 levels in the medium were determined by the ELISA system, using monoclonal antibody for TGFβ1 (Amersham). Quantitative determination was performed within the linear range between input sample amount (0–1000 pg/ml TGFβ1) and optical absorbance at 492 nm, according to the supplier’s protocol. Values are expressed as pg per cell number.

RESULTS

As keratinocyte culture medium contains several growth promoting factors (insulin, EGF, hydrocortisone and BPE) in a conventional culture system, we utilized DME medium for conditioned medium to properly address the effect of keratinocyte-derived factor(s) on fibroblast proliferation. No cellular toxicity was found in the keratinocytes cultured at lower (30°C) or higher (39°C) temperatures for 2 days in DME medium, on the basis of the trypan blue exclusion test (not shown) and unaltered keratinocyte proliferation (see below).

When keratinocytes and fibroblasts were cultured at 30, 34, 37 and 39°C for 2 days, there was no significant difference in mitotic activity in either cells (11.5–12.1×104 and 2.4–2.5×105 per dish, respectively). This result tempted us to explore the possibility whether the keratinocyte culture medium may potentially modulate fibroblast proliferation.

Keratinocyte medium cultured at 30, 34 and 37°C did not exhibit any significant effects on fibroblast proliferation. By contrast, the medium harvested from the culture at 39°C did inhibit fibroblast proliferation in one third of the controls in triplicate experiments.

With the serial dilution of conditioned medium prepared from the keratinocytes cultured at 39°C, the inhibitory effect on fibroblast proliferation was reduced to the control level. When the conditioned medium was heated at 100°C for 2 min, the inhibitory effect was abolished (Fig. 1).

Collagen and total protein syntheses of fibroblasts were unchanged with the treatments of keratinocyte-conditioned medium cultured at 30 and 34°C. The conditioned medium cultured at 37°C or 39°C slightly or significantly stimulated both collagen and total protein syntheses (Fig. 2).

Culture temperature-dependent stimulation of total glycosaminoglycan synthesis in skin fibroblasts was observed by keratinocyte-conditioned medium (Fig. 3). The major components of glycosaminoglycan synthesized by skin fibroblasts were found to be hyaluronic acid and dermatan sulfate. There were no significant differences in the relative synthesis of the two glycosaminoglycans (data not shown).
The TGFβ1 level in the fibroblast cultures was elevated by the keratinocyte-conditioned medium cultured at 39°C, whereas keratinocyte medium cultured below 37°C did not influence the TGFβ1 level, compared with the controls (Fig. 4).

**DISCUSSION**

Cultured keratinocytes have been demonstrated to produce a number of factors affecting dermal fibroblast phenotypes. These include EGF, interleukin (IL)-1, tumor necrosis factor (TNF), TGF-β, interferon (IFN)-γ, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (14). Out of these, only TGFβ and PDGF are known to possess the stimulating activity of extracellular matrix production in skin fibroblasts, but these two growth factors do not exhibit inhibitory effects on fibroblast proliferation (15, 16). This suggests that hyperthermia-induced keratinocyte factor(s) may be unique or comprise more than two growth factors.

It is noted that the conditioned media of keratinocyte cultured at 39°C stimulated the production of TGFβ1. Since TGFβ1 is known to be a potent stimulator of collagen and proteoglycan syntheses (17–19) and is considered to play a key role in the accumulation of connective tissues in scleroderma, keloid and wound healing, it may be postulated that the stimulation of matrix protein synthesis by keratinocyte-conditioned media is mediated at least in part by the stimulation of TGFβ1 production.

Hyperthermia has been utilized in the treatment of human malignancies. Hyperthermia is known to show a direct cytotoxic effect at temperatures above 41°C and to be selectively lethal to cancer cells (20). Our results show that hyperthermal treatment of skin at 39°C can alter the fibroblast function via a soluble factor derived from epidermis. This may provide evidence that skin fibroblasts undergo a critical influence from epidermis during wound healing and fibrotic conditions, such as keloid and scleroderma. Hyperthermal conditions may serve as an accelerant of delayed wound healing.

**REFERENCES**