Endothelin ET\textsubscript{A} Receptor in Human Skins with Keloid and Hypertrophic Scar

Yuichi Hamada\textsuperscript{1)}, Akihiko Himeno\textsuperscript{2)}, Hisa Yamaguchi\textsuperscript{2)}, Akiyoshi Hirano\textsuperscript{1)}, Tohru Fujii\textsuperscript{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\textsubscript{A} receptor was apparently observed in the skin areas, whereas the ET\textsubscript{B} receptor was only slightly detected, as deduced from the finding that mRNA encoding the human ET\textsubscript{A} receptor is expressed in the fibroblast and the vascular bed. The ET\textsubscript{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\textsubscript{A} receptor with a low affinity to ET\textsubscript{A} antagonist of PD151242 and FR139317. The ET\textsubscript{A} receptor of endogenous growth factors possibly play an etiological role in dysfunctions of the fibroblast in hypertrophic scars, and a typical ET\textsubscript{A} receptor may be related to the growth of keloid.

Key Words: endothelin (ET), ET\textsubscript{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\textsubscript{A} and ET\textsubscript{B} of G-protein coupling-type receptors with seven-transmembrane domains.\textsuperscript{2,4} As ETs were originally isolated from cultured porcine aortic endothelial cells, extensive studies have been performed to clarify the regulatory significant 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\textsuperscript{4,5} in collaboration with other growth factors such as platelet-derived growth factor and insulin.\textsuperscript{6} Cultured rat astrocytes proliferate and differentiate through activation of their own ET\textsubscript{B} receptor.\textsuperscript{7} In the case of human dermal fibroblasts, interestingly, the ET\textsubscript{A} receptor operates in the mitogenic effect on these cells.\textsuperscript{8}

Takagi et al. (1994)\textsuperscript{29,30} and Tao et al. (1995)\textsuperscript{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.

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
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 skin 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°C in dry ice-isopentane and stored at -80°C. Informed consent was obtained from each patient after the study procedures had been fully explained.

Table 1. Summary of the patients

<table>
<thead>
<tr>
<th>case No.</th>
<th>sex</th>
<th>age</th>
<th>clinical classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>43</td>
<td>keloid normal skin*</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>57</td>
<td>keloid</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>5</td>
<td>highly hypertrophic scar</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>21</td>
<td>moderately hypertrophic scar</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>38</td>
<td>mildly hypertrophic scar</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>64</td>
<td>normal skin**</td>
</tr>
</tbody>
</table>

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

Quantitative receptor autoradiography for ET receptors binding

Frozen, 16-μm-thick sections of human skins were cut on a cryostat at -20°C, thaw-mounted onto gelatin-coated slides and stored overnight under vacuum at 4°C. Related tissue sections were labelled in vitro with [125I-ET-1 [non-selective radioligand] (specific activity 81.4 TBq/mmol), [125I-PD151242 [ETA radioligand]30], or [125I-IRL1620 [ETB radioligand)]31-(81.4 TBq/mmoll) in 2.0 ml of incubation buffer, according to our method.25,34,39 Briefly, after pre-incubation at room temperature (23°C) for 10 min in incubation buffer, the sections were incubated with a fixed amount or increasing concentrations of radioligands, at 4°C for 48 hr, in the presence or absence of ET receptor-related compounds such as ET-1, ET-3, sarafotoxin S6c [ETB agonist]30, BQ-123 [ETA antagonist]19, BQ-788 [ETB antagonist]19, IRL1620 [ETB antagonist]31, PD151242 [ETB antagonist]27, or FR139317 [ETB 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°C 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-3H (Amersham International plc., UK) and the films were developed with a D19 developer (Eastman Kodak, USA) for 7 min at 4°C.

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 (BaF2-BrEu+)(Bio-imaging analyzer BAS 2000, Fuji Photo Film Co., Japan).11 The dried sections were exposed to a radioluminographic imaging plate with calibrated [125I]-standards ([125I]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 ETα receptor mRNA

We confirmed the presence of ETα receptor mRNAs in the human skin, using our in situ hybridization method with cRNA probes.30 ETα receptor PCR-amplified DNA fragments (ETα, bases 100-911) were subcloned into the multiple cloning site of pBluescript II SK+ flanked by the T3 and T7 promoters. Double-labelled 35S-cRNAs were synthesized from linearized plasmids using α-35S-UTP and α-35S-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°C, 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, 1xDenhart'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 35S-labelled cRNA probe (106 cpm/ml) was applied to each section, and cover-slipped tissue sections were incubated for 16 hr at 55°C. 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°C, 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°C. Tissue sections were finally washed in 0.1 x SSC-10 mM 2-mercaptoethanol for 15 min at 55°C, 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°C for 30 days, developed with a D19 developer, then counterstained with hematoxylin and mounted.

Data analysis and materials

To determine the maximum binding capacity (Bmax), the dissociation constant (Kd), and the inhibition constant (Ki), radioligands binding data were analyzed using the program LIGAND (BIOSOFT, UK). Differences in binding parameters, Kd and Bmax 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-35S-UTP, α-35S-CTP, 125I-ET-1, 125I-IRL1620, and 125I-PD151242 were purchased from New England Nuclear, USA, and Amersham, UK. BQ-123 was a gift from Banyu Pharmaceutical Co., Ltd., Japan, IRL 1620 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 Stratagene 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 125I-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 125I-ET-1 are evident in the left side-panel (Fig. 1A to E), and right side-panel (Fig. 1F to J), respectively. 125I-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 nM) of 125I-ET-1 in the incubation buffer, a considerable amount of specific 125I-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. 125I-ET-1 Binding in the epidermis was irreducible 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 125I-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...
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 ET\(_B\) 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), moderately hypertrophic scar (C, case #4), mildly hypertrophic scar (D, case #5), and normal skin (E, case #6). Receptor autoradiograms were obtained at incubations of 330 nM \(^{125}\)I-IRL1620 [ET\(_B\) radioligand]. Non-specific binding of ET\(_B\) receptor binding sites were determined in the presence of 1.0 \(\mu\)M of unlabelled IRL1620 (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 scar (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 \(\mu\)M 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 ETA 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 compatible to "a" and "b" of the panels A and C.

We confirmed the cellular expression of ETA 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 ETA 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 35S-ETA cRNA were discretely distributed throughout the skin areas. There were many silver grains of 35S-ETA 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 125I-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 125I-ET-1 saturation 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 125I-ET-1 binding to the superficial dermis of keloid was saturable (Fig. 6, left side)

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

Fig. 6. A saturation binding study with increasing concentrations (0.77 pM to 1.7 nM) of 125I-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 125I-ET-1 saturation binding data, analyzed using the program LIGAND.
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 125I-ET-1 binds to a single population of the ET receptor binding sites.

The $K_D$ and $B_{max}$ values of the ETA receptor in the human skin areas; were listed in Tables 2 and 3. As shown, the $K_D$s, 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 ETA 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 ETA receptor (Table 2), and magnitude of the number of ETA 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 $B_{max}$ value between keloid, a group of moderately and mildly hypertrophic scar, and normal skin ($B_{max}$: keloid, $19.5 \pm 2.3$ fmol/kg; moderately and mildly hypertrophic scar, $6.5 \pm 0.9$ fmol/mg; normal skin, $2.7 \pm 0.4$ fmol/mg). Thus, the number of the fibroblastic ETA receptor in keloid was significantly higher than normal skin.

In contrast, we failed to detect significant differences between keloid and highly hypertrophic scar ($12.9 \pm 5.2$ fmol/mg). Furthermore, as shown in Table 3, it seemed likely that there was no difference in the number of the vascular bed ETA receptor among between keloid, hypertrophic scar, and normal skin.

To further characterize the ETA receptor, we did competitive ligand-binding experiments with a fixed amount (60 pM) of 125I-ET-1, in that effects of increasing concentrations of ET-1 and ETA receptor-related compounds such as BQ-123, PD151242, and FR193317 on specific 125I-ET-1 binding were examined (Tables 4 and 5, Fig. 6).

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

<table>
<thead>
<tr>
<th>$K_D$ (pM)</th>
<th>superficial dermis</th>
<th>deep dermis</th>
<th>vascular beds in dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>keloid (case 1)</td>
<td>56</td>
<td>45</td>
<td>53</td>
</tr>
<tr>
<td>keloid (case 2)</td>
<td>48</td>
<td>26</td>
<td>135</td>
</tr>
<tr>
<td>highly hypertrophic scar (case 3)</td>
<td>43</td>
<td>68</td>
<td>23</td>
</tr>
<tr>
<td>moderately hypertrophic scar (case 4)</td>
<td>36</td>
<td>83</td>
<td>65</td>
</tr>
<tr>
<td>mildly hypertrophic scar (case 5)</td>
<td>75</td>
<td>78</td>
<td>41</td>
</tr>
<tr>
<td>normal skin (case 1)</td>
<td>86</td>
<td>58</td>
<td>78</td>
</tr>
<tr>
<td>normal skin (case 6)</td>
<td>53</td>
<td>46</td>
<td>39</td>
</tr>
</tbody>
</table>

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

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

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

Maximum binding capacity ($B_{max}$) was calculated by the program LIGAND. Increasing concentrations (0.77 pM to 1.7 nM) of 125I-ET-1 were used. Statistical differences were observed between $B_{max}$ values (mean ± S.E.M.) of keloid ($19.5 \pm 2.3$ fmol/kg; moderately and mildly hypertrophic scar $6.5 \pm 0.9$ fmol/mg; normal skin $2.7 \pm 0.4$ fmol/mg); $a > c, p < 0.0001; a > d, p < 0.0001$, whereas no difference was detected between keloid and highly hypertrophic scar ($12.9 \pm 5.2$ fmol/mg).
Table 4. Binding parameter; inhibition constant ($K_i$) of ETA receptor-related compounds in ET receptor binding sites in the superficial dermis (fibroblast rich layer) obtained from $^{125}$I-ET-1 competition binding experiments

<table>
<thead>
<tr>
<th></th>
<th>ET-1</th>
<th>BQ-123</th>
<th>PD151242</th>
<th>FR139317</th>
</tr>
</thead>
<tbody>
<tr>
<td>keloid (case 1)</td>
<td>120</td>
<td>600</td>
<td>2700</td>
<td>530</td>
</tr>
<tr>
<td>keloid (case 2)</td>
<td>57</td>
<td>330</td>
<td>2400</td>
<td>410</td>
</tr>
<tr>
<td>highly hypertrophic scar (case 3)</td>
<td>120</td>
<td>600</td>
<td>3300</td>
<td>380</td>
</tr>
<tr>
<td>moderately hypertrophic scar (case 4)</td>
<td>60</td>
<td>360</td>
<td>3100</td>
<td>830</td>
</tr>
<tr>
<td>mildly hypertrophic scar (case 5)</td>
<td>76</td>
<td>610</td>
<td>2600</td>
<td>650</td>
</tr>
<tr>
<td>normal skin (case 1)</td>
<td>68</td>
<td>360</td>
<td>3100</td>
<td>830</td>
</tr>
<tr>
<td>normal skin (case 6)</td>
<td>98</td>
<td>420</td>
<td>2800</td>
<td>900</td>
</tr>
</tbody>
</table>

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 ETA receptor, atypical receptor, may be expressed in the vascular bed of keloid.

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

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

Inhibition constant ($K_i$) was calculated by the program LIGAND. A fixed amount (60 pM) of $^{125}$I-ET-1 was used. a, bNote very high magnitude of $K_i$ values in the vascular bed ETA receptor of keloid (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 ETA receptor, atypical receptor, may be expressed in the vascular bed of keloid.

Discussion

The findings presented here are summarized, as follows: 1) The ETA 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 ETB receptor could be detected in all human skins examined here. 3) The ETA 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 ETA receptor with a low affinity to ETA 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. 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 ETA receptor expressed in human skin.
areas with keloid and hypertrophic scar. The affinity of the ETA receptor binding sites for ET-1, exhibited as K_D (36 pM to 86 pM) obtained here, is in agreement with reported values of the ETA receptor^{21,34,35} As reported by Lawrence et al. (1995)^{46}, ETs in the skin areas have been thought to regulate 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 ETA 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 ETA receptor^{33}, of particular interest is our present finding that amounts of fibroblastic ETA 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 ETA receptor depends on fibroblastic aggregation. Taking note of the present data that the number of ETA 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 ETA 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 ETA receptor. In fact, Kikuchi et al. (1995)^{13} reported that down-regulation of the ETA 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^{33}. Fibroblasts isolated from keloid de novo produce TGF-β, a growth factor functionally linked to the over-expression of procollagen type I mRNA^{10} In the present study, we could not find an apparent difference in the receptor density between skin areas with keloid and hypertrophic scar and keloid. Pathophysiological roles of ETs remain to be elucidated. As the ETA receptor regulates gene expression of other biologically active peptides via multiple G protein-linked pathways^{40} and ET stimulates collagen synthesis^{20}, a collaboration mechanism between ET and growth factors may be operative in keloid formation^{38,41}.

Of two cases of keloid, in one keloid (case #2) we detected a candidate of atypical ETA receptor in the vascular bed, with a very low binding-affinity for two ETA antagonists, PD151242 and FR139317. Dashwood et al. (1994)^{36} 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 ETA and the ETB receptor^{22,29}; however, accumulating evidence revealed functional subtypes of ET receptors which do not fit the present criteria for ETA and ETB receptors, as mainly deduced from pharmacological potencies of ET-1 and ET receptor-related compounds.^{37} Also, in the case of the ETB receptor system, a novel ETB receptor splice variant with a short peptide structure at the carboxy-terminal region was identified from a human placental cDNA library. This alternatively splicing variant of a single gene is functionally distinct from the ETB receptor, lacking in activating intracellular signaling.^{19} Although no data are available on the ETA receptor splice variant, as the unique binding profile for ETA antagonist (FR139317) in vascular beds was detected in proliferative tissues such as keloid and region with neovascularization, the possibility that an atypical ETA 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 ETA 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 ETA receptor participate in wound healing process and scar formation. Experimentally therapeutic approaches with newly synthesized antagonists for ETA receptor would have to be designed. The present finding on the possible existence of an atypical ETA receptor in the vascular beds of keloid seems to be pertinent to the role of ETs in the etiology of keloid.

Acknowledgements

We thank Prof. Masami Niwa, Department of Pharmacology 1, Nagasaki University School of Medicine, Nagasaki, Japan, for collaboration and support. BQ-123 was kindly provided from Dr. M. Yano, Tsukuba Research Institute, Banyu Pharmaceutical Co., Tsukuba 300-33, Japan, IRL 1620 from Dr. T. Okada, International Research Laboratories, Ciba-Geigy Japan, Ltd., Takarazuka 665, Japan, and PD151242 from Dr. A. M. Doherty, Parke-Davis Pharmaceutical Division, Ann Arbor, MI 48105, USA. This work was supported in part by a Grant from the Nagasaki Prefecture Medical Association.
References

8) Glick R P, Gettleman R, Pattel K, Lakshman R, Tsiberis JCM: The current endothelin receptor classifi-
15) McPherson G A: Analysis of radioligand binding experiments. a quantitative receptor autoradiographic analysis using a radiolumino-
26) Shigematsu K, Kawai K, et al: Two subtypes of endothelin receptors and endothelin peptides are expressed in differen-
34) Tsutsui K, Niwa M, Kitagawa N, et al: Enhanced expression of an endothelin ET_{B} receptor in capillaries from human gliblastoma: a quantitative receptor autoradiographic analysis using a radiolumi-
35) Vigne P, Laloux 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 : 599-602, 1991