The Role of the Epidermal Endothelin Cascade in the Hyperpigmentation Mechanism of Lentigo Senilis

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Little is known about the mechanism(s) underlying hyperpigmentation in lentigo senilis. We have previously reported that keratinocyte-derived endothelins are intrinsic paracrine mitogens and melanogens for human melanocytes and that they play an essential role in stimulating ultraviolet-B-induced melanogenesis. In this study, we have used immunohistochemistry and reverse transcriptase polymerase chain reaction analysis to clarify the role of the endothelin cascade, including endothelin production, processing by endothelin-converting enzyme, and expression of the endothelin B receptor, in the hyperpigmentary mechanism(s) involved in lentigo senilis. The number of tyrosinase immunopositive melanocytes in lentigo senilis lesional skin was increased 2-fold over the perilesional epidermis. Immunohistochemistry using antibodies to endothelin-1 demonstrated relatively stronger staining in the lesional epidermis than in the perilesional epidermis. Reverse transcriptase polymerase chain reaction analysis concomitantly demonstrated accentuated expression of transcripts for endothelin-1 and for the endothelin B receptor in lentigo senilis lesional skin, which was accompanied by a similar accentuated expression of tyrosinase mRNA compared with the perilesional control. The endothelin-1-inducible cytokine, tumor necrosis factor α, was consistently upregulated in the lentigo senilis lesional epidermis as determined at the transcriptional level and by immunostaining, whereas interleukin-1α was downregulated. In contrast, endothelin-converting enzyme 1α mRNA was not substantially increased in the lesional epidermis. These findings suggest that an accentuation of the epidermal endothelin cascade, especially with respect to expression of endothelin and the endothelin B receptor, plays an important role in the mechanism involved in the hyperpigmentation of lentigo senilis.

Keywords: endothelin B receptor/interleukin-1α/tumor necrosis factor α. J Invest Dermatol 116:571–577, 2001

Little is known about biologic mechanisms by which melanocytes are activated to produce excessive amounts of melanin in various hyperpigmentary disorders. Recent evidence has suggested that melanocyte function is regulated by several cytokines that are secreted by surrounding epidermal cells (keratinocytes) in a paracrine fashion. In ultraviolet B (UVB) induced pigmentation, human keratinocytes exposed to UVB are stimulated to produce and secrete vasoconstrictive peptides, termed endothelins (ETs), which trigger the activation of melanocytes and act as potent mitogens and melanogens for human melanocytes (Yada *et al*, 1991; Imokawa *et al*, 1992, 1995, 1996a, 1997). Similarly, UVA-induced pigmentation has been shown to be mediated by the action of another mitogen for human melanocytes, termed granulocyte/macrophage colony-stimulating factor (Imokawa *et al*, 1996b). In allergic-contact-dermatitis-induced hyperpigmentation, we have recently found that chemokines, such as growth-related oncogene α, are produced and secreted by epidermal cells under allergic inflammatory conditions, which results in the stimulation of melanocytes (Imokawa *et al*, 1998). No information is available regarding cytokines essential for stimulating melanization of melanocytes in unstimulated epidermal hyperpigmentary disorders, however.

Lentigo senilis (LS) is the skin condition of common aging spots with accentuated epidermal pigmentation. Based upon the observation that large numbers of melanin-producing melanocytes are located in the vicinity of highly proliferating keratinocytes, as seen in hair follicles, we hypothesized that the proliferating keratinocytes in LS trigger the activation of neighboring melanocytes by secreting melanocyte-stimulating cytokines. As, among the known keratinocyte-derived cytokines produced in response to their proliferation, ET-1 is the only cytokine that has dual stimulatory effects on DNA synthesis and melanization of human melanocytes (Yada *et al*, 1991; Imokawa *et al*, 1996a, 1997), it is of considerable interest to clarify whether ET production and secretion is accentuated in the epidermis of LS. Further, ET secretion is known to be regulated by endothelin-converting enzyme (ECE-1α), which converts the propeptide, big-ET, to its active form, ET-1 (Xu *et al*, 1994). In addition to ET produced and secreted by keratinocytes, ET action on melanocytes is mediated via the endothelin B receptor (ET_BR), which is a G-protein-coupled transmembrane receptor (Tada *et al*, 1998). In this study, therefore, we used immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR) analysis to determine whether the epidermal ET cascade, consisting of ET production, ECE-1α (Xu *et al*, 1994), ET secretion, and ET_BR-mediated signaling (Brand *et al*, 1998; Imokawa *et al*, 2000), is accentuated in the LS epidermis.

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Abbreviations: ECE, endothelin-converting enzyme; ET, endothelin; ET_BR, endothelin B receptor; LS, lentigo senilis; SCF, stem cell factor.

Table I. Nucleotide sequences for 5′ and 3′ primers

Endothelin-1	
3′ primer	5′-GACAGGCCCCGAAGTCTGTCA-3′
Tyrosinase	
5′ primer	5′-TATGAATGGAACAATGTCCCAGGT-3′
3′ primer	5′-ATGACCAGATCCGACTCGCTTG-3′
ET_BR	
5′ primer	5′-CGAGCTGTTGCTTCTTGGAGTAG-3′
3′ primer	5′-AACGGAAGTTGTCATATCCGTGAT-3′
ECE-1α	
5′ primer	5′-CGCTGGGGATGTCGACGTACAA-3′
3′ primer	5′-GGGGTCCACTGTGGGGTCCAT-3′
IL-1α	
5′ primer	5′-TTGAAGACCTGAAGAACTGTTACAG-3′
3′ primer	5′-TAAAGTTGTATTTCACATTGCTCAGGA-3′
TNFα	
5′ primer	5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′
3′ primer	5′-GCAATGATCCCATTGTAGACCTCCCAGACT-3′
GAPDH	
5′ primer	5′-GAAGGTGAAGGTCGGAGTCAACG-3′
3′ primer	5′-AGTCCTTCCACGATACCAAAGTTG-3′

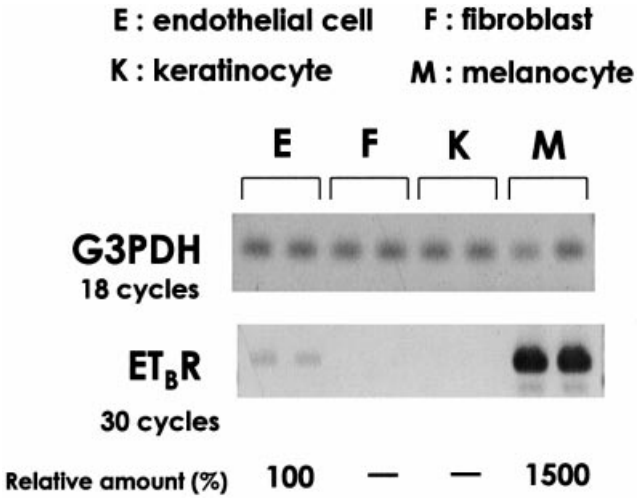

Figure 1. The expression of ET_BR mRNA in various cultured human skin cells.

MATERIALS AND METHODS

Materials Normal human endothelial cells, fibroblasts, keratinocytes, and E300 medium were obtained from Kyokuto (Tokyo, Japan). Serum-free keratinocyte medium (SFM), bovine pituitary extract (BPE), epidermal growth factor (EGF), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Laboratories. Normal human melanocytes and serum-free melanocyte medium (MGM) were purchased from Sankou Pure Chemicals (Tokyo, Japan). Other chemicals were of reagent grade.

Cell culture Cells were maintained at 37°C with 5% CO_2. Human endothelial cells, fibroblasts, keratinocytes, and melanocytes were maintained in E300 medium, DMEM containing 10% fetal bovine serum, modified SFM supplemented with 5 ng per ml EGF and 50 μg per ml BPE, and MGM supplemented with 1 ng per ml recombinant basic fibroblast growth factor, 5 μg per ml insulin, 0.5 μg per ml hydrocortisone, 10 ng per ml phorbol 12-myristate 13-acetate, antibiotics (50 μg per ml streptomycin), and 0.2% (vol/vol) BPE, respectively.

Chemicals Streptavidin-biotin immunoperoxidase staining systems were obtained from Shandon-Lipshaw (PA).

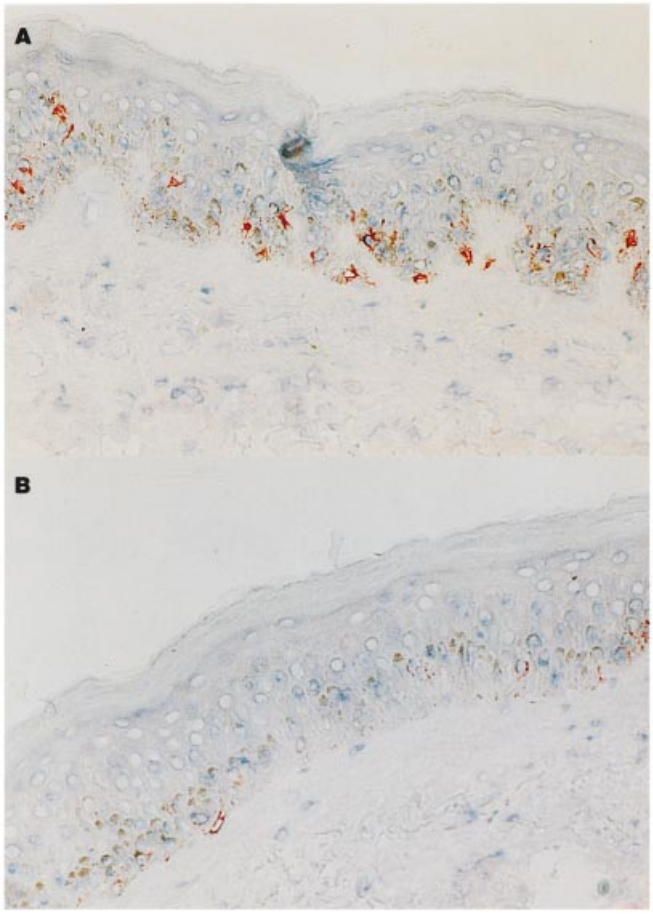

Figure 2. Immunohistochemistry with tyrosinase antibodies in the epidermis of LS. (*A*) Lesional skin (180×); (*B*) non-lesional skin (180×).

Antibodies Polyclonal antibodies against recombinant human ET-1, tyrosinase, interleukin-1α (IL-1α), and tumor necrosis factor α (TNFα) were purchased from Immuno Biological Laboratories (Gunma, Japan).

Samples Skin biopsy specimens were obtained from patients with LS ($n = 19$; Japanese, six males, 13 females) and perilesional skin was considered as nonlesional normal skin. These patients ranged in age from 45 to 80 y old (average, 62 y). The sites of skin excisions were in the abdomen, lower legs, buttocks, and back, which are generally sun-protected skin areas, and there were no symptoms of sun exposure when the biopsies were taken. The main portion of each specimen was subjected to routine histopathologic examination. Additional sample specimens were embedded in Tissue-Tek OCT compound (Miles, IN), snap-frozen in liquid nitrogen, and then stored at –80°C until use.

Immunohistochemistry Cryostat sections (4 μm thick) were air-dried, fixed in cold acetone, and, after washing in 0.01 M phosphate-buffered saline (PBS), incubated in 3% H_2O_2 for 5 min at room temperature. After further washing in 0.01 M PBS, the sections were incubated with protein blocking agent for 20 min at room temperature. The sections were then washed again in 0.01 M PBS and incubated overnight at 4°C in 0.01 M PBS, pH 7.2, with antibodies to ET-1, TNFα, or IL-1α (1:50). After further washing in 0.01 M PBS, the sections were incubated for 30 min at room temperature with rabbit IgG antibody, washed again in 0.01 M PBS, and incubated for 30 min at room temperature with standard Omnitags streptavidin-peroxidase. The reaction was then developed with aminoethylcarbazole (AEC). Sections were incubated for 10–15 min at room temperature with Omnitags plus/HRP kit (consisting of two drops of sodium acetate buffer, two drops of AEC chromogen concentrate, and two drops of 3% H_2O_2 in distilled water), washed in PBS, and stained with hematoxylin and eosin.

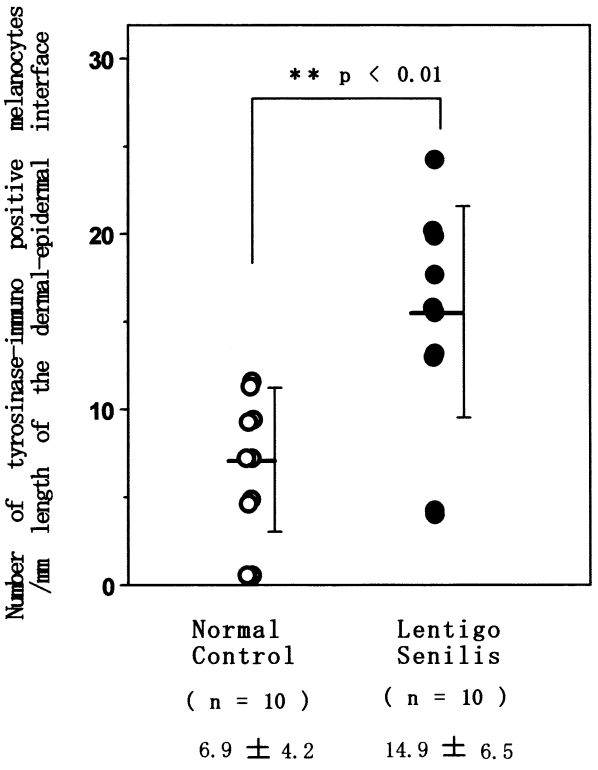

Figure 3. The increased number of tyrosinase immunopositive melanocytes in the epidermis of LS.

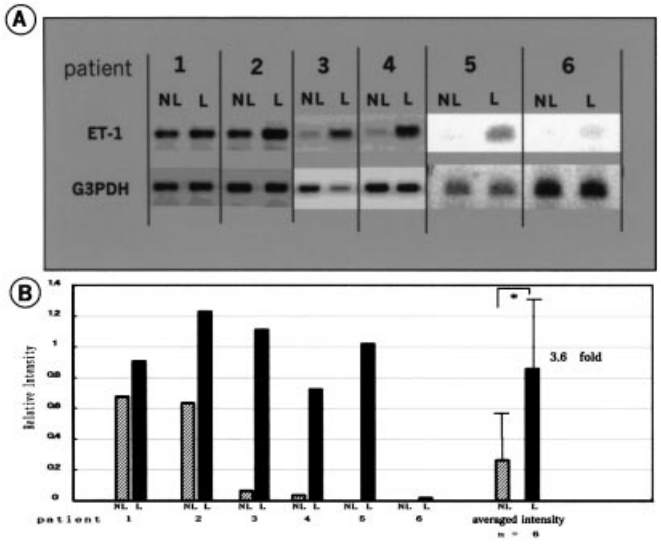

Figure 4. RT-PCR analysis of ET-1 mRNA in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS. Fluorograms are shown at 35 or 37 cycles of PCR for ET-1 and at 22 or 23 cycles for GAPDH. *p < 0.05.

Detection of ECE-1α, ET-1, ET_BR, and tyrosinase gene transcripts in cultured cells or human epidermis from LS and perilesional normal skin Biopsy specimens were obtained from LS and perilesional skin as detailed above. The epidermal sheet of each specimen was separated by heating for 3–4 min at 60°C. The residue of separated tissue was examined after periodic acid-Schiff staining and we confirmed that the separation took place within the epidermis, leaving no dermal components on the epidermal sheet. Total cellular RNA was extracted from cultured human keratinocytes, endothelial cells, fibroblasts, and melanocytes, or from the separated epidermis, using acid guanidinium phenol chloroform with only a single precipitation. The RNA was quantified by measuring the optical density at 260 nm. About 1 μg of total RNA was obtained from each tissue sample. To synthesize oligo-(dT16)-primed cDNA, total RNA was heated to 65°C for 3 min, and then chilled on ice. Reverse transcription was performed in 20 μl reaction mixtures containing 2 μl 10 × PCR buffer II, 4 μl $MgCl_2$ (25 mM), 1 μl oligo-(dT16) (50 μM), 8 μl deoxynucleotides (2.5 mM each), 1 μl RNase inhibitor (20 U per μl), 1 μl Moloney murine leukemia virus reverse transcriptase (50 U per μl), and 3 μl RNA

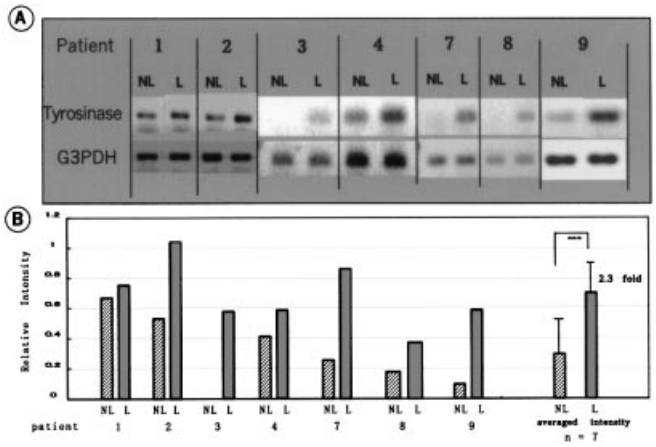

Figure 5. RT-PCR analysis of tyrosinase mRNA in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS. Fluorograms are shown at 31 or 35 cycles of PCR for tyrosinase and at 23 or 25 cycles for GAPDH. ***p < 0.005.

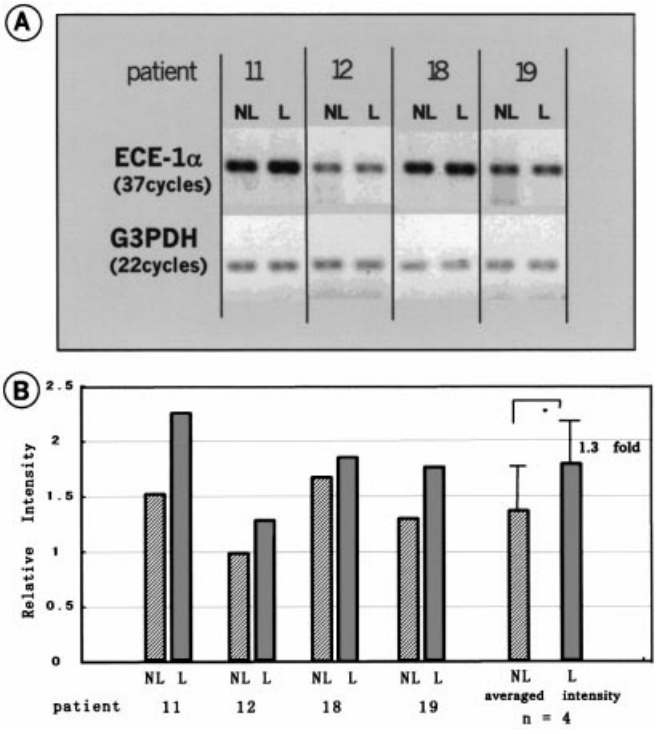

Figure 6. RT-PCR analysis of ECE-1α mRNA in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS. *p < 0.05.

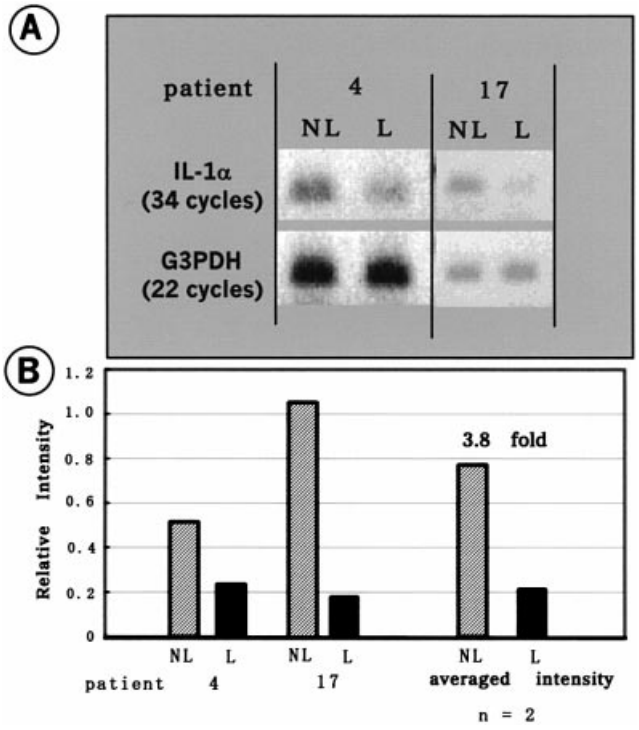

Figure 7. RT-PCR analysis of IL-1α in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS.

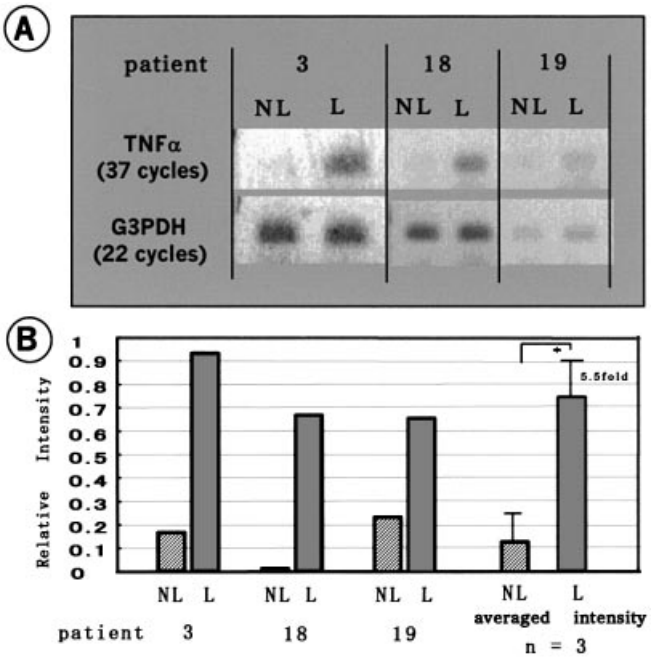

Figure 8. RT-PCR analysis of TNFα mRNA in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS. *p < 0.05.

(300 ng in DEPC-treated water). Reaction mixtures were incubated at 42°C for 60 min and 52°C for 30 min, and then heated to 99°C for 5 min to inactivate the reverse transcriptase. For PCR amplification, 2 μl of the cDNA reaction mixture was added to 8 μl of an 80 μl PCR mixture containing 4 μl $MgCl_2$ (25 mM), 8 μl 10 × PCR buffer II, 1 μl each of the 3′ and 5′ primers (20 μM each), and 0.5 μl Taq polymerase (Cetus). Reaction mixtures were overlaid with mineral oil, and then amplified using a Perkin-Elmer/Cetus thermal cycler. The PCR cycle conditions were melting for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C. Reaction products (5 μl) were resolved on 1%–1.5% agarose gels and were visualized by ethidium bromide staining. Primers for ET-1, tyrosinase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized on a DNA synthesizer (Applied Bio Systems). The sequences of the 3′ and 5′ primer pairs used in this study are shown in **Table I**. As negative controls, we conducted PCR without reverse transcriptase and confirmed that there was no amplification of the transcripts. To quantitate the expression of the transcripts, the intensities of PCR bands were measured by densitometry and are expressed as intensities relative to GAPDH.

Statistics The level of significance of the difference was calculated by the Student *t* test.

RESULTS

ET_BR gene expression in human keratinocytes and melanocytes To examine whether the ET_BR gene is exclusively expressed by human melanocytes among the various types of epidermal cells, we used RT-PCR to compare the levels of expression of that gene by human keratinocytes, melanocytes, fibroblasts, and endothelial cells in culture. RT-PCR analysis of ET_BR mRNA demonstrated its distinct or slight expression by human melanocytes or endothelial cells, respectively, but not by human keratinocytes and human fibroblasts (**Fig 1**).

Melanocyte population To determine whether the epidermal hyperpigmentation of LS is associated with an increased proliferation of melanocytes, we first studied the population of melanocytes by measuring their number following immunostaining with a tyrosinase antibody. The immunostaining revealed immunoreactivity localized to a few dendritic cells along the basal layer (**Fig 2**), suggesting an increased proliferation of melanocytes in the LS epidermis. Quantitation along the interface between the epidermis and the dermis revealed that the number of tyrosinase immunopositive melanocytes was increased 2-fold in the lesional LS epidermis compared with the perilesional epidermis (**Fig 3**).

ET-1, ECE-1α, IL-1α, TNFα, and ET_BR gene transcripts in LS To examine whether the increased melanogenesis was associated with accentuation of the ET cascade in LS, we examined expression of the ECE-1α, ET-1, and ET_BR genes using RT-PCR. Analysis of ET-1 mRNA demonstrated an increased expression (average 3.6-fold, $n = 6$) of the ET-1 transcript in LS skin compared with that in perilesional normal skin (**Fig 4**) and there was also a marked increase (average 2.3-fold, $n = 7$) in tyrosinase transcripts in the lesional LS skin (**Fig 5**). As the production of ET-1 is biologically regulated by ECE-1α (Xu *et al*, 1994), we determined whether the ECE-1α transcript was also upregulated in LS, which would result in the increased secretion of ET-1. RT-PCR analysis of ECE-1α mRNA, however, revealed no substantial difference in expression (average 1.3-fold, $n = 4$) of the ECE-1α transcript in the lesional LS skin (**Fig 6**). As IL-1α and TNFα are known as ET-1-inducible cytokines in cultured human keratinocytes (Imokawa *et al*, 1992; Tsuboi *et al*, 1994), we also examined the expression of IL-1α ($n = 2$) and TNFα mRNA ($n = 3$) in the LS epidermis. RT-PCR of these mRNAs demonstrated a decrease (average 3.8-fold, $n = 2$) and an increase (average 5.5-fold, $n = 3$) in the expression of IL-1α and TNFα, respectively, in the lesional LS epidermis (**Figs 7, 8**), suggesting the involvement of TNFα as a primary cytokine in the accentuated production of ET.

In human melanocytes, ET-1 secreted by keratinocytes triggers the activation of the intracellular protein kinase C dependent signaling pathway through ET_BRs (Imokawa *et al*, 2000). Therefore, we determined whether the expression of ET_BRs was also accentuated in melanocytes in the LS

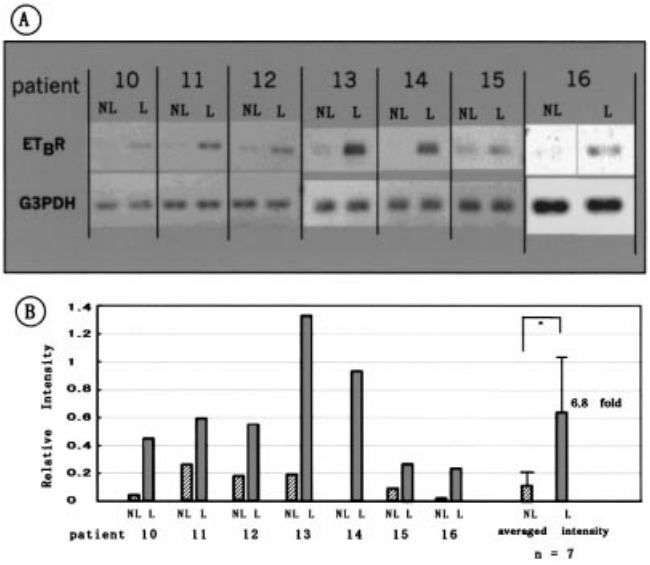

Figure 9. RT-PCR analysis of ET_BR mRNA in the epidermis of LS and in perilesional skin. (*A*) Fluorogram; (*B*) densitometric analysis. NL, non-lesional epidermis; L, lesional epidermis of LS. Fluorograms are shown at 34 or 37 cycles of PCR for ET_BR and at 22 or 25 cycles for GAPDH. *$p < 0.05$.

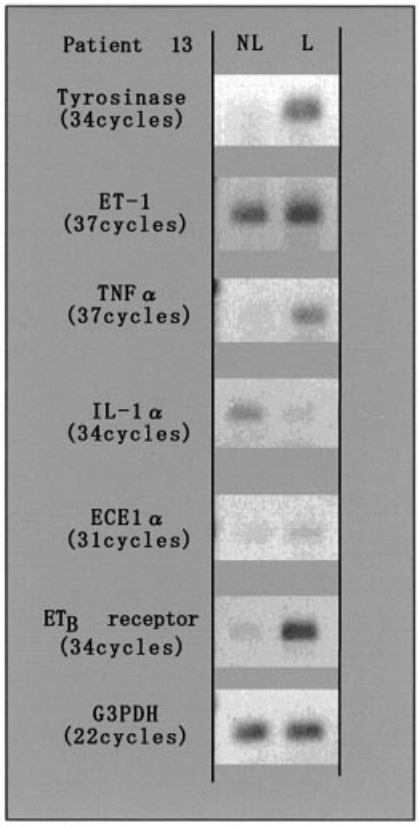

Figure 10. RT-PCR analysis of all transcripts used in the epidermis of the same donor with LS and the perilesional skin. NL, non-lesional epidermis; L, lesional epidermis of LS.

epidermis. RT-PCR of ET_BR mRNA revealed an increased expression (average 6.8-fold, $n = 7$) of ET_BR transcripts (**Fig 9**), suggesting the coordinated increase in expression of

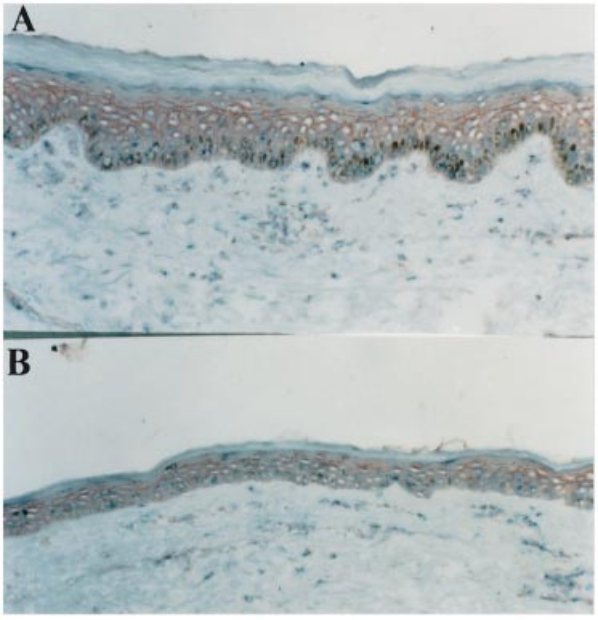

Figure 11. Immunostaining with anti-ET-1 in the epidermis of LS. (*A*) Lesion (180×); (*B*) non-lesion (180×).

the ET-1/ET_BR linkage. To examine whether there were variations in the patterns of expression of the transcripts tested, we compared the expression of ET-1, ECE-1α, tyrosinase, ET_BR, IL-1α, and TNFα transcripts in the epidermis of the same donor with LS. RT-PCR analysis of these mRNAs revealed a reproducible expression pattern for each transcript that was similar to the above results (**Fig 10**). The ET-1, tyrosinase, ET_BR, and TNFα transcripts were upregulated, whereas the IL-1α transcript was downregulated, in the epidermis of LS relative to the perilesional normal epidermis. On the other hand, there was no substantial difference in the expression of ECE-1α transcript between LS and the perilesional skin.

Immunohistochemistry To confirm the upregulated production of ET-1 in LS, we examined whether the production of this cytokine in LS actually reflected the changes observed in their transcript levels. Staining with antibodies against ET-1 revealed a distinct positive immunoreactivity throughout the basal layers and along the lower epidermis in LS (**Fig 11**), whereas there was only a weakly positive staining in the perilesional normal control. As IL-1α and TNFα are known to stimulate the secretion of ET-1 in cultured human keratinocytes (Imokawa *et al*, 1992; Tsuboi *et al*, 1994), we also evaluated the levels of IL-1α and TNFα by immunohistochemistry. Staining with antibodies against TNFα revealed a positive immunoreactivity throughout the LS epidermis (**Fig 12**), whereas there was only a weakly positive staining in the perilesional normal control. In contrast, staining with IL-1α antibodies was decreased in LS relative to the perilesional skin (**Fig 13**).

DISCUSSION

LS is a skin condition comprising slightly hyperplastic pigmented spots, which appear over the entire body, especially on sun-exposed areas. The clinical features of LS are distinct from those of UVB-induced pigmentation, the latter being strictly localized in UVB-irradiated areas and generally accompanied by cutaneous inflammation (Imokawa *et al*, 1992). Therefore, the pathogenesis of LS is poorly characterized, particularly with respect to the mechanism involved in the hyperpigmentation. Because of the potential role of ET as a paracrine cytokine in the activation of epidermal

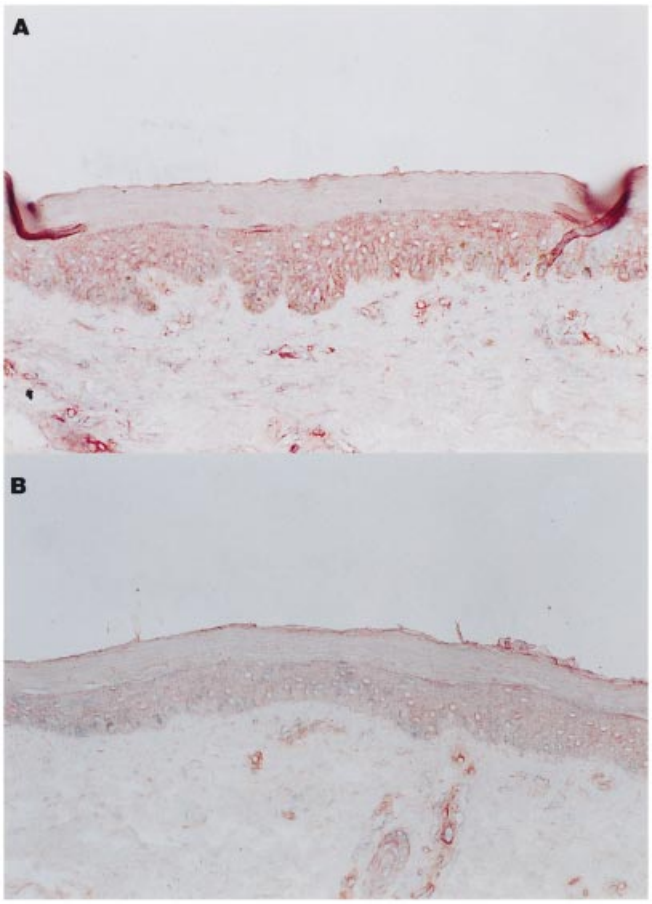

Figure 12. Immunostaining with anti-TNFα in the epidermis of LS. (*A*) Lesion (180×); (*B*) non-lesion (180×).

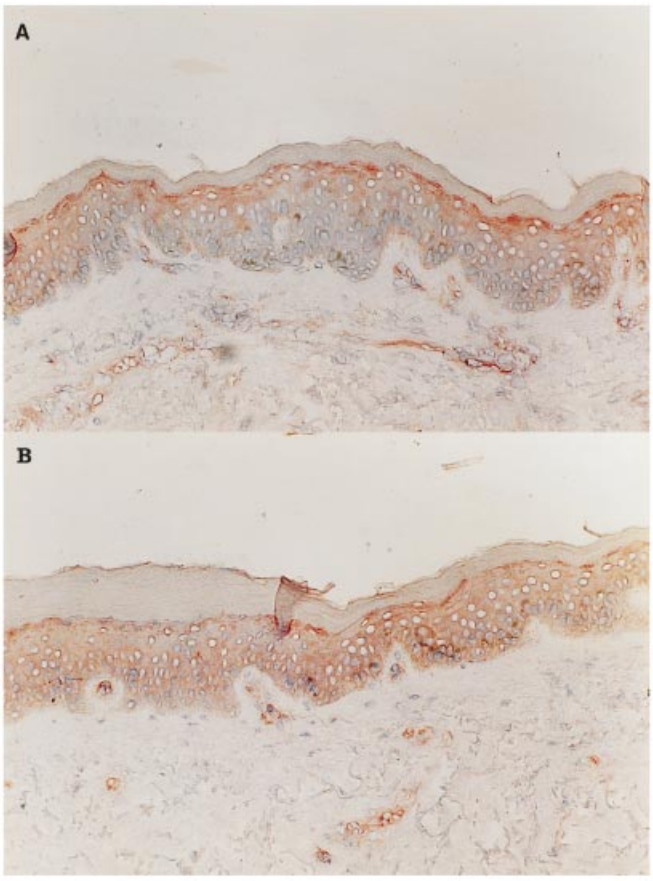

Figure 13. Immunostaining with anti-IL-1α in the epidermis of LS. (*A*) Lesion (180×); (*B*) non-lesion (180×).

melanocytes, as occurs in UVB pigmentation (Imokawa *et al*, 1992, 1995), it is of particular interest to clarify the potential involvement of the ET cascade in the hyperpigmentation associated with LS. Although ET plays an essential role in activating melanocyte function following stimulation such as in UVB-exposed skin where ET is overproduced by irradiated keratinocytes (Imokawa *et al*, 1992), this study has provided a new insight into the role of ET as a regulator of human melanocytes in the epidermal hyperpigmentation involved in a variety of pigmentary disorders. The potential of keratinocytes located in the LS lesional epidermis to produce ET-1 was significantly higher than in the perilesional normal controls. The increased production and localization of ET-1, as assessed in LS epidermis by immunostaining, were paralleled by increased amounts of tyrosinase in melanocytes revealed by immunostaining with tyrosinase antibody. This suggests that melanocyte activation, including their stimulated proliferation and increased production of tyrosinase, occurs in LS lesional skin concomitant with the stimulation of ET-1 production by surrounding keratinocytes. Consistent with these immunohistochemical observations, RT-PCR analysis of mRNAs isolated from the epidermis of lesional LS skin revealed that, whereas ET-1 and tyrosinase mRNA signals are weak in perilesional normal skin, there is a marked increase in the expression of both genes in LS lesional epidermis. ET-1 is a potent vasoconstrictive peptide that was discovered in the conditioned medium of endothelial cells (Yanagisawa *et al*, 1988). Recently, ET-1 has been defined as a strong mitogen and melanogen for human melanocytes (Imokawa *et al*, 1996a, 1997). UVB exposure stimulates autocrine production of ET-1 through IL-1α secretion in keratinocytes, which in turn leads to the activation of melanocyte function seen in UVB-induced hyperpigmentation (Imokawa *et al*, 1995). Based on these findings, the accentuated production and expression of ET-1 and ET-1 mRNA, respectively, in LS epidermis strongly suggests that the increased production of ET-1 is a major factor responsible for the hyperpigmentation seen in LS.

There is another regulatory factor involved in the secretion of ET-1 by keratinocytes. ETs are synthesized in endothelial cells as a 36 amino acid prepropeptide (Yanagisawa *et al*, 1988), which is subsequently converted by ECE-1α to its active form (Xu *et al*, 1994). Thus, production of the active form of ET depends on the activity of ECE-1α, probably based on its level of expression in the epidermis. RT-PCR analysis of ECE-1α previously demonstrated that human keratinocytes in culture express the same ECE-1α transcript as do endothelial cells and that its expression is not altered by UVB exposure, in contrast to an increased expression of the ET-1 transcript.[1] This suggests that, as in endothelial cells, ECE-1α plays an important role in the secretion of the active form of ET-1 by human keratinocytes and that UVB exposure does not trigger increases in the expression of ECE-1α. RT-PCR analysis revealed that the LS lesional epidermis has no substantial increase in the expression of ECE-1α transcripts compared with the perilesional control, which suggests no involvement of ECE-1α in the stimulated secretion of ET-1 in the lesional skin.

There is yet another mechanism known to be involved in the stimulation of ET-1 production in the epidermis. The cytokine IL-1α is stimulated and released by keratinocytes following UVB irradiation, which triggers the production of ET-1 by keratinocytes in an autocrine fashion (Imokawa *et al*, 1992). The observation that large numbers of melanin-producing melanocytes are localized in

[1]Manaka I, Tajima S, Teraki E, Kawashima M, Miyagishi M, Imokawa G: Role of endothelin-1 in hyperpigmentation in seborrheic keratosis. *Pigment Cell Res. Supplement* 5:81 1998 (abstr.)

the general vicinity of highly proliferating keratinocytes, as seen in hair follicles or in seborrhoeic keratosis (Teraki *et al*, 1996), led us to suppose that keratinocytes may secrete large amounts of IL-1α during the slightly proliferative conditions of LS epidermis, which might trigger the autocrine stimulation of ET-1 production. Immunohistochemistry with IL-1α antibodies, however, unexpectedly demonstrated downregulation of IL-1α in the LS lesional epidermis compared with the perilesional epidermis. In contrast, a similar ET-inducible cytokine, TNFα (Tsuboi *et al*, 1994), is expressed at high levels in the lesional LS epidermis relative to the perilesional epidermis. The expression of transcripts encoding IL-1α or TNFα was consistently downregulated or upregulated, respectively, in the lesional LS epidermis. Thus it is likely that the accentuated expression of ET-1 in LS epidermis is mediated via the increased production of TNFα**,** although the specific mechanisms involved in the high expression of TNFα remain unclear.

Because of the essential role of the ET_BR in intracellular signaling elicited by ET within melanocytes (Tada *et al*, 1998; Imokawa *et al*, 2000), it is probable that expression of the ET_BR on melanocytes in the LS epidermis is also involved in the stimulation by ET that leads to the accentuation of melanin production. In the epidermis, it has been documented that keratinocytes also express the ET_BR (Tsuboi *et al*, 1994; Kelley *et al*, 1994). There is a negligible expression of ET_BR by cultured human keratinocytes, however, in contrast to their marked expression by cultured human melanocytes, whereas the *in vitro* expression of ET_BR in phorbol-ester-treated melanocytes may not necessarily reflect their *in vivo* expression. In this connection, it should be noted that there is a distinct immunostaining with polyclonal antibodies to ET_BR in melanocytes, but not in keratinocytes in the normal human epidermis.[2] Because the expression of ET_BR mRNA in the epidermis substantially reflects the production per epidermal cell unit, the upregulated expression of the ET_BR in the LS epidermis suggests that the increased secretion of ET-1 and its receptor are stimulated in a manner coordinated with epidermal hyperpigmentation in LS. As the expression of the ET_BR has also been shown to be directly regulated by TNFα in human endothelial cells (Smith *et al*, 1998), the increased expression of the ET_BR may also result from the upregulation of TNFα in LS.

In UVB-induced pigmentation, we have recently found that, in addition to ET-1, membrane-bound stem cell factor (SCF) plays an important role in stimulating the proliferation and melanogenesis of melanocytes, and that this is accompanied by an increase in the expression of the SCF receptor, c-kit.[3] Further, we have recently reported that there is a synergistic crosstalk between ET-1 and SCF in the stimulation of mitogenesis and melanogenesis in human melanocytes (Imokawa *et al*, 2000). Therefore, it will be of considerable interest in the future to determine whether the SCF/c-kit cascade is also upregulated in LS epidermis, which would support the stimulatory effect of ET-1 on melanogenesis in LS in concert with SCF.

In conclusion, ET-1 is the only keratinocyte-derived cytokine reported to date that can stimulate both proliferation and melanization of human melanocytes at concentrations as low as 1 nM (Yada *et al*, 1991; Imokawa *et al*, 1997). Thus, it seems likely that the increased secretion of ET-1 noted in LS epidermis is the major factor responsible for the hyperpigmentation found in this pigmentary disorder. Taken together, our findings suggest that upregulation of the ET cascade, which consists of the production and secretion of ET-1 by keratinocytes and the ET/ET_BR binding-mediated signal transduction pathway in melanocytes, plays an important role in the stimulated pigmentation in LS.

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