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*Cutaneous Biology*

# The mechanism of epidermal hyperpigmentation in café-au-lait macules of neurofibromatosis type 1 (von Recklinghausen's disease) may be associated with dermal fibroblast-derived stem cell factor and hepatocyte growth factor

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**Summary**

*Background* The mechanism of the accentuated melanization in café-au-lait macules (CALMs) in patients with neurofibromatosis type 1 (NF1; von Recklinghausen's disease) has not been elucidated.

*Objectives* To clarify the mechanism involved in the hyperpigmentation of CALMs in NF1.

*Methods* Using enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cultured cells, we measured the levels of cytokines produced and secreted by keratinocytes and fibroblasts derived from CALMs (group RC: *R*ecklinghausen *C*ALM) skin, compared with cells derived from the skin of normal individuals (group NN: *N*ormal skin of *N*ormal individuals) and cells derived from non-CALM skin of NF1 patients (group RN: *R*ecklinghausen *N*on-CALM).

*Results* ELISA revealed that the secretion of hepatocyte growth factor (HGF) and stem cell factor (SCF) by cultured fibroblasts was significantly elevated in group RC compared with groups RN and NN. In parallel, semiquantitative real-time RT-PCR of HGF and SCF mRNAs demonstrated increased expression of both types of transcripts by cultured fibroblasts in group RC compared with group NN. In contrast, the secretion of endothelin-1 and granulocyte/macrophage colony-stimulating factor by cultured keratinocytes occurred at a similar level among all three groups, RC, RN and NN.

*Conclusions* These findings suggest that increased secretion of HGF and SCF by dermal fibroblasts may be associated with the accentuated epidermal melanization observed in CALMs in the skin of NF1 patients.

*Key words*: café-au-lait macules, fibroblasts, hepatocyte growth factor, neurofibromatosis type 1, stem cell factor, von Recklinghausen's disease

Café-au-lait macules (CALMs) are light to dark brown, well-circumscribed cutaneous macular areas with no hair. CALMs are the best-known cutaneous sign of neurofibromatosis type 1 (NF1; von Recklinghausen's disease) and they are present in almost 100% of patients with NF1.[1] Histological studies of CALMs in NF1 skin show increased epidermal melanization and an increased number of melanocytes with normal amounts of tyrosinase activity.[2–4] The *NF1* gene is localized to chromosome 17,[5] and encodes neurofibromin, a tumour-suppressor protein.[6–8] It has been suggested that a reduced neurofibromin level in the

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epidermis of NF1 patients is responsible for the elevated melanogenesis and the increased density of melanocytes.[9] However, this is not the complete mechanism by which melanization is locally stimulated in CALMs of NF1 skin, because the reduction in neurofibromin levels is systemic, not localized.

Recently, it has been reported that paracrine linkages among keratinocytes, fibroblasts and melanocytes within the skin play important roles in regulating epidermal melanization. In response to various stimuli, human keratinocytes secrete various cytokines that serve as mitogens or melanogens for human melanocytes, including endothelin (ET)-1[10–12] and granulocyte/macrophage colony-stimulating factor (GM-CSF).[13] These cytokines have been suggested to be intrinsic melanogenic factors involved in ultraviolet (UV) B[11,12] and UVA melanosis.[13] In UVB melanosis, the epidermis of lentigo senilis expresses an increased amount of ET at the gene and protein levels, which suggests an essential role for ET in the stimulation of epidermal pigmentation in lentigo senilis.[14] Although hepatocyte growth factor (HGF), stem cell factor (SCF) and basic fibroblast growth factor (bFGF) have been shown to stimulate melanocyte proliferation *in vitro*[15–21] and *in vivo*,[22,23] keratinocytes do not secrete them at concentrations sufficient to stimulate melanocyte proliferation, even following exposure to various stimuli.[16] In contrast to keratinocytes, human fibroblasts do secrete several melanogenic cytokines, such as bFGF, HGF and SCF, when they are rapidly growing or during inflammation,[16,17] which suggests the possibility that overexpression of these cytokines by dermal fibroblasts may activate melanocytes in the overlying epidermis. In dermatofibroma, where the epidermis overlying the fibroblastic tumour is highly pigmented, rapidly growing fibroblasts are stimulated to secrete SCF and HGF, which suggests an important involvement of these cytokines in the accentuated epidermal pigmentation.[24] Based upon the paracrine cytokine network now known to function within the skin to regulate epidermal pigmentation, it is interesting to examine the patterns of cytokine secretion in cells located in CALMs of NF1 skin. In this study, we determined whether keratinocytes or fibroblasts derived from CALM skin of NF1 patients differed in their production and secretion of several melanogenic cytokines, including ET-1, GM-CSF, HGF, SCF and bFGF, compared with cells derived from healthy control skin or from non-CALM skin of NF1 patients.

## Materials and methods

### *Histochemistry*

Specimens of normal skin, non-CALM and CALM skin were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were stained with haematoxylin and eosin or toluidine blue using standard techniques.

### *Cell isolation and cell culture*

Skin biopsies were obtained from seven Japanese patients with NF1 (patients 1–7; mean ± SD age 27·1 ± 14·5 years) who fulfilled the diagnostic criteria for NF1 according to the National Institutes of Health Consensus Development Conference Statement.[25] CALM skin (group RC: *R*ecklinghausen *C*ALM) was obtained from five patients (patients 1–5; mean ± SD age 29·8 ± 16·9 years) and non-CALM skin (group RN: *R*ecklinghausen *N*on-CALM) was obtained from five patients (patients 3–7; mean ± SD age 31·4 ± 12·6 years). In three patients (patients 3–5) both non-CALM skin and CALM skin was biopsied (Table 1). Normal skin specimens (group NN: *N*ormal skin of *N*ormal individuals) were obtained from Japanese patients during plastic surgery; fibroblasts were cultured from 19 such patients (eight males and 11 females; mean ± SD age 26·7 ± 5·6 years), and keratinocytes were cultured from 16 patients (six males and 10 females; mean ± SD age 28·0 ± 17·1 years).

Human keratinocytes were isolated using a modification of the method reported previously.[26] Briefly, the specimens were washed three times in phosphate-buffered saline (PBS), were finely shredded with scissors, and then incubated with 0·25% trypsin and 0·02% ethylenediamine tetraacetic acid in PBS for 16–24 h at 4 °C. The epithelium was separated from the dermis with forceps, and keratinocytes were isolated from the

**Table 1.** Profiles of patients with neurofibromatosis type 1 used in this study

| Patient no. | Age (years)/sex | Skin | Donor site |
|---|---|---|---|
| 1 | 4/M | CALM | Chest |
| 2 | 29/F | CALM | Face |
| 3 | 30/F | CALM | Abdomen |
| | | Non-CALM | Abdomen |
| 4 | 35/M | CALM | Arm |
| | | Non-CALM | Arm |
| 5 | 51/M | CALM | Arm |
| | | Non-CALM | Arm |
| 6 | 20/M | Non-CALM | Face |
| 7 | 21/F | Non-CALM | Back |

CALM, café-au-lait macule.

subepithelial side. Keratinocytes were grown in modified serum-free keratinocyte growth medium (KGM; Kyokuto Seiyaku, Tokyo, Japan) which consists of MCDB 153 with high concentrations of amino acids, transferrin (final concentration 10 μg $mL^{-1}$), insulin (5 μg $mL^{-1}$), hydrocortisone (0·5 μg $mL^{-1}$), phosphorylethanolamine (14·1 μg $mL^{-1}$) and bovine pituitary extract (40 μg $mL^{-1}$). The final concentration of $Ca^{2+}$ in the medium was 0·03 mmol $L^{-1}$. Fibroblasts were isolated for explant after the dermis was separated from the epidermis, and were grown in fibroblast growth medium (FGM), which consists of Dulbecco's modified Eagle's medium, 0·6 mg $mL^{-1}$ glutamine and 10% fetal calf serum (FCS).

### *Measurement of cytokines by enzyme-linked immunosorbent assay*

Cytokine secretion by cultured keratinocytes and fibroblasts was compared among the three groups in order to elucidate which cytokine(s) might be involved with the melanocyte activation in CALM skin.

*Cytokine secretion into fibroblast-conditioned medium.* The third passage of fibroblast cultures was used for these experiments. Fibroblasts were seeded in 60-mm culture dishes (three dishes per specimen) at a density of $5 \times 10^5$ cells per 5 mL and were cultured in FGM. After the fibroblasts had been cultured for 96 h at 37 °C in a 5% $CO_2$ atmosphere, the medium was collected and 0·05 mL from each dish was used to quantify HGF, 0·1 mL was used for SCF and 0·2 mL was used for measurement of bFGF by enzyme-linked immunosorbent assay (ELISA).

*Cytokine secretion into keratinocyte-conditioned medium.* The second passage of keratinocyte cultures was used for these experiments. For the ELISA assay, the keratinocytes were seeded in 60-mm culture dishes (three dishes per specimen) at a density of $1·5 \times 10^5$ cells per 5 mL, and were cultured in KGM supplemented with 0·5% FCS. After they had been cultured at 37 °C in a 5% $CO_2$ atmosphere for 72 h, the keratinocyte-conditioned medium was collected and 0·1 mL per well for ET-1, 0·1 mL per well for GM-CSF, 0·05 mL for HGF and 0·2 mL for SCF were used for ELISA.

The ET-1 ELISA kit was purchased from Immuno-Biological Laboratories (Gunma, Japan), and the other ELISA kits were purchased from TECHNE Corporation (Minneapolis, MN, U.S.A.). These ELISA kits employ solid-phase enzyme immunoassays using the multiple antibody sandwich principle. Purified polyclonal antibodies specific for human ET-1, SCF, HGF or bFGF and a purified monoclonal antibody specific for human GM-CSF were attached to 96-well microtitre plates. Levels of immunoreactive cytokines were measured at A490 by an ELISA microplate reader (Model 550; Bio-Rad Laboratories, Hercules, California, USA). Cytokine levels in the culture samples were determined by comparing their absorbance with that produced by standards. The standard curve was linear from 3·13 to 100 pg $mL^{-1}$ for ET derivatives, from 3·9 to 250 pg $mL^{-1}$ for GM-CSF derivatives, from 62·5 to 4000 pg $mL^{-1}$ for HGF derivatives, from 31·2 to 2000 pg $mL^{-1}$ for SCF derivatives, and from 0·5 to 64 pg $mL^{-1}$ for bFGF derivatives. Comparison of cytokine concentrations was carried out using Bonferroni's multiple comparison tests.

### *Real-time polymerase chain reaction*

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed using real-time quantitative PCR (Sequence Detection System ABI PRISM 7700; Applied Biosystems, Perkin Elmer, Foster City, CA, U.S.A.) as reported previously.[27,28] After the fibroblast-conditioned media were collected, fibroblasts were used for RT-PCR analyses to quantify the expression of HGF and SCF transcripts. The oligonucleotide primers used for PCR were as follows: HGF 5′-GGT ACG CTA CGA AGT CTG TGA CA-3′ and 5′-CGC TGA CAA ATC TTG CCT GAT-3′, and SCF 5′-GCC GCT GTT CGT GCA ATA T-3′ and 5′-CTG CGA TCC AGC ACA AAC AGT-3′. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (TaqMan GAPDH Control Reagents, Perkin Elmer Biosystems). As negative controls, we conducted PCR without RT and confirmed that there was no amplification of the transcripts.

### *Semiquantitative reverse transcriptase-polymerase chain reaction*

Next, semiquantitative RT-PCR analysis was carried out to examine whether the expression of HGF and SCF transcripts by cultured fibroblasts was increased in group RC compared with group NN. After the fibroblast-conditioned media were collected for ELISA analysis, fibroblasts were used for RT-PCR analyses to quantify the expression of HGF and SCF transcripts. Total cellular RNA was extracted from cultured fibroblasts using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.), and was quantified by measur-

ing the optical density at 260 nm. About 1 μg total RNA was obtained from each tissue sample. Reverse transcription was performed in 20-μL reaction mixtures, each containing 2 μL 10 × PCR buffer, 4 μL $MgCl_2$ (25 mmol $L^{-1}$), 1 μL oligo-(dT) adaptor primer (2·5 μmol $L^{-1}$), 0·5 μL RNase inhibitor (40 U $\mu L^{-1}$), 2 μL deoxynucleotide mixture (10 mmol $L^{-1}$), 1 μL avian myeloblastosis virus RT (5 U $\mu L^{-1}$) and 9·5 μL RNA [1 μg in diethylpyrocarbonate (DEPC)-treated water]. Reaction mixtures were incubated at 42 °C for 30 min, and were heated to 99 °C for 5 min to inactivate the RT. For PCR amplification, 0·5 μL of each cDNA reaction mixture was added to 49·5 μL PCR mixture containing 5 μL $MgCl_2$ (25 mmol $L^{-1}$), 5 μL 10 × PCR buffer, 1 μL deoxynucleotide mixture (10 mmol $L^{-1}$), 0·5 μL of each of the 3′ and 5′ primers (50 μmol $L^{-1}$ each), 0·25 μL *Taq* polymerase and 37·25 μL DEPC-treated water. Reaction mixtures were amplified using a Microplate Gradient Thermal Cycler PC-960G (Corbett Research, Sydney, Australia). The PCR cycle conditions were: melting for 30 s at 94 °C, annealing for 30 s at 61 °C, and extension for 1·5 min at 72 °C. Reaction products (2 μL) were resolved on 2% agarose gels and were visualized by ethidium bromide staining. The sequences of the 3′ and 5′ primer pairs used in this study are shown in Table 2. As negative controls, we conducted PCR without RT and confirmed that there was no amplification of the transcripts. To quantify the expression of the transcripts, the intensities of the PCR bands were measured by densitometry using Image-Pro Plus (version 3·0; Media Cybernetics, Silver Spring, MA, U.S.A.), and are expressed as intensities relative to GAPDH.

## Results

*Clinical appearance and histochemistry*

The clinical appearance of NF1 is shown in Fig. 1. Haematoxylin and eosin-stained sections of CALM and non-CALM areas of skin showed no significant difference in tissue architecture or in the number or morphology of fibroblasts in the dermis (Fig. 2A,B). Histochemistry with toluidine blue staining revealed a marked increase in the number of mast cells in CALM (mean 28 cells per 0·2 $mm^2$, $n = 20$) and in

**Table 2.** Nucleotide sequences for 5′ and 3′ primers used

| |
|---|
| **Hepatocyte growth factor, 396 bp** |
| 5′ primer, 5′-GTT ATC GTG GGA ATG GCA A-3′ |
| 3′ primer, 3′-G TAT AGA CGC CTC CTA GTA-5′ |
| **Stem cell factor, 351 bp** |
| 5′ primer, 5′-GAT GTT TTG CCA AGT CAT TGT TGG-3′ |
| 3′ primer, 3′-GGA CTC TTT CTA AGG TCT CAG TCA-5′ |
| **Glyceraldehyde-3-phosphate dehydrogenase, 226 bp** |
| 5′ primer, 5′-GAA GGT GAA GGT CGG AGT C-3′ |
| 3′ primer, 3′-CT TTA GGG TAG TGG TAG AAG-5′ |

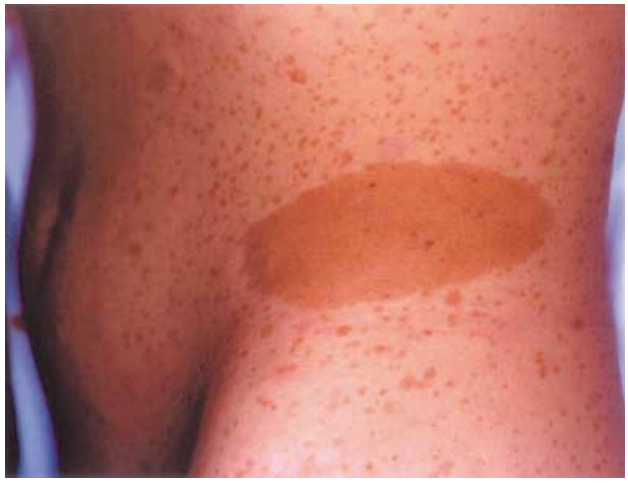

**Figure 1.** Clinical appearance of patient 3.

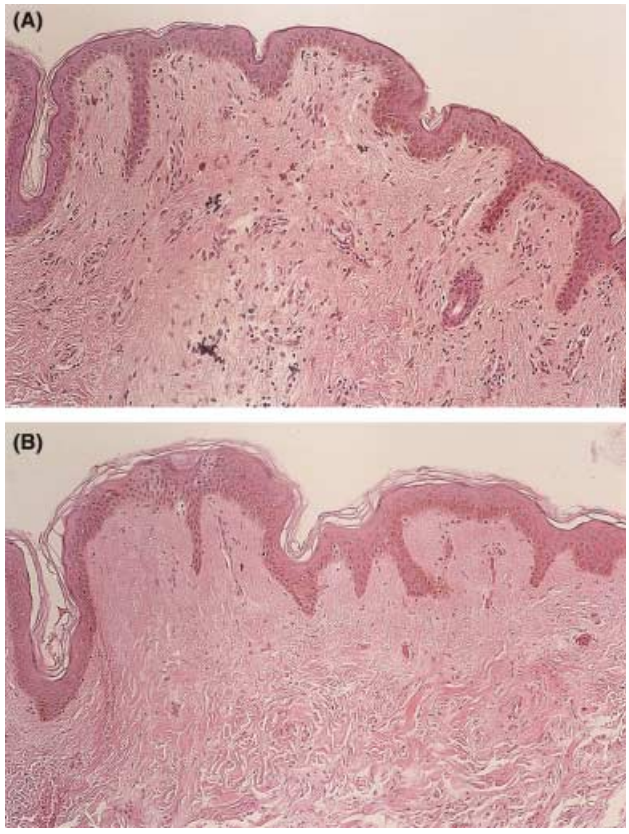


**Figure 2.** Histology of (A) café-au-lait macule (CALM) (patient 2) and (B) non-CALM skin (patient 3) (haematoxylin and eosin).

non-CALM (mean 10 cells per 0·2 $mm^2$, $n = 20$) skin compared with normal skin (mean three cells per 0·2 $mm^2$, $n = 20$) (Fig. 3A–C).

*Measurement of cytokines by enzyme-linked immunosorbent assay*

*Cytokine secretion into keratinocyte-conditioned medium.* ELISA of factors released into the keratinocyte-conditioned medium showed no significant differences between the three groups in levels of ET-1 and GM-CSF (Fig. 4A,B). HGF, bFGF and SCF were not detectable in any of the conditioned media (data not shown).

*Cytokine secretion into fibroblast-conditioned medium.* ELISA of factors released into the fibroblast-conditioned medium showed significantly higher concentrations of HGF and SCF in group RC than in groups RN and NN (Fig. 5A,B, Table 3). Levels of bFGF did not differ significantly among the three groups (Fig. 5C).

*Real-time reverse transcriptase-polymerase chain reaction*

Real-time RT-PCR of HGF and SCF mRNA revealed an increased expression of HGF (mean 14·4-fold, $n = 2$) and SCF (mean 4·6-fold, $n = 2$) transcripts in group RC compared with group NN (Fig. 6).

*Semiquantitative reverse transcriptase-polymerase chain reaction*

Semiquantitative RT-PCR of HGF and SCF mRNA revealed that the expression of HGF transcripts was

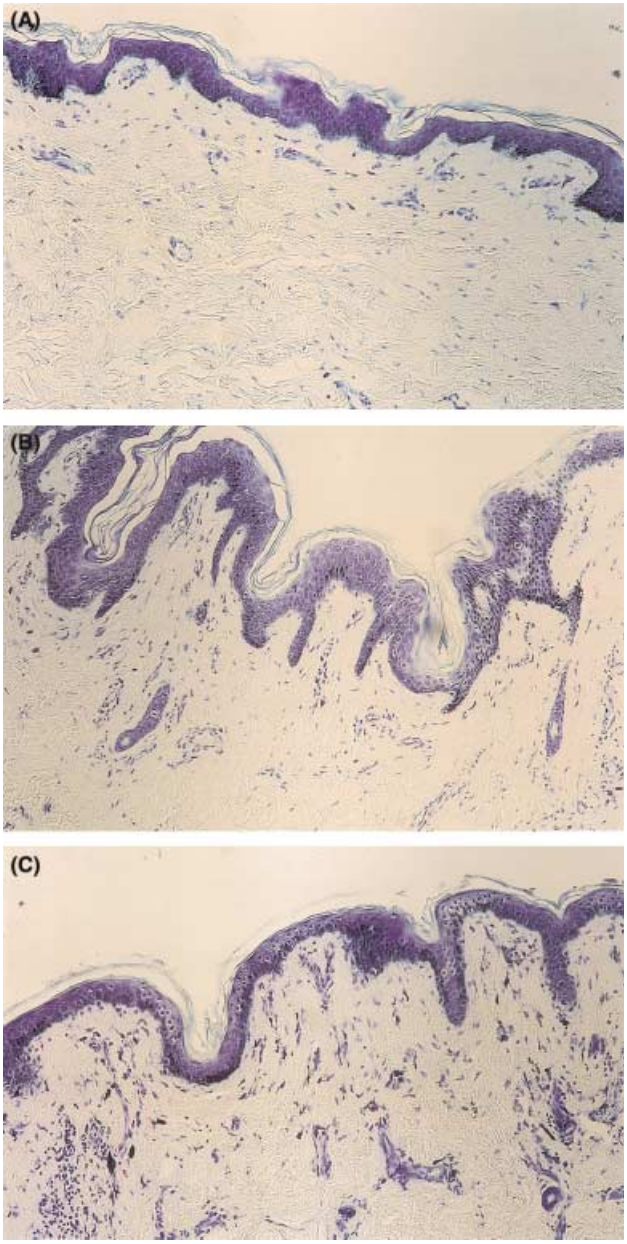


**Figure 3.** Histochemistry of (A) normal skin, (B) non-café-au-lait macule (CALM) skin, and (C) CALM skin (toluidine blue; original magnification × 200).

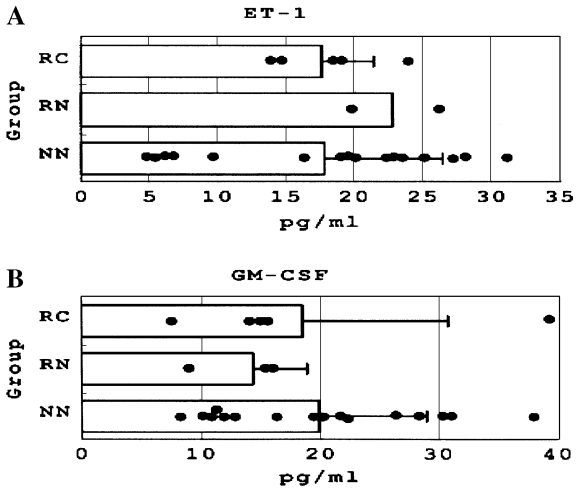


**Figure 4.** Cytokine secretion by keratinocytes after 3 days in culture. After human keratinocytes were cultured for 3 days at 37 °C in a 5% $CO_2$ atmosphere, the keratinocyte-conditioned medium was collected and used to quantify secreted cytokines using an enzyme-linked immunosorbent assay (ELISA). Values reported are mean ± SD. Group RC (*R*ecklinghausen *C*ALM), $n = 5$; group RN (*R*ecklinghausen *N*on-CALM), $n = 2–3$; group NN (*N*ormal skin of *N*ormal individuals), $n = 16$. CALM, café-au-lait macule. In group RN, measurement of endothelin-1 (ET-1) (A) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (B) by ELISA was performed in two or three specimens, because no significant difference was observed between group RC and group RN in preliminary experiments.

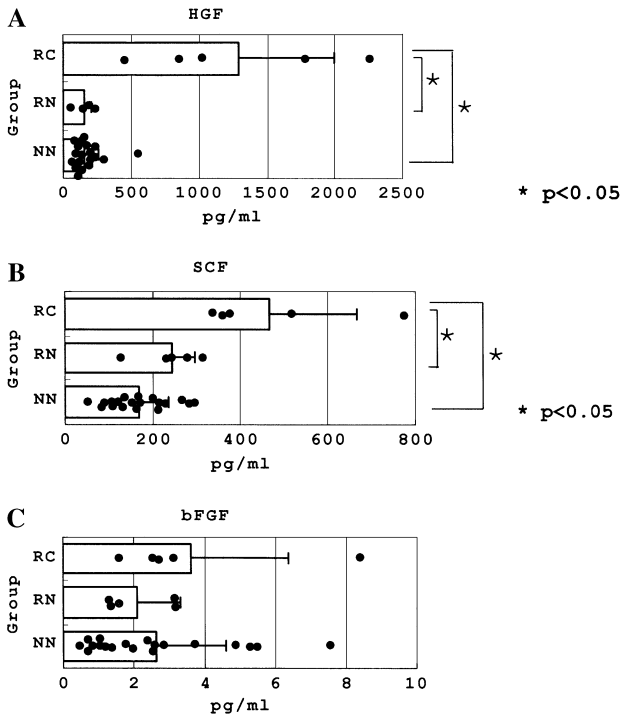


**Figure 5.** Cytokine secretion by fibroblasts after 4 days in culture. After human fibroblasts were cultured for 4 days at 37 °C in a 5% $CO_2$ atmosphere, the fibroblast-conditioned medium was collected and used to quantify secreted cytokines by enzyme-linked immunosorbent assay. Values reported are mean ± SD. Group RC (*R*ecklinghausen *C*ALM), $n = 5$; group RN (*R*ecklinghausen *N*on-CALM), $n = 5$; group NN (*N*ormal skin of *N*ormal individuals), $n = 19$. CALM, café-au-lait macule. $^*P < 0{\cdot}05$ compared with NN. (A) Hepatocyte growth factor (HGF). (B) Stem cell factor (SCF). (C) Basic fibroblast growth factor (bFGF).

markedly increased in group RC compared with group NN, while the expression of SCF transcripts was increased in two of the three specimens of group RC (Fig. 7).

## Discussion

Histological studies of CALM skin in NF1 patients showed an increased number of melanocytes with normal morphology.[6–8] As NF1 is a congenital skin disease and as CALMs of NF1 skin have an accentuated melanization in their epidermis due to an increased number of epidermal melanocytes undergoing stimulated melanogenesis, we first examined the possibility that keratinocytes in the lesional epidermis secrete larger amounts of melanogenic cytokines than do keratinocytes in the nonlesional or healthy control epidermis. Regarding known cases where epidermal keratinocytes produce and secrete larger amounts of melanogenic cytokines that lead to epidermal hyperpigmentation, we recently reported that such a mechanism occurs *in vivo* in UVB melanosis[11,12] and lentigo senilis,[14] where ET-1 functions as a paracrine melanogenic cytokine. Further, we have hypothesized that UVA melanosis primarily results from a stimulated secretion of GM-CSF by irradiated epidermal keratinocytes based upon an *in vitro* study which showed the stimulatory effect of UVA-exposed keratinocyte-conditioned medium on human melanocytes.[13] Those studies have shown that melanogenic cytokines secreted by cultured human keratinocytes have so far been confined to ET-1 and GM-CSF. The present study consistently demonstrated that SCF and HGF proteins were not detectable in the lesional keratinocyte-conditioned

**Table 3.** Mean ± SD secretion of hepatocyte growth factor (HGF) or stem cell factor (SCF) into the medium of cultured fibroblasts after 24 h incubation

| Patient | Origin of fibroblasts | HGF (pg $mL^{-1}$) | SCF (pg $mL^{-1}$) |
|---|---|---|---|
| Patient 1 RC ($n = 3$) | Lesional skin | 1765 ± 560 | 793 ± 96 |
| Patient 2 RC ($n = 3$) | Lesional skin | 1069 ± 47 | 365 ± 16 |
| Patient 3 RC ($n = 3$) | Lesional skin | 2237 ± 702 | 326 ± 8 |
| Patient 4 RC ($n = 3$) | Lesional skin | 479 ± 38 | 342 ± 12 |
| Patient 4 RN ($n = 3$) | Nonlesional skin | 204 ± 25 | 239 ± 9 |
| Patient 5 RC ($n = 3$) | Lesional skin | 917 ± 165 | 519 ± 36 |
| Patient 5 RN ($n = 3$) | Nonlesional skin | 156 ± 12 | 274 ± 9 |
| Patient 6 RN ($n = 3$) | Nonlesional skin | 175 ± 30 | 157 ± 24 |
| Patient 7 RN ($n = 3$) | Nonlesional skin | 79 ± 6 | 234 ± 18 |
| Total RC ($n = 5$) | Lesional skin | 1293 ± 701 | 469 ± 37 |
| Total RN ($n = 4$) | Nonlesional skin | 154 ± 53 | 226 ± 49 |
| Healthy controls NN ($n = 19$) | Healthy normal skin | 153 ± 98 | 168 ± 69 |

The isolated fibroblasts were seeded in three culture dishes and cultured for 24 h. The medium in each culture dish was assayed by enzyme-linked immunosorbent assay for HGF and SCF. RC, *R*ecklinghausen *C*ALM; RN, *R*ecklinghausen *N*on-CALM; NN, *N*ormal skin of *N*ormal individuals; CALM, café-au-lait macule.

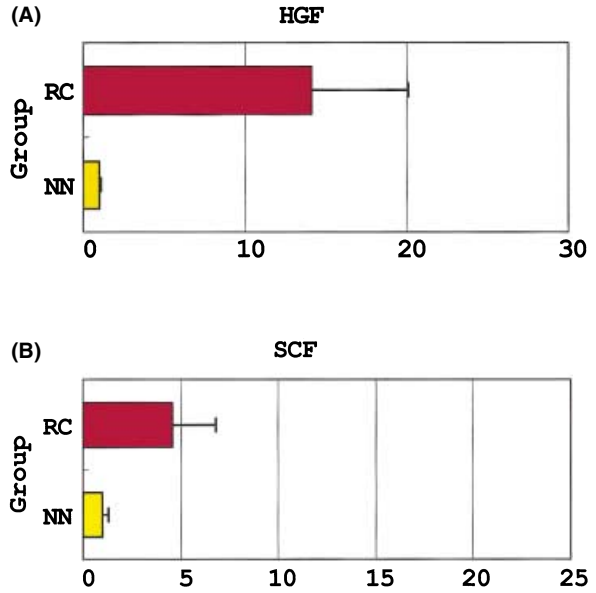


**Figure 6.** Real-time reverse transcriptase-polymerase chain reaction of (A) hepatocyte growth factor (HGF) and (B) stem cell factor (SCF) transcripts. Values reported are mean ± SD; $n = 2$ in each group. RC, *R*ecklinghausen *C*ALM; NN, *N*ormal skin of *N*ormal individuals; CALM, café-au-lait macule.

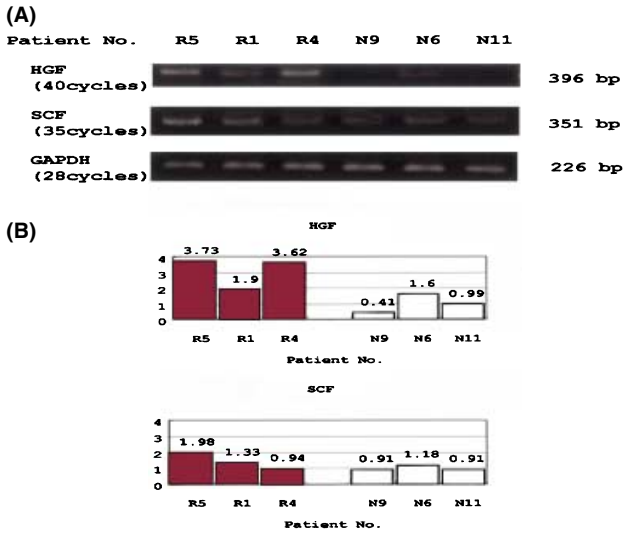


**Figure 7.** Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of hepatocyte growth factor (HGF) and stem cell factor (SCF) mRNA expression. (A) Fluorogram. (B) Densitometric analysis. Relative values are shown. Semiquantitative RT-PCR analysis was performed in three representative specimens of group RC (*R*ecklinghausen *C*ALM) and in three specimens of group NN (*N*ormal skin of *N*ormal individuals). CALM, café-au-lait macule. R1, R4 and R5 indicate the specimen number of CALM skin from patients with neurofibromatosis type 1. N6, N9 and N11 indicate the specimen number of normal skin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

medium (data not shown). Further, comparison of secreted amounts of ET-1 and GM-CSF using ELISA revealed that there was no significant difference in the levels of ET-1 and GM-CSF secreted by keratinocytes isolated from CALMs of NF1 skin, the nonlesional NF1 skin and the healthy controls. This suggests that lesional keratinocytes have no significant alteration in their potential to secrete melanogenic cytokines and may not be involved in the hyperpigmentary mechanism of CALMs in NF1, although an alternative possibility that lesional melanocytes have increased sensitivity to cytokines through receptors remains to be clarified.

As epidermal melanogenic factors derived from dermal fibroblasts are known mainly to be SCF, HGF and/or bFGF in culture,[16,17] we next compared the secretion of SCF, HGF and bFGF as intrinsic cytokines leading to epidermal pigmentation by fibroblasts derived from CALM, non-CALM and healthy control skin. Regarding known cases where dermal fibroblasts produce and secrete larger amounts of melanogenic cytokines that lead to accentuated pigmentation in epidermis overlying fibroblasts in the dermis, we have recently found that such a mechanism occurs *in vivo* in dermatofibroma, which is associated with the secretion of HGF and SCF by fibroblasts as melanogenic paracrine cytokines.[24] In the present study, determination of cytokine levels demonstrated that in patients with NF1, the potential of dermal fibroblasts localized in CALMs to secrete HGF and SCF was significantly higher than that of fibroblasts derived from non-CALM skin or from normal control skin. These increases are accompanied by increased expression of mRNAs encoding HGF and SCF by cultured fibroblasts of CALMs from NF1 skin compared with healthy control skin. In contrast, the secretion of bFGF was not increased in fibroblasts derived from CALMs of NF1 skin compared with non-CALM skin and healthy control skin. These findings suggest the hypothesis that high levels of HGF and SCF secretion by dermal fibroblasts located in CALMs correlate with the stimulated melanogenesis in epidermal melanocytes.

The *NF1* gene was cloned in 1990,[6–8] and belongs to the family of tumour-suppressor genes. Neurofibromin is encoded by the *NF1* gene and is a major negative regulator of the Ras pathway, a key signal transduction pathway in cells. Loss of neurofibromin leads to increased levels of activated Ras, and thus increased downstream mitogenic signalling. It has been suggested that the reduction of neurofibromin in the epidermis of NF1 patients is responsible for the abnormal physiology such as the elevated melanogenesis and the increased density of melanocytes.[9] However, this does not necessarily

account for the localized hyperpigmented areas (CALMs) seen in NF1 because the reduction of neurofibromin is systemic, not localized. Our observation that fibroblasts localized beneath the CALMs, but not those in other areas, are stimulated to secrete SCF and HGF, suggests that the reduction of neurofibromin is not directly associated with the hyperpigmentation of CALM skin, but that additional unknown factors associated with neurofibromin stimulate fibroblasts to secrete such cytokines. Thus, the relation between reduced neurofibromin in patients with NF1 and the mechanisms of increased secretion of HGF and SCF by dermal fibroblasts remains unclear.

Available evidence indicates that there is a significant increase in mast cells in neurofibromas of patients with NF1 and neurofibromatosis type 5 compared with the dermis of normal individuals, and that the density of mast cells in neurofibromas is independent of the neurofibromatosis type and the age of patients.[29] In our study, an increase in the number of mast cells in CALM skin was observed compared with non-CALM skin and normal skin. Increased secretion of SCF by dermal fibroblasts derived from CALM skin of patients with NF1 may lead to the increased number of mast cells in neurofibromas of NF1. This is also corroborated by the fact that in dermatofibromas there are increased numbers of mast cells in the dermis overlying the fibroblastic tumours, and those cells secrete larger amounts of SCF than does nonlesional dermis.[24] Thus, the fact that in addition to the increased numbers of activated melanocytes in the CALM epidermis, the number of mast cells located beneath the epidermis is increased over that in the nonlesional skin supports the suggestion that at least SCF is secreted in a soluble and diffusible form by fibroblasts at concentrations capable of directly influencing mast cells to proliferate. This strongly suggests that at least soluble SCF plays an important role in the epidermal hyperpigmentation of CALM skin.

In conclusion, our findings suggest that the increased secretion of HGF and SCF by dermal fibroblasts in CALM skin of NF1 patients may be associated with the accentuated cutaneous pigmentation seen in the lesions. However, the association of reduced neurofibromin levels in NF1 patients and the mechanism(s) of increased HGF and SCF secretion by dermal fibroblasts remains to be further clarified.

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