Biological characterization of human fibroblast-derived mitogenic factors for human melanocytes

Genji IMOKAWA¹, Yukihiro YADA, Naoko MORISAKI and Mitsutoshi KIMURA
Biological Science Laboratories, Kao Corporation, Ichikaimachi 2606, Haga, Tochigi 321-34, Japan

INTRODUCTION
Paracrine linkage between keratinocytes and melanocytes within the epidermis secreting and responding to cytokines plays an important role in accentuating melanization in UV-light-induced cutaneous pigmentation [1–4]. Keratinocytes produce various cytokines in response to specific stimuli. Of these cytokines, a few are known mitogens for human melanocytes, including basic fibroblast growth factor (bFGF), stem cell factor (SCF), granulocyte–macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF) and endothelin-1 (ET-1) [1,5–8]. Keratinocytes secrete GM-CSF and ET-1 in response to various stimuli at concentrations sufficient to stimulate adjacent melanocytes via specific receptors [1–3], leading to an increase in epidermal pigmentation in UV-light-exposed or injured skin. Although bFGF, SCF and HGF have a high capacity to stimulate melanocyte proliferation [6–8], keratinocytes do not secrete them at concentrations sufficient to stimulate melanocyte proliferation even during response to various stimuli [9], making it impossible to link epidermal pigmentation with the action of these cytokines through paracrine linkage between keratinocytes and melanocytes within the epidermis. While screening different human fibroblasts, we observed that human fibroblasts derived from older skin produce much higher levels of factors that stimulate DNA synthesis of human melanocytes than do those derived from younger skin. The goal of the present investigation was to clarify the paracrine linkage between human fibroblasts and melanocytes in cutaneous pigmentation by characterizing human fibroblast-derived factors responsible for stimulating DNA synthesis of human melanocytes.

MATERIALS AND METHODS

Materials
Normal human melanocytes and serum-free melanocyte growth medium (MGM) were obtained from Sankou Pure Chemicals (Tokyo, Japan). Normal human fibroblasts were purchased from Kyokutou Pharmaceutical Corp. (Tokyo, Japan). Endothelin ELISA kits and interleukin derivatives were purchased from International Reagents Corp. (Kobe, Japan). Interleukin (IL)-1α ELISA kits were purchased from Otsuka Pharmaceutical Corp. (Tokyo, Japan). Other ELISA or EIA kits were obtained from Amersham International, Amersham, Bucks., U.K. Anti-bFGF antibody was purchased from Oncogene Science (Uniondale, NY, U.S.A.). Anti-SCF was from Immunobiological Laboratories (Gunma, Japan). Bovine pituitary extract (BPE) was obtained from Gibco Laboratories (Tokyo, Japan). Other chemicals were of reagent grade.

Cell culture
Human fibroblasts were maintained in fibroblast growth medium (FGM) (Clonetics) supplemented with 5 µg/ml insulin, 1 ng/ml recombinant bFGF, antibiotics (50 µg/ml gentamicin and 0.25 µg/ml amphotericin B) and 10% fetal calf serum (FCS) at 37 °C under a 5% CO₂ atmosphere. Human melanocytes were maintained in modified MCDB 153 supplemented with 1 ng/ml recombinant bFGF, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml PMA, antibiotics (50 µg/ml gentamicin and 0.25 µg/ml amphotericin B), 0.5% FCS, and 0.2% BPE (MGM) at 37 °C

Abbreviations used: IL, interleukin; ET, endothelin; HGF, hepatocyte growth factor; SCF, stem cell factor; bFGF, basic fibroblast growth factor; TGFβ, transforming growth factor β; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNFα, tumour necrosis factor α; IFNγ, interferon γ; BPE, bovine pituitary extract; MGM, serum-free melanocyte growth medium; FGM, fibroblast growth medium; FCS, fetal calf serum; MAP, mitogen-activated protein; BPE, bovine pituitary extract.

¹ To whom correspondence should be addressed.
under a 5% CO₂ atmosphere. In experiments to measure the cellular effects of conditioned medium, melanocytes were seeded in a 24- or 48-well culture tray, cultured in modified MCDB 153 supplemented with antibiotics and 0.2% BPE (without PMA and bFGF) for 24 h, then treated with the conditioned medium.

**Assay of mitogen-activated protein (MAP) kinase**

Melanocytes were seeded in a 24-well culture tray and cultured in modified MCDB 153 supplemented with antibiotics and 0.2% BPE extract (without PMA and bFGF) for 24 h, after which the medium was aspirated, exchanged for fresh modified MCDB 153 (without supplements) and after 2 h culture, cells were treated with the fibroblast-conditioned medium or cytokines. MAP kinase activity was measured using the p42/p44 MAP kinase enzyme assay system (Amersham International) as the rate of phosphorylation of a synthetic peptide substrate that is highly selective for p42/p44 MAP kinase. Briefly, after 0–60 min of treatment with the fibroblast-conditioned medium or HGF (10 nM) and SCF (10 nM), cells were rinsed twice with ice-cold PBS and lysed at 4 °C in buffer consisting of 10 mM Tris/HCl, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 µM orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotonin, at pH 7.4. Cellular debris was precipitated at 25,000 g for 20 min and the supernatants were obtained as cytosolic extracts for MAP kinase assay. The cytosolic extracts (15 µl) were mixed at 30 °C with the substrate buffer (10 µl, containing Hepes, sodium orthovanadate, 0.05% NaN₃ and the synthetic peptide, at pH 7.4), the assay buffer (containing Hepes, ATP and MgCl₂, pH 7.4) and 1 µCi [γ-³²P]ATP. Reactions were terminated after 0, 2, 4, 6 and 10 min by adding 10 µl of stop reagent containing orthophosphoric acid. The terminated reaction mixture was spotted on to the centre of paper discs, which were washed three times with 250 µl of 75 mM orthophosphoric acid, twice with water, air-dried and counted in a liquid-scintillation counter. MAP kinase activity was expressed as pmol of phosphate transferred/min per mg of protein.

**Measurement of cytokines**

Human fibroblasts were seeded in six-well trays at a density of 1 × 10⁴–2 × 10⁴ cells/ml and cultured in FGM supplemented with 5 µg/ml insulin, 1 ng/ml recombinant bFGF, antibiotics (50 µg/ml gentamicin and 0.25 µg/ml amphotericin B) and 10% FCS for 24 h. The medium was aspirated and exchanged for fresh FGM supplemented with antibiotics (50 µg/ml gentamicin and 0.25 µg/ml amphotericin B) and 1% FCS (without bFGF). After human fibroblasts had been cultured for 4 days at 37 °C under a 5% CO₂ atmosphere, the fibroblast-conditioned medium was collected, and 100 µl was used to quantify ET-1 and Big-ET-1, and 200 µl was used for measurement of bFGF, SCF, HGF, IL-1α, IL-2, IL-6 and IL-8 by ELISA. The ET ELISA kit is a solid-phase enzyme immunoassay using the multiple-antibody sandwich principle. A human purified polyclonal antibody specific for human ET-1, and human purified monoclonal antibodies specific for bFGF, SCF, HGF, IL-1α, IL-2, IL-6 or IL-8 were attached to 96-well microtitre plates. Cytokines present in standards or unknowns were captured by the solid-phase antibody. Horseradish peroxidase-labelled rabbit anti-human ET-1 (or other cytokines) IgG was added, which bound to multiple epitopes on ET-1 (or the other cytokines) attached to the solid phase. Levels of immunoreactive cytokine were measured as A₄₀₀ by an ELISA plate reader (Bio-Rad; model 3550). Cytokine levels in the cultures were determined by comparing their absorbance with that produced by standards. The standard curve was linear from 5.0 to 1000 pg/ml for ET derivatives, IL-2, IL-6 and IL-8, and from 5.0 to 500 pg/ml for IL-1 derivatives, bFGF, SCF and HGF.

**Effect of human fibroblast-conditioned medium on thymidine incorporation by human melanocytes**

Melanocytes were seeded in 24- or 48-well trays at a density of 4 × 10⁴–1 × 10⁵ cells/ml and cultured in modified MCDB 153 supplemented with 0.2% BPE (without PMA and bFGF) for 24 h. The fibroblast-conditioned medium was obtained by culturing human fibroblasts for 4 days in FGM (without bFGF) supplemented with 1% FCS. The fibroblast-conditioned medium was then concentrated 5-fold by lyophilization. Half the medium (600 µl) was aspirated from the well containing human melanocytes, and 200 µl of the 5-fold concentrated fibroblast-conditioned medium was added. To determine the effects of SCF (or other) antibody, 2.0–5.0 µg/ml anti-human SCF (or other) IgG was added to the culture medium. After 20 h, the cells were labelled for 4 h with 1 µCi/ml [³H]thymidine. After three washes with Mg²⁺/Ca²⁺-free PBS, the cells were lysed with 2 M NaOH, then neutralized with 2 M HCl. Acid-insoluble material was precipitated with 4 vol. of 10%, trichloroacetic acid, collected on glass filters, washed three times with 10% trichloroacetic acid, once with ethanol, and then dried. The radioactivity on the filters was determined in a liquid-scintillation counter.

**RESULTS**

**Stimulation of DNA synthesis in melanocytes by fibroblast-conditioned medium**

The culture medium conditioned for 4 days by human fibroblasts significantly stimulated DNA synthesis by human melanocytes when incubated for 24 h (Table 1). The stimulatory effect was significantly higher in medium conditioned with human fibroblasts from old skin than those from young skin. DNA synthesis was most markedly stimulated when the conditioned medium was concentrated 5-fold and then added at 2:4 dilution to the melanocyte culture (results not shown).

**Table 1 Effects of fibroblast-conditioned medium on DNA synthesis of cultured human melanocytes**

<table>
<thead>
<tr>
<th>Conditioned medium</th>
<th>[³H]Thymidine incorporation (d.p.m./well)</th>
<th>Increase over control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1249 ± 51</td>
<td>—</td>
</tr>
<tr>
<td>Young fibroblast-1 (6 years old)</td>
<td>1656 ± 71</td>
<td>33</td>
</tr>
<tr>
<td>Young fibroblast-2 (7 years old)</td>
<td>1596 ± 139</td>
<td>29</td>
</tr>
<tr>
<td>Young fibroblast-3 (9 years old)</td>
<td>1340 ± 107</td>
<td>7</td>
</tr>
<tr>
<td>Young fibroblast-4 (10 years old)</td>
<td>1480 ± 133</td>
<td>18</td>
</tr>
<tr>
<td>Old fibroblast-1 (61 years old)</td>
<td>2022 ± 182</td>
<td>62</td>
</tr>
<tr>
<td>Old fibroblast-2 (68 years old)</td>
<td>2767 ± 71</td>
<td>122</td>
</tr>
<tr>
<td>Old fibroblast-3 (72 years old)</td>
<td>3236 ± 167</td>
<td>159</td>
</tr>
<tr>
<td>Old fibroblast-4 (81 years old)</td>
<td>2562 ± 129</td>
<td>105</td>
</tr>
</tbody>
</table>
Table 2 Effects of protein kinase inhibitors on fibroblast-conditioned-
medium-induced stimulation of DNA synthesis in cultured human melanocytes

<table>
<thead>
<tr>
<th>Conditioned medium</th>
<th>[3H]Thymidine incorporation (d.p.m./well)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>551 ± 41</td>
<td>100</td>
</tr>
<tr>
<td>Old fibroblast-3-conditioned medium</td>
<td>2037 ± 128</td>
<td>0</td>
</tr>
<tr>
<td>Old fibroblast-3-conditioned medium + H-7 (10 µM)</td>
<td>1656 ± 47</td>
<td>25.3</td>
</tr>
<tr>
<td>Old fibroblast-3-conditioned medium + Genistein (10 µM)</td>
<td>1819 ± 101</td>
<td>14.3</td>
</tr>
<tr>
<td>Old fibroblast-3-conditioned medium + Herbimycin (10 µM)</td>
<td>918 ± 51</td>
<td>75.2</td>
</tr>
<tr>
<td>Old fibroblast-3-conditioned medium + Tyrophostin (10 µM)</td>
<td>776 ± 163</td>
<td>84.8</td>
</tr>
</tbody>
</table>

Effects of protein kinase inhibitors on fibroblast-conditioned-
medium-induced DNA synthesis

To clarify the activation mechanism(s) underlying agonist-
receptor-mediated signalling pathways caused by the fibroblast-
conditioned medium, we determined which protein kinase inhibitors inhibited fibroblast-conditioned-medium stimulation of
DNA synthesis by human melanocytes. The stimulatory effect
was markedly interrupted by inhibitors of tyrosine kinase, such
as tyrphostin B42 (10 µM), genistein (10 µM) and herbimycin (10 µM), but not by inhibitors of protein kinases C and A, such
as H-7 (10 µM) and phloretin (1 µM) (Table 2).

Effects on MAP kinase activity

Assay of MAP kinase activity after incubation with human
melanocytes revealed that when 5-fold concentrated fibroblast-
conditioned medium was added at 2:4 dilution, rapid activation
of MAP kinase occurred within 5 min, reached a peak within
10 min, and declined to the previous control level 60 min later
(Figure 1A). Activation was much greater with old-fibroblast-
conditioned medium than with young-fibroblast-conditioned medium. Ligands for tyrosine kinase, such as HGF (10 nM) and
SCF (10 nM), elicited the activation of MAP kinase in a pattern
similar to that by old-fibroblast-conditioned medium (Figure 1B).

Cytokine secretion into fibroblast-conditioned medium

ELISA of factors released into the conditioned medium revealed
that concentrations of HGF and SCF were significantly increased
in old-fibroblast-conditioned medium compared with young-
fibroblast-conditioned medium (Table 3). In contrast, levels of
bFGF did not differ significantly. IL-1α, IL-2, IL-6, ET-1 and
Big-ET-1 were not detected in medium conditioned with either

![Figure 1](image_url)
old or young fibroblasts, but IL-8 was just detectable in one sample of old fibroblasts (Table 3).

### Abrogation of the stimulated DNA synthesis by antibodies

When conditioned medium was treated with SCF, HGF and/or bFGF antibodies, the stimulation of DNA synthesis was markedly abrogated by HGF antibodies and to a greater extent by HGF + SCF antibodies (Table 4).

### Effect of cytokines on the secretion of HGF by young fibroblasts

To clarify the possibility of the involvement of an autocrine cytokine loop in the increased secretion of SCF and HGF by old fibroblasts, we determined the effects of cytokines on the secretion of HGF by young fibroblasts. ELISA of medium conditioned for 4 days after cytokine application demonstrated that, of the cytokines tested, IL-1α (10 units) and TNFα (10 nM) are highly effective in stimulating the secretion of HGF. The stimulation of SCF and HGF secretion, the young fibroblast conditioned in the absence of IL-1α, although the levels of their secretion were already much higher than in young fibroblasts. IL-8, ET-1 and Big ET-1 were not detected in either IL-1α-treated or untreated old or young fibroblast-conditioned medium. Consistent with IL-1α-induced stimulation of SCF and HGF secretion, the young fibroblast medium conditioned for 4 days in the presence of IL-1α elicited a significant increase in DNA synthesis by human melanocytes compared with medium conditioned in the absence of IL-1α (Table 6). This stimulatory effect was also abrogated by tyrophostin B42 (10 μM). Old fibroblast medium conditioned for 4 days in the presence of IL-1α (10 units) did not stimulate DNA synthesis above the level observed using old-fibroblast-medium conditioned in the absence of IL-1α.

### DISCUSSION

The present findings demonstrate that human fibroblasts derived from old skin secrete higher levels of mitogenic factors for human melanocytes than do fibroblasts derived from young skin. The stimulatory effect of these factors on DNA synthesis by human melanocytes was interrupted by tyrosine kinase inhibitors, such as genistein, herbimycin and tyrophostin B42, but not by other protein kinase inhibitors, such as H-7 and phloretin. Furthermore, these factors were able to activate MAP kinase, which is a major cascade downstream of tyrosine kinase signalling. These findings suggest that these factors, which are present at higher levels in old-fibroblast-conditioned medium, stimulate DNA synthesis by human melanocytes through tyrosine kinase ligand–receptor-mediated signal-transduction pathways. To determine which tyrosine kinase ligands are responsible for this stimulation, we studied the effects of antibodies against c-Kit or c-Met ligands, such as bFGF, SCF and HGF. The stimulation of DNA synthesis elicited by old-fibroblast-conditioned medium could be decreased to 20% of the elevated level by HGF antibody and by HGF + SCF antibodies, indicating that the mitogenic factors detected in old-fibroblast-conditioned medium are mainly HGF and SCF. Consistent with this finding, quan-
titative analysis of cytokines in fibroblast-conditioned medium by ELISA revealed that HGF and SCF were significantly increased in old-fibroblast-conditioned medium compared with young-fibroblast-conditioned medium. In contrast, bFGF did not differ between the two, although there is a much lower level of secretion in both old- and young-fibroblast-conditioned media compared with those of HGF and SCF. Since HGF and SCF are known potent mitogens for human melanocytes [7,9,10], it is possible that old fibroblasts accentuate the cellular activities of human melanocytes by secreting more HGF and SCF than young fibroblasts.

As a possible mechanism for the high level of secretion of HGF and SCF by old fibroblasts, we explored whether any fibroblast-derived cytokines stimulate HGF secretion in an autocrine fashion. Of the cytokines tested, IL-1α and TNFα are the only ones that significantly stimulate HGF secretion by young fibroblasts. Further studies on the IL-1α-inducible effects demonstrated that HGF and SCF, but not bFGF, secretion was markedly stimulated in culture medium by treatment of young fibroblasts with IL-1α. In contrast, there was no marked increase over the original elevated levels by IL-1α treatment of old fibroblasts. Consistent with the IL-1α-inducible stimulation of SCF and HGF secretion, young-fibroblast medium conditioned for 4 days in the presence of IL-1α elicited an increase in DNA synthesis compared with medium conditioned in the absence of IL-1α. This stimulatory effect was also abrogated by the tyrosine kinase inhibitor tyrphostin B42. In contrast, old-fibroblast medium conditioned for 4 days in the presence of IL-1α did not stimulate DNA synthesis above the level observed for old-fibroblast medium conditioned without IL-1α. This cytokine loop, observed as the stimulation of SCF and HGF secretion by IL-1α or TNFα, may lead to the hypothesis that the increased SCF and HGF secretion by old fibroblasts is the result of constitutively accentuated secretion of IL-1α or TNFα by old fibroblasts compared with young fibroblasts.

Aging human fibroblasts with 90% of their lifespan completed in culture have been reported to produce IL-1α constitutively with an increased level of IL-1α mRNA [11]. However, under our culture conditions, IL-1α was not detected in the conditioned medium from young or old human fibroblasts. It should be noted that the human fibroblasts used in the present studies were isolated from skin of various chronological age and subjected to these experiments within five passages in culture. Thus it is unlikely that IL-1α is involved as an autocrine loop in the increased HGF and SCF observed in old-fibroblast-conditioned medium. Further experiments in which mRNA levels or secretory processing for HGF and SCF are compared between young and old human fibroblasts may elucidate the role of these cytokines in cellular aging.

The physiological role of the increased SCF and HGF secretion by old human fibroblasts is at present not known. Although human keratinocytes are reported to produce SCF and HGF mRNA [12], our previous study on their secretion demonstrated that no SCF or HGF is detected in human keratinocyte medium conditioned for 2 days, even in response to stimuli such as UVB irradiation (25 mJ/cm²) [9]. In the same series of studies, no bFGF secretion could be detected in human keratinocyte-conditioned medium, and was not induced by UVB irradiation. Thus, it is not likely that keratinocytes trigger melanocyte proliferation by secreting SCF or HGF in epidermal pigmentation. Paradoxically, it is possible that human fibroblasts trigger melanocyte proliferation by secreting potent mitogens for human melanocytes, thereby influencing epidermal pigmentation. Aged skin becomes highly sensitive to pigmentary changes in response to several stimuli, including UV exposure. This may be accounted for by the present evidence that old human fibroblasts actively secrete SCF and HGF, coupled with the additive or synergistic interaction with other cytokines known to be secreted by keratinocytes responding to several stimuli [10]. In fact, even at concentrations insufficient to induce melanocyte proliferation, SCF and HGF can enhance ET-1-induced stimulation of DNA synthesis in cultured human melanocytes through convergence of the different signalling pathways, including the co-activation of MAP kinase [13]. This may account for the susceptibility of aged skin to the elicitation of cutaneous pigmentation by UV irradiation which is known to stimulate ET-1 production by keratinocytes.

The physiological importance of the stimulation of HGF and SCF secretion by IL-1α or TNFα is at present not known. Many types of cell are capable of producing IL-1α. These include keratinocytes [2], macrophages and fibroblasts [9,11] in the skin. In most cells, IL-1 is produced in response to external stimuli. Experiments involving IL-1α treatment of young human fibroblasts demonstrated that the conditioned medium, which contained several IL-1α-inducible cytokines in addition to SCF and HGF as well as the added IL-1α, acquired an increased potential to stimulate DNA synthesis by cultured human melanocytes. This was accompanied by a parallel increase in the secretion of HGF and SCF, thus mimicking the cytokine network initiated by IL-1α in skin tissue. On the basis of the fact that IL-1α is a primary mediator that responds to inflammation and injury, the major source being monocytes/macrophages, the paracrine linkage between fibroblasts and melanocytes secreting and responding to SCF or HGF may be part of a regulatory mechanism for increases in cutaneous pigmentation during inflammation and aging.

REFERENCES