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Endothelin ET_A Receptor in Human Skins with Keloid and Hypertrophic Scar

Yuichi HAMADA[1], Akihiko HIMENO[2], Hisa YAMAGUCHI[2], Akiyoshi HIRANO[1], Tohru FUJII[1]

1) Department of Plastic Surgery, Nagasaki University School of Medicine
2) Department of Pharmacology 1, Nagasaki University School of Medicine

Endothelins (ETs) work as growth factors for fibroblastic growth. Keloids are clinically unpleasant scars within the skin that grow beyond the confines of the original wound. We compared the expression of ET receptors between human skins with hypertrophic scars and keloids, by using our quantitative receptor imaging system. The ET receptor binding sites were distributed in skin areas anatomically corresponding to the epidermis, the superficial and deep dermis, and the vascular bed. The ET_A receptor was apparently observed in the skin areas, whereas the ET_B receptor was only slightly detected, as deduced from the finding that mRNA encoding the human ET_A receptor is expressed in the fibroblast and the vascular bed. The ET_A receptor densities of keloid and highly hypertrophic scar were significantly higher than those of normal skin and moderately and mildly hypertrophic scar. There seemed to be no differences in the density between keloid and highly hypertrophic scar. In a keloid skin we noted a possible existence of atypical vascular bed ET_A receptor with a low affinity to ET_A antagonist of PD151242 and FR139317. The ET_A receptor of endogenous growth factors possibly play an etiological role in dysfunctions of the fibroblast in hypertrophic scars, and a typical ET_A receptor may be related to the growth of keloid.

Key Words: endothelin (ET), ET_A receptor, human skin, wound healing, keloid, hypertrophic scar (human), *in situ* hybridization, quantitative receptor autoradiography

Introduction

Endothelins (ETs), family peptides of biologically active ET-1, ET-2 and ET-3 consisting of 21 amino acid residues, work as autocrine and paracrine transmitters, by interacting with two receptors; ET_A and ET_B of G-protein coupling-type receptors with seven-transmembrane domains.[2,23,40] As ETs were originally isolated from cultured porcine aortic endothelial cells, extensive studies have been performed to clarify the regulatory significance in maintaining the function of cardiovascular-endothelial system. In addition to a role in modulation of vascular tone, ETs function as autocrine /paracrine growth factors. The peptides stimulate DNA synthesis of rat vascular smooth muscle cells, astrocyte, C6 glioma cells[14,15,20,21], in collaboration with other growth factors such as platelet-derived growth factor and insulin.[8] Cultured rat astrocytes proliferate and differentiate through activation of their own ET_B receptor.[9] In the case of human dermal fibroblasts, interestingly, the ET_A receptor operates in the mitogenic effect on these cells.[13]

Takagi et al. (1994)[29,30] and Tao et al. (1995)[32] found that in primary culture of rabbit and bovine corneal epithelium ET-1 stimulated and accelerated wound closure in vivo. Keloids are clinically unpleasant scars within the skin that growth beyond the confines of the original wound, and dys- and/or hyperfunction of dermal fibroblasts during wound-healing seems to be an event related to the pathophysiology of keloids. Therefore, to investigate the pathophysiological significance of ETs on wound healing process and keloid formation, we compared the expression of ET receptors in human skins, keloid and hypertrophic scar.

Address Correspondence: Yuichi Hamada, M.D.,
Department of Plastic Surgery,
Nagasaki University School of Medicine,
1-7-1 Sakamoto, Nagasaki 852-8523, Japan

Materials and Methods

Human skins

Human skins with keloid, and hypertrophic scar were obtained from 5 patients undergoing keloid and hypertrophic scar removal in university-affiliated clinics

(Table 1). Lesions were classified according to clinical findings into; 1) keloid (cases #1 and #2), 2) highly hypertrophic scar (case #3), 3) moderately hypertrophic scar (case #4), and 4) mildly hypertrophic scar (case #5). Normal parts of skins were dissected from skins of patients with keloid (case #1) and also obtained from trimmed excess site from free flap transfer (case #6). Collected tissues were immediately frozen at -30℃ in dry ice-isopentane and stored at -80℃. Informed consent was obtained from each patient after the study procedures had been fully explained.

Table 1. Summary of the patients

case No.	sex	age	clinical classification
1	M	43	keloid
			normal skin*
2	F	57	keloid
3	F	5	highly hypertrophic scar
4	F	21	moderately hypertrophic scar
5	F	38	mildly hypertrophic scar
6	M	64	normal skin**

*Normal skin was obtained from trimmed excess site after scar revision.
**Normal skin was obtained from trimmed excess site after free flap tranfer.

Quantitative receptor autoradiography for ET receptors binding

Frozen, 16-μm-thick sections of human skins were cut on a cryostat at -20℃, thaw-mounted onto gelatin-coated slides and stored overnight under vacuum at 4℃. Related tissue sections were labelled *in vitro* with ^{125}I-ET-1 [non-selective radioligand] (specific activity -81.4 TBq/mmol), ^{125}I-PD151242 [ET_A radioligand][7], or ^{125}I-IRL1620 [ET_B radioligand])[37](-81.4 TBq/mmol) in 2.0 ml of incubation buffer, according to our method.[25,34,39] Briefly, after pre-incubation at room temperature (23℃) for 10 min in incubation buffer, the sections were incubated with a fixed amount or increasing concentrations of radioligands, at 4℃ for 48 hr, in the presence or absence of ET receptor-related compounds such as ET-1, ET-3, sarafotoxin S6c [ET_B agonist][38], BQ-123 [ET_A antagonist][11], BQ-788 [ET_B antag-onist][12], IRL1620 [ET_B agonist][31], PD151242 [ET_A antagonist][7], or FR139317 [ET_A antagonist][27], in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 10 mM EDTA-2Na, 1 mg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 10 μM phosphoramidon, and 0.3% protease-free bovine serum albumin. After incubation, the slides were washed three times (1 min each) at 4℃ in 50 mM Tris-HCl buffer, pH 7.4, and rinsed quickly in ice-cold distilled water. Tissue sections were dried under a stream of cold air. The dried sections were apposed against Hyperfilm-^{3}H (Amersham International plc., UK) and the films were developed with a D19 developer (Eastman Kodak, USA) for 7 min at 4℃.

Quantitation of radioligands binding was made using our method, taking advantage of the high sensitivity of the computerized radioluminographic system with imaging plates coated with fine photostimulable phosphor crystals (BaF- Br:Eu^{2+}) (Bio-imaging analyzer BAS 2000, Fuji Photo Film Co., Japan).[1] The dried sections were exposed to a radioluminographic imaging plate with calibrated ^{125}I-standards ([^{125}I]micro-scales, Amersham). The values for photostimulated luminescence obtained directly from the imaging plates by the computerized scanning system were converted to the bound radioactivity of the section and the results were expressed as the mean ± S.E. in fmol/mg, based on a comparison with standard curves for sets of standards run for each autoradiogram. We measured the photostimulated luminescence at four spots in each area, and automatic computer averages were attained.

In situ hybridization for human ET_A receptor mRNA

We confirmed the presence of ET_A receptor mRNAs in the human skin, using our *in situ* hybridization method with cRNA probes.[26] ET_A receptor PCR-amplified DNA fragments (ET_A, bases 100-911) were subcloned into the multiple cloning site of pBluescript II SK^+ flanked by the T3 and T7 promotes. Double-labelled ^{35}S-cRNAs were synthesized from linearized plasmids using α-^{35}S-UTP and α-^{35}S-CTP (~47.73 TBq/mmol) with T7 RNA polymerase. The probes were alkaline-hydrolyzed to an average length of 350 bp.

Frozen, 16-μm-thick sections of the human skin adjacent to the sections used for quantitative receptor autoradiographic studies were processed for *in situ* hybridization techniques. Briefly, related tissue sections were fixed in 4.0% paraformaldehyde-phosphate-buffered saline, digested with 10 μg/ml proteinase K for 10 min at 37℃, and soaked for 10 min in 0.5% acetic anhydride-0.1 M triethanolamine (pH 8.0) and 0.9% NaCl. Tissue sections were dehydrated through a graded series of ethanol, defatted in chloroform, and air-dried. Subsequently, the hybridization buffer, 10 mM Tris-HCl buffer, pH 7.5, containing 50% deionized formamide, 1 x Denhart's solution, 600 mM NaCl, 1.0 mM EDTA, 10% dextran sulfate, 0.25% sodium dodecyl sulfate, 50 mM dithiothreitol (DTT), 500 μg/ml tRNA, 250 μg/ml herring sperm DNA, and ^{35}S-labelled cRNA probe (10^7 cpm/ml) was applied to each section, and cover-slipped tissue sections were incubated for 16 hr at 55℃. Slides were washed several times in 4 x NaCl-sodium citrate (SSC) with 10 mM DTT to remove the cover slides and hybridization

buffer, and then incubated in 50% deionized formamide-2 x SSC with 10 mM DTT for 30 min at 55℃, and in 10 mM Tris-HCl buffer, pH 7.6, containing 20 μg/ml RNase, 500 mM NaCl, and 1.0 mM EDTA for 30 min at 37℃. Tissue sections were finally washed in 0.1 x SSC-10 mM 2-mercaptoethanol for 15 min at 55℃, and dehydrated through a graded series of ethanol. The sections were dipped into NTB-3 nuclear emulsion (Eastman Kodak), stored in the dark at 4℃ for 30 days, developed with a D19 developer, then counterstained with hematoxylin and mounted.

Data analysis and materials

To determine the maximum binding capacity (B_{max}), the dissociation constant (K_D), and the inhibition constant (K_i), radioligands binding data were analyzed using the program LIGAND (BIOSOFT, UK).[17,18] Differences in binding parameters, K_D, and B_{max} were assessed by one-way analysis of variance (ANOVA), followed by Bonferroni /Dunn multiple range test (StatView 4.5, Abacus Concepts, Inc., U.S.A.) at a p value of less than 0.05.

All radiolabelled compounds used here, α-^{35}S-UTP, α-^{35}S-CTP, ^{125}I-ET-1, ^{125}I-IRL1620, and ^{125}I-PD151242 were purchased from New England Nuclear, USA, and Amersham, UK. BQ-123 was a gift from Banyu Pharmaceutical Co., Ltd., Japan, IRL1620 was from Ciba-Geigy Japan Ltd., Japan, PD151242 was from Parke-Davis Pharmaceutical Research Division, USA., and FR139317 was from Fujisawa Pharmaceutical Co., Ltd., Japan. Peptides were purchased from Peptide Institute Co., Ltd., Japan. pBluescript II SK$^+$ and T7 RNA polymerase were obtained from Strategene Inc., USA, and Takara Shuzo Co., Ltd., Japan, respectively.

Results

Distribution of endothelin receptors in keloid and hypertrophic scar tissues

Under binding conditions, we observed a considerable amount of ^{125}I-ET-1 [non-selective radioligand] binding to the human skin with a significant amount of density. Total binding and non-specific binding (Fig. 1F to J) of ^{125}I-ET-1 are evident in the left side-panel (Fig. 1A to E), and right side-panel (Fig. 1F to J), respectively. ^{125}I-ET-1 binding sites were distributed in skin areas anatomically corresponding to the epidermis, superficial and deep dermis, and vascular beds. Even with a low concentration (0.77 pM) of ^{125}I-ET-1 in the incubation buffer, a considerable amount of specific ^{125}I-ET-1 binding to the human skin tissue could be detected and was calculated to be 1.1 fmol/mg of total binding (Fig. 1E) and 0.01 fmol/mg of non-specific binding (Fig. 1J) in the dermis of normal skin. Thus, we confirmed that this receptor autoradiographic method coupled to the radioluminographic imaging plate system makes feasible quantitative determinations of the ET receptors in the human skin, with a considerable increase in sensitivity. ^{125}I-ET-1 Binding in the epidermis was irreplaceable with large amounts of non-radioactive ET-1, suggesting it to be non-specific binding.

As shown in Fig. 1A, we noted the highest density of ^{125}I-ET-1 binding to the dermis of keloid, among human skins with highly (Fig. 1B), moderately (Fig. 1C), and mildly hypertrophic scars (Fig. 1D). Taking note of a very low density in the dermis of normal skin (Fig. 1E), The amount of ET receptors expressed in the dermis seemed to depend upon the degree of

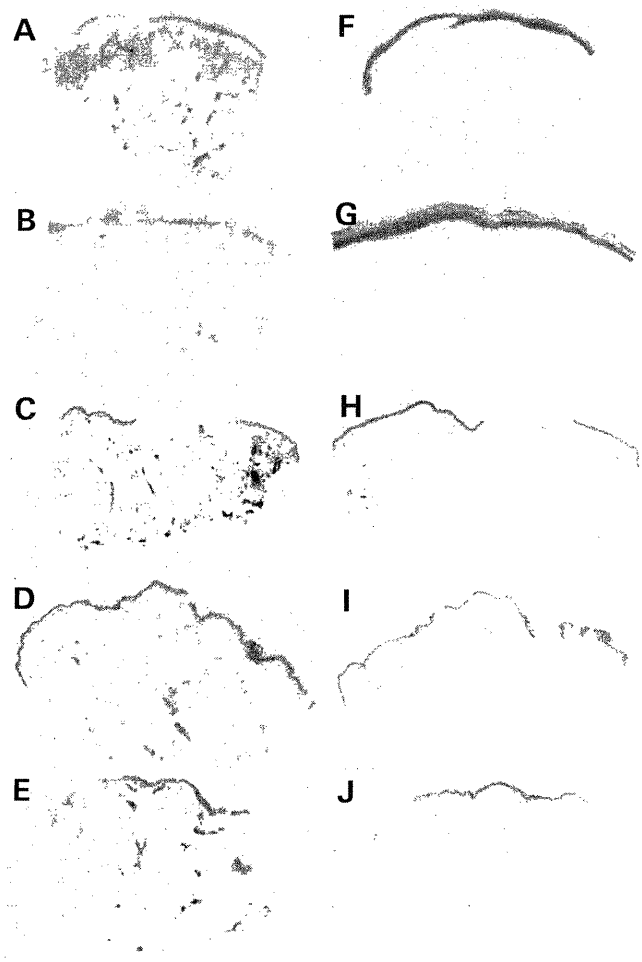

Fig. 1. Evidence for the existence of ET receptor binding sites in skin areas of keloid (A, case #2), highly hypertrophic scar (B, case #3), mederately hypertrophic scar (C, case #4), mildly hypertrophic sar (D, case #5), and normal skin (E, case #6). Receptor autoradiograms were obtained at incubations of 1.7 nM ^{125}I-ET-1 [non-selective radioligand]. Non-specific binding of ET receptor binding sites (F to J, right side panel) were determined in the presence of 1.0 μM of labelled ET-1.

cicatrization.

To characterize ET receptor binding sites, we used two selective radioligands; ^{125}I-PD151242 [ET_A radioligand], and ^{125}I-IRL1620 [ET_B radioligand]. Figure 2 shows results of investigation using ^{125}I-IRL1620, which binds specifically to endothelin ETB receptor. We could not detect significant amounts of specific ^{125}I-IRL1620 binding to the human skin areas.

Contrary to ^{125}I-IRL1620 binding, there were rich binding sites of ^{125}I-PD151242 [ET_A radioligand] in the human skin areas; superficial and deep dermis, and vascular beds (Fig. 3), corresponding to ^{125}I-ET-1 binding sites. Thus, the ET_A receptor seemed to predominantly exist in the human skin areas.

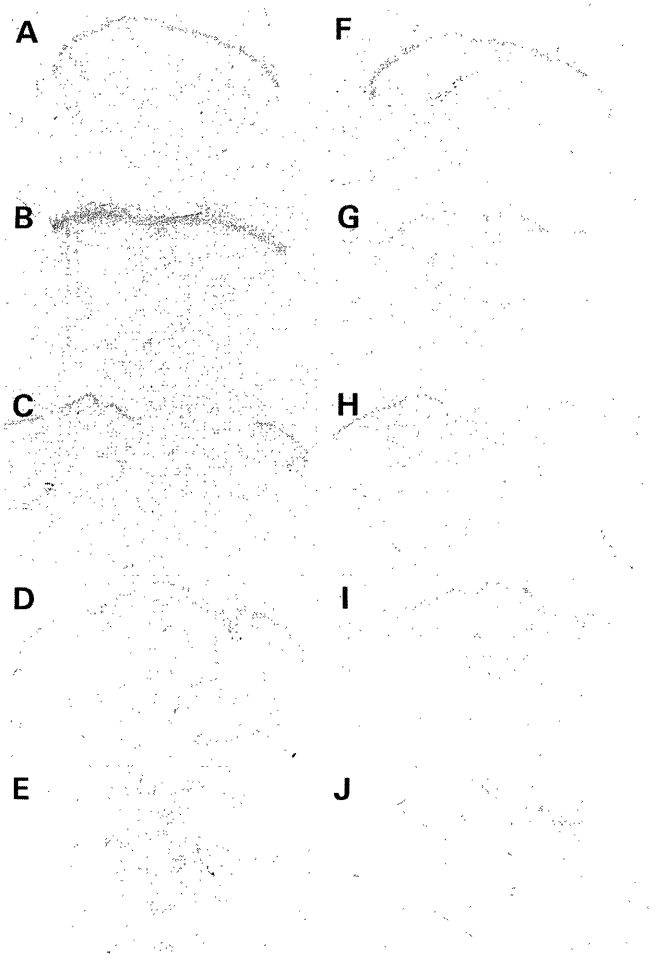

Fig. 2. No evidence for the existence of ET_B receptor binding sites in skin areas of keloid (A, case #2), highly hypertrophic scar (B, case #3), mederately hypertrophic scar (C, case #4), mildly hypertrophic sar (D, case #5), and normal skin (E, case #6). Receptor autoradiograms were obtained at incubations of 330 pM ^{125}I-IRL1620 [ET_B radioligand]. Non-specific binding of ET_B receptor binding sites were determined in the presence of 1.0 μM of unlabelled IRL1620 (F to J, right side panel). Note no differences in the density between total binding (left side panel) and non-specific binding (right side panel).

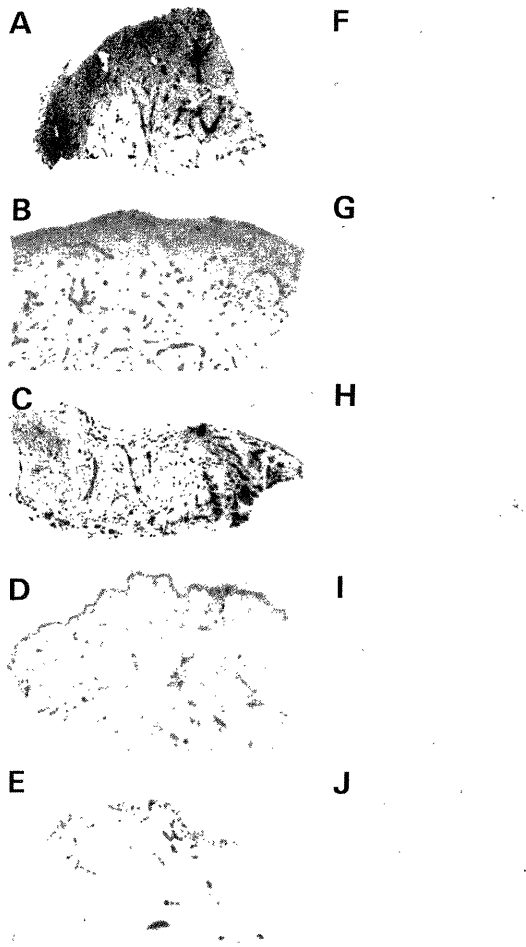

Fig. 3. Evidence for the existence of ET_A receptor binding sites in skin areas of keloid (A, case #2), highly hypertrophic scar (B, case #3), moderately hypertrophic scar (C, case #4), mildly hypertrophic sar (D, case #5), and normal skin (E, case #6). Receptor autoradiograms were obtained at incubations of 1.0 nM ^{125}I-PD151242 [ET_A radioligand]. Non-specific binding of ET_A receptor binding sites were determined in the presence of 1.0 μM of unlabelled PD151242.

Microscopic observation of endothelin ET receptor

Cellular distribution of the ET receptor expressed in the human skin areas was also investigated (Figs. 4 and 5). As shown in Fig. 4, we microscopically confirmed that fibroblasts richly aggregated in the superficial dermis of human skins with keloid (Fig. 4A and B), and hypertrophic scar (Fig. 4C and D). The area with a rich aggregation of fibroblasts (fibroblasts rich area) (Fig. 4Aa and Ca) was compared with the autoradiographic localization of ^{125}I-PD151242 binding sites (Fig. 4Ba and Da). Binding of ^{125}I-PD151242 occurred at the fibroblast rich layer of the dermis, suggesting that the ET_A receptor existed in fibroblasts. Similarly, belt-shaped binding sites in the deep dermis (Fig. 4Bb and Db) corresponded with vascular beds (Fig. 4Ab and Cb).

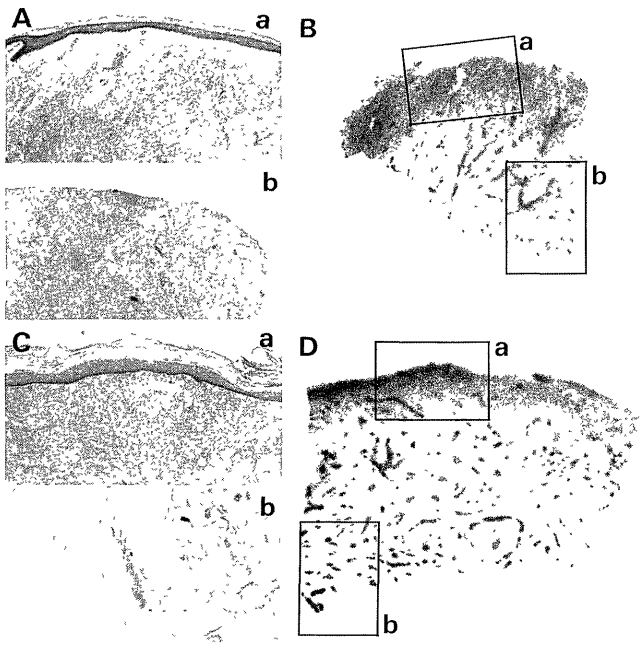

Fig. 4. Comparison of microscopic evidence (A and C, hematoxylin and eosin) on fibroblastic aggregations (a) and vascular beds-distribution (b) with receptor autoradiographic localization of ET_A receptor binding sites (B and D) in the superficial dermis of keloid (A and B, case #2) and highly hypertrophic scar (C and D, case #3). The squares "a" and "b" of the panels B and D are compartible to "a" and "b" of the panels A and C.

We confirmed the cellular expression of ET_A receptor mRNA in the human skin, using our *in situ* hybridization method with cRNA probe and an emulsion autoradiographic technique (Fig. 5). 35S-labelled cRNA probe derived from the ET_A receptor cDNA specifically hybridized to the human skin section (Fig. 5A and B), as evidenced by no significant hybridization signals in parallel experiments performed under conditions with the same probe in the presence of a 50-fold excess of unlabelled probes (Fig. 5C and D). Light microscopic autoradiograms revealed that silver grains of ^{35}S-ET_A cRNA were discretely distributed throughout the skin areas. There were many silver grains of ^{35}S-ET_A cRNA in vascular beds (Fig. 5A), and also the silver grains were concentrated in spotty areas with a cluster of fibroblasts (Fig. 5B).

Characterization and Quantitation of ET receptor in human skin

Related, 16-μm-thick sections were incubated in the presence of increasing and nine different concentrations of ^{125}I-ET-1, ranging from 0.77 pM to 1.7 nM, in the absence (total binding) or presence of 1.0 μM ET-1 (non-specific binding) (Fig. 6). In ^{125}I-ET-1 saturation

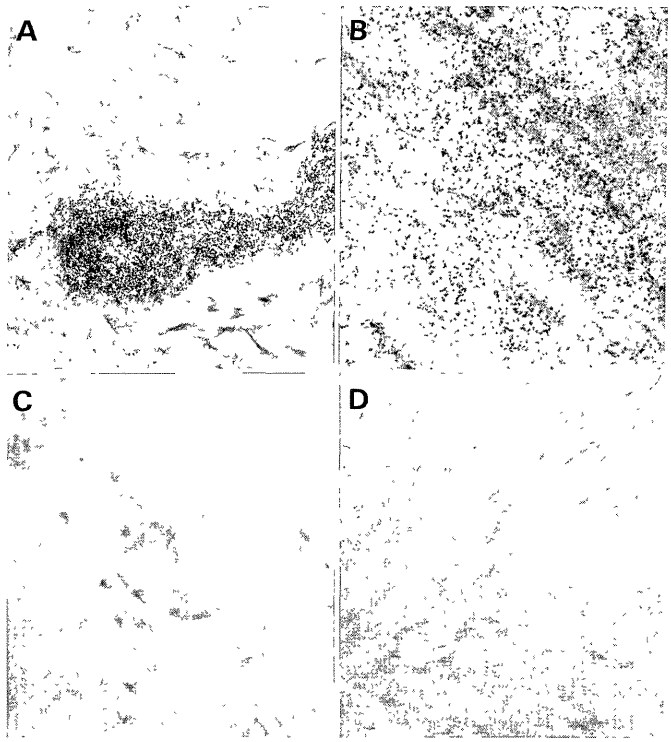

Fig. 5. Cellular localization of the expression of mRNA encoding ET_A receptor in vascular beds (A) and fibroblasts (B) of human skins with keloid (case #2). Silver grains of ^{35}S-ET_A cRNA are heterogeneously distributed throughout the human skin areas. Non-specific signals (C and D) were determined at competition of ^{35}S-labelled cRNA probes with a 50-fold excess of unlabelled ET_A cRNA.

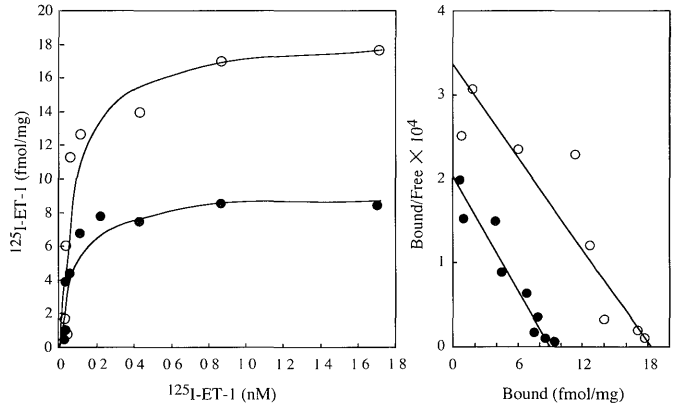

Fig. 6. A saturation binding study with increasing concentrations (0.77 pM to 1.7 nM) of ^{125}I-ET-1 (left side panel) carried out in the human skin sections of keloid (case #2, open circles) and of highly hypertrophic scar (case #3, closed circle). Scatchard plots (right side panel) were obtained by ^{125}I-ET-1 saturation binding data, analyzed using the program LIGAND.

binding experiments done on use of consecutive tissue sections obtained from patient with keloid (case #1 of Table 1, open circles), and hypertrophic scar (case #3, closed circles), specific ^{125}I-ET-1 binding to the superficial dermis of keloid was saturable (Fig. 6, left side

panel), and Scatchard analysis of the data obtained from a LIGAND computer program gave a straight line (Fig. 6, right side panel), thereby indicating that ^{125}I-ET-1 binds to a single population of the ET receptor binding sites.

The K_D, and B_{max} values of the ET_A receptor in the human skin areas; were listed in Tables 2 and 3. As shown, the K_Ds, a parameter of ligand-binding affinity, were of the same order of magnitude, from 36 pM of the minimum value to 86 pM of the maximum, with the exception of 125 pM in the vascular beds of case #2, a finding which supports the idea that a single and homogenous population of the ET_A receptor exists in the human skin areas, even in hypertrophic scar and keloid with fibroblastic proliferation.

Taking note of the present data that we detected a single and homogenous populations of the ET_A receptor (Table 2), and magnitude of the number of ET_A receptor (expressed as B_{max}), we divided the B_{max} values of the receptor in the superficial and deep dermis into four groups; a) keloid, b) highly hypertrophic scar, c) moderately and mildly hypertrophic scar, d) normal skin. Then, we compared statistically the apparent B_{max} calculated from values listed in Table 3, among four groups, and found a significant difference in the Bmax value between keloid, a group of moderately and mildly hypertrophic scar, and normal skin [keloid, 19.5 ± 2.3 fmol/kg[a] (mean ± S.E.M., n = 4); moderately and mildly hypertrophic scar hypertrophic scar, 6.5 ± 0.9 fmol/mg[c] (n = 4); normal skins, 2.7 ± 0.4 fmol/mg[d] (n = 4); a > c, $p < 0.0001$; a > d, $p < 0.0001$)]. Thus, the number of the fibroblastic ET_A receptor in keloid was significantly higher than normal skin and low grade (moderately and mildly) hypertrophic scar.

In contrast, we failed to detect significant differences between keloida and highly hypertrophic scarb [12.9 ± 5.2 fmol/mg (n = 2)]. Furthermore, as shown in Table 3, it seemed likely that there was no difference in the number of the vascular bed ET_A receptor among between keloid, hypertrophic scar, and normal skin.

Table 2. Binding parameter; dissociation constant (K_D) obtained from ^{125}I-ET-1 saturation binding experiments done on use of consecutive human skin tissue sections

	K_D (pM)		
	superficial dermis	deep dermis	vascular beds in dermis
keloid (case 1)	56	45	53
keloid (case 2)	48	26	125
highly hypertrophic scar (case 3)	43	68	23
moderately hypertrophic scar (case 4)	36	83	65
mildly hypertrophic scar (case 5)	75	78	41
normal skin (case 1)	86	38	78
normal skin (case 6)	53	46	39

Dissociation constant (K_D) was calculated by the program LIGAND. Increasing concentrations (0.77 pM to 1.7 nM) of ^{125}I-ET-1 were used.

Table 3. Binding parameter; maximum binding capacity (B_{max}) obtained from ^{125}I-ET-1 saturation binding experiments done on use of consecutive human skin tissue sections

	B_{max} (fmol/mg)		
	superficial dermis	deep dermis	vascular beds in dermis
keloid (case 1)	19a	14a	10
keloid (case 2)	25a	20a	18
highly hypertrophic scar (case 3)	18b	7.7b	12
moderately hypertrophic scar (case 4)	8.7c	7.0c	16
mildly hypertrophic scar (case 5)	4.6c	5.6c	8
normal skin (case 1)	3.6d	2.0d	13
normal skin (case 6)	2.3d	2.9d	10

Maximum binding capacity (B_{max}) was calculated by the program LIGAND. Increasing concentrations (0.77 pM to 1.7 nM) of ^{125}I-ET-1 were used. Statistical differences were observed bewteen Bmax values (mean ± S.E.) of keloid[a] [19.5 ± 2.3 fmol/kg (n=4)], moderately and mildly hypertrophic scar[c] [6.5 ± 0.9 fmol/mg (n=4)] and normal skins[d] [2.7 ± 0.4 fmol/mg (n=4)]; a>c, $p<0.0001$; a>d, $p<0.0001$, whereas no diiference was detected between keloid[a] and highly hypertrophic scar[b] [12.9 ± 5.2 fmol/mg (n=2)].

A possible heterogeneity of the ET_A receptor expressed in the vascular beds of keloid

To further characterize the ET_A receptor, we did competitive ligand-binding experiments with a fixed amount (60 pM) of ^{125}I-ET-1, in that effects of increasing concentrations of ET-1 and ET_A receptor-related compounds such as BQ-123, PD151242, and FR193317 on specific ^{125}I-ET-1 binding were examined (Tables 4 and 5, Fig. 6). ^{125}I-ET-1 binding to the superficial dermis was competitively displaced by incubation in the presence of increasing concentrations of ET-1 with K_i at values ranging from 57 pM to 120 pM (Table 4). BQ-123, ET_A receptor antagonist, was also a potent displacer for specific ^{125}I-ET-1 bindings to the superficial dermis. Although PD151242 and FR139317 had a much weak potency in displacing binding, compared to the potency of BQ-123, the both ET_A antagonists significantly displaced binding. We observed the same potencies of ET-1, BQ-123, PD151242, and FR139317 to displace ^{125}I-ET-1 binding to the superficial dermis of all cases examined.

In the case of vascular bed ET_A receptor (Fig. 6, Table 6), BQ-123 and ET-1 equipotently displaced ^{125}I-ET-1 binding to the vascular ET_A receptor in all cases examined, however, we noted a very phenomenal finding in the data obtained from the competition study with PD151242 and FR139317 in the vascular bed ET_A receptor of keloid

Table 4. Binding parameter; inhibition constant (K_i) of ET_A receptor-related compounds in ET receptor binding sites in the superficial dermis (fibroblast rich layer) obtained from ^{125}I-ET-1 competition binding experiments

	K_i (pM)			
	ET-1	BQ-123	PD151242	FR139317
keloid (case 1)	120	600	2700	530
keloid (case 2)	57	330	2400	410
highly hypertrophic scar (case 3)	120	600	3300	380
moderately hypertrophic scar (case 4)	100	490	1500	400
mildly hypertrophic scar (case 5)	76	610	2600	650
normal skin (case 1)	68	360	3100	830
normal skin (case 6)	98	420	2800	900

Inhibition constant (K_i) was calculated by the program LIGAND. A fixed amount (60 pM) of ^{125}I-ET-1 was used

(case 2). As shown in the autoradiograms of Fig. 6, ^{125}I-ET-1 binding images of the keloid (case 2) vascular bed obtained by displacements of PD151242 and FR139317 at the concentrations of 0.1 nM ("-10" in the figure and an arrow) and 1.0 mM ("-6" and an arrow) were seen more clearly, than those of the normal skin. In fact, the K_i of PD151242 and FR139317 were calculated to be 31 nM and 7.8 nM, respectively, values which are ten times as large as hypertrophic keloid and normal skin (Table 5). The case #1 of keloid had the same potencies of PD151242 and FR139317 as hypertrophic scars and normal skins. Thus, Among two cases of keloid, the case #2 had a unique binding profile of PD151242 and FR139317. A different type of the ET_A receptor, atypical receptor, may be expressed in the vascular bed of keloid.

Table 5. Binding parameter; inhibition constant (K_i) of ET_A receptor-related compounds in ET receptor binding sites in the vascular beds obtained from ^{125}I-ET-1 competition binding experiments

	K_i (pM)			
	ET-1	BQ-123	PD151242	FR139317
keloid (case 1)	210	1300	3700	980
keloid (case 2)	67	410	31000a	7800b
highly hypertrophic scar (case 3)	41	250	3800	830
moderately hypertrophic scar (case 4)	170	1600	1700	770
mildly hypertrophic scar (case 5)	170	1100	1300	780
normal skin (case 1)	120	1000	2100	640
normal skin (case 6)	89	600	3600	800

Inhibition constant (K_i) was calculated by the program LIGAND A fixed amount (60 pM) of ^{125}I-ET-1 was used. [a], [b]Note very high magnitude of K_i values in the vascular bed ET_A receptor of keloid (case

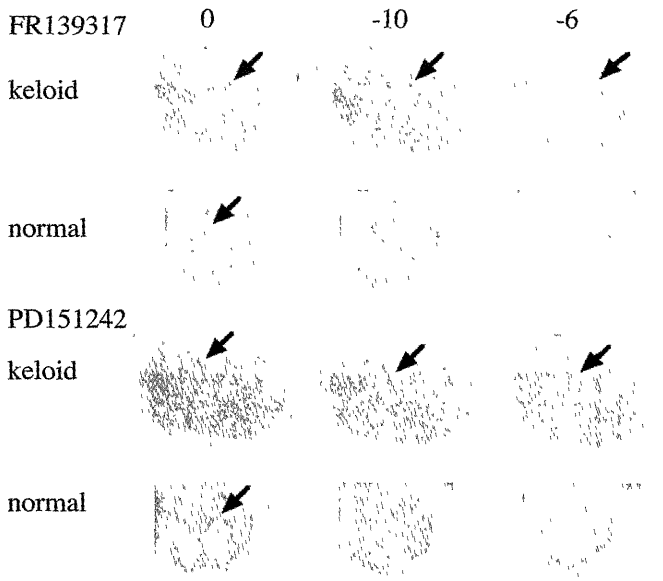

Fig. 7. Receptor autoradiographic evidences of a possible existence of atypical ET_A receptor in the vascular bed of keloid (case #2). The autoradiograms were obtained from competition binding studies at incubations of 60 pM of ^{125}I-ET-1 in the absence ("0") and presence of 0.1 nM ("-9") and 1.0 mM ("-6") of FR139317 and PD 151242. Arrows indicate the ET_A receptor binding sites. Note the findings that in the presence of 1.0 μM ("-6") of FR139317 and PD 151242, the binding sites cannot be detected in the autoradiograms of normal skins, whereas more clearly seen in keloids, as indicated by arrows.

Discussion

The findings presented here are summarized, as follows; 1) The ET_A receptor was found to be overexpressed on fibroblasts in the dermis and on vascular beds of human skins with all grades (keloid, highly hypertrophic scar, moderately hypertrophic scar, mildly hypertrophic scar and normal skin). 2) No significant amounts of the ET_B receptor could be detected in all human skins examined here. 3) The ET_A receptor densities of keloid and highly hypertrophic scar were significantly higher than those of normal skin and moderately and mildly hypertrophic scar. There seemed to be no differences in the density between keloid and highly hypertrophic scar. 4) We noted a possible existence of atypical ET_A receptor with a low affinity to ET_A antagonist of PD151242 and FR139317 in the vascular bed of keloid.

Biological active peptide / growth factors such as angiotensin II and IGF-1 are thought to participate in wound healing process.[36,41] In this study, we found a possible role of ETs, endogenous biological active peptides, in the formation of hypertrophic scar and keloid. This is apparently the first demonstrations and characterization of the ET_A receptor expressed in human skin

areas with keloid and hypertrophic scar. The affinity of the ET_A receptor binding sites for ^{125}I-ET-1, exhibited as K_D (36 pM to 86 pM) obtained here, is in agreement with reported values of the ET_A receptor.[24,34,35] As reported by Lawrence et al. (1995)[16], ETs in the skin areas have been thought to regulates cutaneous blood flow, the present finding sheds some light on the new role of endothelins within skin. Taking together with the present finding of the expression of ET_A mRNA in fibroblasts and vascular beds of the human skin areas, our results suggest a potential pathophysiological role of ETs during wound healing process and scar formation.

As ETs exerts the mitogenic effects to cultured human dermal fibroblasts through the ET_A receptor[13], of particular interest is our present finding that amounts of fibroblastic ET_A receptor expressed in hypertrophic scar were closely related to the degree of severity in scar formation. This may mean that an increase in the number of ET_A receptor depends on fibroblastic aggregation. Taking note of the present data that the number of ET_A receptor in keloid dermis was equivalent to that in highly hypertrophic scar, as the both have the same density of fibroblastic proliferation, it seems likely that there are no changes in the expression amount of ET_A receptor in one fibroblast aggregating in scar formation. Therefore, we tentatively conclude that an excessive proliferation of fibroblasts, an etiological factor in scar formation is due to the overexpression of the ET_A receptor. In fact, Kikuchi et al. (1995)[13] reported that down-regulation of the ET_A receptor decreases cell-growth of fibroblasts isolated from patients with scleroderma, a disease with fibroblastic hypofunction.

In addition to fibroblastic hyperfunction, dysfunctions of these cells are thought to participate in keloid formation as an etiological factor.[3,33] Fibroblasts isolated from keloid *de novo* produce TGF-β, a growth factor functionally linked to the over-expression of procollagen type I mRNA[5] In the present study, we could not find an apparent difference in the receptor density between skin areas with fibroblastic aggregations of highly hypertrophic scar and keloid. Pathophysiological roles of ETs remain to be elucidated. As the ET_A receptor regulates gene expression of other biologically active peptides via multiple G protein-linked pathways[10] and ET stimulates collagen synthesis[22], a collaboration mechanism between ET and growth factors may be operative in keloid formation.[28,41]

Of two cases of keloid, in one keloid (case #2) we detected a candidate of atypical ET_A receptor in the vascular bed, with a very low binding-affinity for two ET_A antagonists, PD151242 and FR139317. Dashwood et al. (1994)[6] found the same receptor of heterogeneity with a low affinity of FR139317 in regions of neovascularization of atherosclerotic human coronary artery (46). The present concept is that there are only two cDNA clones encoding the mammalian ET_A and the ET_B receptor[2,23,40], however, accumulating evidence revealed functional subtypes of ET receptors which do not fit the present criteria for ET_A and ET_B receptors, as mainly deduced from pharmacological potencies of ET-1 and ET receptor-related compounds.[4] Also, in the case of the ET_B receptor system, a novel ET_B receptor splice variant with a short peptide structure at the carboxyl-terminal region was identified from a human placental cDNA library. This alternatively splicing variant of a single gene is functionally distinct from the ET_B receptor, lacking in activating intracellular signaling.[19] Although no data are available on the ET_A receptor splice variant, as the unique binding profile for ET_A antagonist (FR139317) in vascular beds was detected in proliferative tissues such as keloid and region with neovacularization, the possibility that an atypical ET_A receptor is expressed during wound healing process and exerts tumorigenic neoangiogenesis or microcirculatory disturbances to develop keloid would have to be considered. Thus, the present finding on the possible existence of atypical ET_A receptor in the vascular bed of keloid shed some light on the pathophysiological role of ETs in keloid formation.

In summary, we obtained what seems to be the first evidence that fibroblasts with the ET_A receptor participate in wound healing process and scar formation. Experimentally therapeutic approaches with newly synthesized antagonists for ET_A receptor would have to be designed. The present finding on the possible existence of an atypical ET_A receptor in the vascular beds of keloid seems to be pertinent to the role of ETs in the etiology of keloid.

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References

1) Amemiya Y and Miyahara J: Imaging plate illuminates many fields. Nature 336 : 89-90, 1988
2) Arai H, Hori S, Aramori I, Ohkubo H and Nakanishi S: Cloning and expression of a cDNA encoding an endothelin receptor. Nature 34 8 : 730-732, 1990
3) Babu M, Diegelmann, and Oliver N: keloid fibroblats exhibited an altered response to TGF beta. J Invest Dermatol 99 : 203-212
4) Bax W A and Saxena P R: The current endothelin receptor classification: time for reconsideration? Trend Pharmacol Sci 15 : 379-386, 1994
5) Bettinger DA, Yager DR, Diegelmann RF, Cohen IK: The effect of TGF-b on keloid fibroblats proliferation and collagen synthesis. Plast. Reconstr. Surg. 98 : 827-833, 1996
6) Dashwood M R, Allen S P, Luu T N and Muddle J R: The effect of the ET_A receptor antagonist, FR 139317, on [^{125}I]-ET-1 binding to the atherosclerotic human coronary artery. Br J Pharmacol 112 : 386-389, 1994
7) Davenport A P, Kuc R E, Fitzgerald F, Maguire J J, Berryman K, and Doherty A. M: 125I-PD151242: a selective radioligand for human ET_A receptors. Br J Pharmacol 111 : 4-6, 1994
8) Glick R P, Gettleman R, Pattel K, Lakshman R, Tsibris JCM: Insulin and insulin-like growth factor I in brain tumors: Binding and in vitro effects. Neurosurgery 27 : 791-797, 1989
9) Hama H, Sakurai T, Kasuya Y, Fujiki M, Masaki T, and Goto K: Action of endothelin-1 on rat astrocytes through the ET_B receptor. Biochem Biophy Res Commun 186 : 355-362, 1992
10) Hilal-Dandan R, Ramirez MT, Villegas S, *et al.*: Endothelin ET_A receptor regulates signaling and ANF gene expression via multiple G protein-linked pathways. Am J Physiol 272 : H130-H137, 1997
11) Ihara M, Noguchi K, Saeki T, *et al*: Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. Life Sci 50 : 247-255, 1992
12) Ishikawa K, Ihara M, Noguchi K, *et al*: Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc Natl Acad Sci USA 91 : 4892-4896, 1994
13) Kikuchi K, Kadono T, Sato S, Tamaki K and Tkehara K: Impaired growth response to endothelin-1 in scleroderma fibroblasts. Biochem Biophys Res Commun 207 : 829-838, 1995
14) Komuro I, Kurihara H, Sugiyama T, Takaku F and Yazaki Y: Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. FEBS lett 238 : 249-252, 1988
15) Kurihara M, Ochi A, Kawaguchi T, Niwa M, Kataoka Y and Mori K: Localization and characterization of endothelin receptors in human gliomas: A growth factor? Neurosurgery 27 : 275-281, 1990
16) Lawrence E, Siney L, Wilsoncroft P, *et al*: Evidence for ET_A and ET_B receptors in rat skin and an ivestigation of their function in the cutaneous microvasculature. Brit J Pharmacol 115 : 840-844, 1995
17) McPherson G A: Analysis of radioligand binding experiments. a collection of computer programs for the IBMPC. J Pharmacol Methods 14 : 213-228, 1985
18) Munson P J and Rodbard D: LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107 : 220-239, 1980
19) Elshourbagy NA, Adamou JE, Gagnon AW, Wu HL, Pullen M and Nambi P: Molecular Characterization of a Novel Human Endothelin Receptor Splice Variant. J Biol Chem 271 : 25300-25307, 1996
20) Nakaki T, Nakayama M, Yamamoto S and Kato R: Endothelin-mediated stimulationof DNA synthesis in vascular smooth muscle cells. Biochem Biophys Res Commun 158 : 880-883, 1989
21) Nister M, Heldin CH and Wetermark B: Clonal variation in the production of a platelet-derived growth factor-like protein and expression of corresponding receptors in a human malignant glioma. Cancer Res 494 : 332-340, 1986
22) Rizvi M A D, Katwa L, Spadone D P and Myers P R: The Effects of Endothelin-1 on Collagen Type I and Type III Synthesis in Cultured Porcine Coronary Artery Vascular Smooth Muscle Cells. J Mol Cell Cardiol 28 : 243-252, 1996
23) Sakurai T, Yanagisawa M, Takuwa Y, *et al*: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348 : 732-735, 1990
24) Shibata S, Himeno A, Shigematsu K, Tsutsumi K, Sakurai-Yamashita Y and Yamashita K: Endothelin receptors in rat pituitary gland. Cell and Mol Neurobiol 17 : 89-100, 1997
25) Shibata S, Niwa M, Himeno A, *et al*: The endothelin ET_A receptor exists in the caudal solitary tract nucleus of the rat brain. Cell Mol Neurobiol 17 : 151-156, 1997
26) Shigematsu K, Nakatani A, Kawai K, *et al*: Two subtypes of endothelin receptors and endothelin peptides are expressed in differential cel types of the rat placenta: in vitro receptor autoradiographic and in situ hybridization studies. Endocrinology 137 : 738-748, 1996
27) Sogabe K, Nirei H, Shoubo M, Nomoto A, Ao S, Notsu Y and Ono T: Pharmacological profile of FR139317, a novel, potent endothelin ET_A receptor antagonist. J Pharmacol Exp Therap 264 : 1040-1046, 1993
28) Swope V B, Medrano E E, Smalara D and Abdel-Malek Z A: Log-Term Proliferation of Human Melanocytes Is Supported by the Physiologic Mitogens α-Melanotropin, Endothelin-1, and Basic Fibroblast Growth Factor. Exper Cell Res 217 : 453-459, 1995
29) Takagi H, Reinach PS, Tachado SD and Yoshimura N: Endothelin-mediated cell signaling and proliferation in cultured rabbit corneal epithelial cells. Invest Ophthalmol Vis Sci 35 : 134-142, 1994
30) Takagi H, Reinach PS, Yoshimura N and Honda Y: Endothelin-1 promotes wound healing of rabbit corneal epithelium. Curr Eye Res 13 : 625-628, 1994
31) Takai M, Umemura I, Yamasaki K, *et al*: A potent and specific agonist, Suc-[Glu9, Ala11, 15]-Endothelin-1 endothelin-1 (8-21), IRL 1620, for the ET_B receptor. Biochem Biophy Res Commun 1 84 : 953-959, 1992
32) Tao W, Liou G I, Wu X and Reinach P S: ET_B and Epidermal Growth Factor Receptor Stimulation of Wound Closure in Bovine Corneal Epithelial Cells. Invest Ophthalmol Vis Sci 36 : 2614-2622, 1995
33) Tredget EE, Nedelec B, Scott PG, Ghahary A: Hypertrophic scars, keloids, and contractures. The cellular and molecular basis for therapy. Surg Clin N Am 77 : 701-730, 1997
34) Tsutsumi K, Niwa M, Kitagawa N, *et al*: Enhanced expression of an endothelin ET_A receptor in capillaries from human glioblastoma: a quantitative receptor autoradiographic analysis using a radioluminographic imaging plate system. J Neurochem 63 : 2240-2247, 1994
35) Vigne P, Ladoux A, and Frelin C: Endothelins activate Na+/H+ exchange in brain capillary endothelial cells via a high affinity endothelin-3 receptor that is not coupled to phospholipase C. J Biol Chem 266 : 599-5928, 1991
36) Viswanathan M and Saavedra J M: Expression of Angiotensin II AT_2 Receptors in the Rat Skin During Experimental Wound Healing. Peptides 13 : 783-786, 1992
37) Watakabe T, Urade Y, Takai M, Umemura I, and Okada T: A reversible radioligand specific for the ET_B receptor: [^{125}I] Tyr13-Suc-[Glu9, Ala11, 15]-Endothelin-1(8-21)endothelin-1(8-21), [^{125} I]IRL 1620. Biochem Biophy Res Commun 185 : 867-873, 1992
38) Williams D L JR, Jones K L, Pettibone D J, Lis E V and Clineschmidt B V: Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem Biophys Res Commun 175 : 556-561, 1991
39) Yamashita K, Niwa M, Kataoka Y, *et al*: Microglia with an endothelin ET_B receptor aggregate in rat hippocampus CA1 subfields following transient forebrain ischemia. J. Neurochem 63 : 1042-1051, 1994
40) Yanagisawa M, Kurihara H, Kimura S, *et al*: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332 : 411-415, 1991
41) Yoshimoto H, Ishihara H, Ohtsuru A, *et al*: Overexpression of insulin-like growth factor-I (IGF-I) receptor and the invasiveness of keloid fibroblasts. Am J Pathol (in press) 1999