Full Paper

Involvement of Endothelin and ET\textsubscript{A} Endothelin Receptor in Mechanical Allodynia in Mice Given Orthotopic Melanoma Inoculation

Masahide Fujita\textsuperscript{1}, Tsugunobu Andoh\textsuperscript{1}, Ikuo Saiki\textsuperscript{2}, and Yasushi Kuraishi\textsuperscript{1,*}

\textsuperscript{1}Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences and \textsuperscript{2}Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

Received November 13, 2007; Accepted December 12, 2007

Abstract. We investigated whether endothelin (ET) would be involved in skin cancer pain in mice. Orthotopic inoculation of B16-BL6 melanoma cells into the plantar region of the hind paw produced marked mechanical allodynia in C57BL/6 mice. Intraplantar injections of the ET\textsubscript{A} receptor antagonist BQ-123 (0.3 – 3 nmol/site), but not the ET\textsubscript{B} receptor antagonist BQ-788 (1 and 3 nmol/site), inhibited mechanical allodynia in mice with grown melanoma. In naive mice, an intraplantar injection of tumor extract (1 and 3 mg/site), which was prepared from the grown melanoma in the paw, produced mechanical allodynia, which was inhibited by BQ-123 and BQ-788 at doses of 3 and 10 nmol/site. An intraplantar injection of ET-1 (1 and 10 pmol/site) elicited licking behavior, which was increased in the melanoma-bearing hind paw. BQ-123 (3 and 10 nmol/site) inhibited licking induced by ET-1 (10 pmol/site). The level of mRNA of ET\textsubscript{A}, but not ET\textsubscript{B}, receptor, was significantly increased in the dorsal root ganglia on the inoculated side. Cultured B16-BL6 cells contained ET, and the melanoma mass increased the concentration of ET as it grew bigger. These results suggest that ET-1 and ET\textsubscript{A} receptor are at least partly involved in the induction of pain induced by melanoma cell inoculation.

Keywords: cancer pain, endothelin, endothelin receptor, allodynia, licking

Introduction

Pain is the most disruptive experience for cancer patients. One-third of cancer patients complain of pain and 80% of patients with advanced or terminal cancer suffer from severe pain, which decreases quality of life (1, 2). Laboratory animal studies have shown that pain enhances the growth and metastasis of tumors (3, 4). These findings suggest that pain relief is important for not only quality of life but also cancer therapy, and it is important to know the mechanisms of cancer pain for proper pain management.

Orthotopic inoculation of melanoma cells induces spontaneous pain (aversive behavior), thermal hyperalgesia, and mechanical hyperalgesia/allodynia in mice (4 – 6). Thermal and mechanical hyperalgesia are slight from day 5 to day 10 after inoculation (early phase) and become grave thereafter (late phase). Thermal hyperalgesia at the early phase is inhibited by morphine at a dose of 1 mg/kg and the aspirin-like drug diclofenac sodium at a dose of 3 mg/kg (4). On the other hand, thermal hyperalgesia at the late phase is inhibited by morphine at higher doses of 5 mg/kg or more, but they are not inhibited by diclofenac sodium even at the higher dose of 30 mg/kg (4). Mechanical allodynia (pain-like response to non-noxious mechanical stimulation) and spontaneous aversive behavior (licking of the melanoma-bearing region) are not obvious at the early phase, and it becomes marked at the late phase (4, 6). The allodynia is suppressed by gabapentin and partially suppressed by ketamine, but it is not inhibited by diclofenac, mexiletine, and baclofen (7). The distribution of nerve fibers in the skin is not altered at the early phase, but it increases and disappears in the skin of peripheral and middle regions, respectively, of the melanoma mass at the late phase (6). In addition, an intraplantar injection of an extract of tumor mass isolated at the late phase, but not at the early phase,
induces aversive behavior and mechanical hyperalgesia/allodynia in naive mice (5). These findings suggest that the mechanisms of cancer pain are different between the early and late phases. It is also suggested that the melanoma mass increases the production of some algesiogenic substance(s) at the late phase. This may be a cause of the increase of cancer-associated pain at the late phase. The present study was carried out to elucidate candidate algesiogenic substance(s) in the melanoma mass.

Endothelin-1 (ET-1) is a potent vasoconstrictor 21–amino acid peptide and a member of the endothelin family (ET-1, 2, and 3), which are derived from the precursor big endothelin. ET-1 is secreted from a variety of cells including endothelial cells and macrophages; has high binding affinity for two receptor subtypes, ET\(_{A}\) and ET\(_{B}\) receptors, and exerts various biological actions mediated by these receptors (8–10). ET-1 is partly involved in inflammatory and neuropathic pain (11–15). It was shown to be involved in the bone pain of patients with prostate cancer (16) and bone cancer pain in mice (17, 18). However, it is unclear whether the role of ET-1 in cancer pain is dependent on the type of tumor cells. Therefore, the present study was conducted to determine whether ET-1 would be involved in pain of mice with melanoma.

**Materials and Methods**

**Animals**

All experiments were performed using male C57BL/6 mice (6-week-old at the start of the experiments). They were kept under controlled temperature (22 ± 1°C) and humidity (55 ± 10%). The room was lighted from 7:00 AM to 7:00 PM. Food and water were freely available. Procedures for animal experiments were approved by the Committee for Animal Experiments at the University of Toyama and were conducted in accordance with the guidelines of The Japanese Pharmacological Society.

**Agents**

The ET\(_{A}\) endothelin-receptor antagonist BQ-123 and the ET\(_{B}\) endothelin-receptor antagonist BQ-788 were purchased from Phoenix Pharmaceutical Inc. (St. Joseph, MO, USA) and were dissolved in physiological saline. ET-1 was purchased from Peptide Institute, Inc. (Osaka) and was dissolved in 0.1% acetic acid solution. All these compounds were injected intraplantarly in a volume of 10 \(\mu\)l.

**Tumor inoculation**

The B16-BL6 cells, melanoma derived from C57BL/6 mouse, were cultured in modified Eagle’s medium containing 10% fetal bovine serum at 37°C and in a humidified atmosphere of 5% CO\(_2\). The cells (2 \(\times\) 10\(^5\) cells/site) were injected into the plantar region of the hind paw in a volume of 20 \(\mu\)l.

**Tumor extract for injection**

The extract from the tumor mass was prepared by the previously described procedure (5). Melanoma mass was isolated from the hind paw under anesthesia on day 18–24 post-inoculation. It was homogenized in purified water and centrifuged at 17,500 \(\times\) g for 30 min at 4°C. The supernatant was lyophilized and stored at –80°C until use. The lyophilized extract was dissolved in physiological saline at a concentration of 50 or 150 mg/ml and was injected intraplantarly in a volume of 20 \(\mu\)l.

**Behavioral test**

Pain tests were performed according to the guidelines published in a Guest Editorial in Pain on ethical standards for investigations of experimental pain in animals. Mechanical allodynia of the hind paw was assessed using a von Frey filament as described (5). Briefly, the mice were placed individually in an acrylic cage (11 cm \(\times\) 18 cm \(\times\) 15 cm) with a wire mesh bottom. After the acclimation period of at least 10 min, von Frey filament with a bending force of 0.69 mN was pressed perpendicularly against the plantar skin and held for 3–5 s with it slightly buckled. The responses to this stimulus were ranked as follows: 0, no response; 1, move away from the stimulus; 2, immediate flinching or licking of the hind paw. The stimulation was applied six times to the hind paw at intervals of several seconds and the mean score served as the pain-related score.

The percentage of the inhibitory effect of ET-receptor antagonists was calculated as follows:

\[
\text{Inhibitory effect (\%) = } \frac{\text{PS (pre-injection)} - \text{PS (post-injection)}}{\text{PS (pre-injection)} - \text{PS (contralateral side)}} \times 100
\]

, where PS is the pain-related score and PS (pre- or post-injection) is the score of the treated hind paw.

For assessing the algesiogenic action of ET-1, mice were placed in the acrylic cage for at least 1 h for acclimation and then were given an intraplantar injection of ET-1. Immediately after injection, the animals were put back into the cage and their behaviors were video-taped for 1 h with no experimenter present in the observation room during this period. Playback was used to measure the amount of time an animal spent licking the hind paw.
Reverse transcriptase and polymerase chain reaction (RT-PCR)

The dorsal root ganglia (DRGs) at the level of L4 and 5 were isolated from mice on day 15 – 18 post-inoculation. Total RNA was obtained from the pooled DRGs and was used for the determination of ET-receptor mRNA with RT-PCR, as described (19). The sequences of primers used were as follows: ET\textsubscript{A} endothelin receptor, 5'-aagcctcatgacctcggtcc-3' (sense) and 5'-tctgtgtctagcaagggcg-3' (antisense); ET\textsubscript{B} endothelin receptor, 5'-tgaaggcaagaacactgcgg-3' (sense) and 5'-tggcatgtgaagacgactagg-3' (antisense); \(\beta\)-actin, 5'-tcagaaggactctatatgtgg-3' (sense) and 5'-tctctttgatgtcacgcacg-3' (antisense). To determine the expression levels, the density of the bands was measured with NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Immun assay for ET

Melanoma mass isolation and extract preparation were done as described above. Melanoma masses from three mice were pooled and used for the determination of the concentration of ET with an ET EIA kit (Cayman Chem., Ann Arbor, MI, USA), which measures all isoforms of ET. The concentration of ET was normalized to the concentration of protein.

Immunocytochemistry for ET-1

The cultured B16-BL6 cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After being treated with phosphate buffer containing 0.3% triton X-100 and 1% fetal bovine serum, the cells were incubated with anti-ET antibody (1:100; Phoenix Pharmaceuticals, Inc.) at 4°C overnight; the antibody is specific for ET-1 (cross-reactivity: 100%) but it cross-reacts with ET-2 (3.5%) and ET-3 (28%) also, according to the manufacturer’s information. The cells were washed with the phosphate buffer and were reacted with donkey anti-rabbit IgG antibody conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA). The signal was detected using a confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA).

Data processing

Data are presented as means and S.E.M. Results were analyzed with Student’s \(t\)-test, paired \(t\)-test, Mann-Whitney U-test, or Kruskal-Wallis analysis of variance on ranks followed by Dunnett’s multiple comparisons; \(P<0.05\) was considered significant.

Results

Effects of ET receptors antagonists on tumor-induced allodynia

Orthotopic inoculation of melanoma cells produced allodynia in the melanoma-bearing hind paw; allodynia became apparent from day 11 post-inoculation, increased until day 16, and was relatively constant at least until day 20 (Fig. 1A). The contralateral non-inoculated hind paw did not show allodynia. In the following experiments, mice that showed the pain-related score of 1.8 or higher on day 15 – 18 post-inoculation were used.

Tumor-induced allodynia was significantly inhibited by intraplantar injections of the ET\textsubscript{A}-receptor antagonist BQ-123 at doses of 0.3 – 3 nmol/site (Fig. 1B); the
effects peaked 45 min after injection (data not shown). A higher dose (10 nmol/site) was without effect (Fig. 1B). The allodynia was not affected by intraplantar injections of the ET\(_B\)-receptor antagonist BQ-788 at doses of 1 and 3 nmol/site, and there was an inhibited tendency after a higher dose of 10 nmol/site (Fig. 1B). These antagonists at the doses tested did not produce any abnormal behaviors in mice.

**Effects of ET-receptor antagonists on tumor extract–induced allodynia**

Intraplantar injections of tumor extract (1 and 3 mg/site) into the hind paw produced a dose-dependent mechanical allodynia in naive mice; the effect of a dose of 3 mg/site peaked at 15 min post-injection and persisted for at least 6 h (Fig. 2A). The tumor extract at the doses tested did not induce alldynia in the contralateral hind paw.

**Fig. 2.** Effects of endothelin (ET)-receptor antagonists on the mechanical allodynia induced by an injection of tumor extract in naive mice. Melanoma mass was isolated from the hind paw on day 18–24 post-inoculation. An extract of the melanoma mass was injected into the plantar region of the hind paw of naive mice. A: Time course of mechanical allodynia induced by an injection of tumor extract (1 or 3 mg/site). B and C: Effects of endothelin-receptor antagonists on the mechanical allodynia induced by tumor extract. BQ-123 (ET\(_A\) endothelin-receptor antagonist), BQ-788 (ET\(_B\) endothelin-receptor antagonist), and vehicle (saline) were injected intraplantarly 10 min before the injection of tumor extract (3 mg/site). The results were shown as time-course (B) and area under the curve (AUC) (C) from 0 to 120 min after the extract injection. In panel B, the dose of BQ-123 and BQ-788 was 10 nmol/site. Data are presented as the means and S.E.M. of six to seven animals. *P<0.05, as compared with the vehicle.

Figure 2B shows the effects of pretreatment with BQ-123 (10 nmol/site) and BQ-788 (10 nmol/site) on alldynia following an injection of tumor extract at a dose of 3 mg/site; these antagonists inhibited the maximum alldynia without effects on the duration of alldynia. BQ-123 (3 and 10 nmol/site) and BQ-788 (3 and 10 nmol/site) produced a dose-dependent and partial inhibition of alldynia; BQ-123 (10 nmol/site)
and BQ-788 (10 nmol/site) produced 36% and 28% inhibition, respectively (Fig. 2C).

**ET-1–elicited licking behavior**

Intraplantar injections of ET-1 (1 and 10 pmol/site) into the melanoma-bearing hind paw elicited licking behaviors in a dose-dependent manner (Fig. 3A); the effects peaked at 5–15 min and almost subsided by 30 min after the injection. Although ET-1 also elicited licking in naive mice, the effects were more marked in the melanoma-bearing paw than in healthy paw (Fig. 3B). In melanoma-bearing mice, licking elicited by ET-1 (10 pmol/site) was dose-dependently suppressed by pretreatment with the ET<sub>A</sub>-receptor antagonist BQ123 at doses of 3 and 10 nmol/site (Fig. 3C).

**Expression levels of ET-receptor mRNAs in the DRG**

The expression levels of mRNAs encoding ET<sub>A</sub> and ET<sub>B</sub> receptors were determined by using the RT-PCR method. ET<sub>A</sub>- and ET<sub>B</sub>-receptor mRNAs were detected in the DRG on the non-inoculated side (Fig. 4A). The expression level of ET<sub>A</sub>-receptor mRNA, but not ET<sub>B</sub>-receptor mRNA, on the melanoma-bearing side was significantly increased on day 15–18 post-inoculation, as compared with the non-inoculated side (Fig. 4).

**ET in melanoma cells and melanoma mass**

Imunochemical staining showed that the cultured melanoma cells expressed ET, probably ET-1 (Fig. 5A). The tumor mass contained ET and the contents were significantly and time-dependently increased after inoculation (Fig. 5B). The content of ET in the cultured cells was higher than the cutaneous content, and the content in the tumor mass day 20 post-inoculation was higher than that of the cultured cells (Fig. 5B).

---

Fig. 4. **Expression of mRNA encoding endothelin (ET)-receptor subtypes in the dorsal root ganglia.** Mice were given orthotopic inoculation of melanoma cells into the hind paw. Total RNA was obtained from the L4 and 5 dorsal root ganglia on day 15–18 post inoculation and RT-PCR was performed. A: Typical examples of the bands of ET<sub>A</sub>-endothelin receptor (ET<sub>A</sub>-R), ET<sub>B</sub>-endothelin receptor (ET<sub>B</sub>-R), and β-actin mRNAs. B: ET<sub>A</sub>-R mRNA in the dorsal root ganglia. C: ET<sub>B</sub>-R mRNA in the dorsal root ganglia. The expression levels of mRNAs of ET<sub>A</sub>-R and ET<sub>B</sub>-R were normalized to that of β-actin mRNA. Contra., contralateral side; Ipsi., inoculated side. The data are presented as the means and S.E.M. of three samples. *P<0.05, as compared with non-inoculated side.

Fig. 5. **The presence of endothelin-1 in the cultured melanoma cells and the tumor mass.** A: ET-1 immunoreactivity in cultured B16-BL6 melanoma cells. Scale bar: 20µm. B: The content of ET in the melanoma mass and cultured melanoma cells. Melanoma cells were injected into the plantar region of the hind paw and melanoma mass was removed on the indicated day. Day 0 and culture indicate the skin of the plantar region and cultured melanoma cells, respectively. The data are presented as the means and S.E.M. of three samples. *P<0.05, as compared with day 0.
Discussion

Mechanical allodynia was not observed until day 10 post-inoculation and thereafter rapidly increased until day 16. Submaximal allodynia was observed from day 16 to 19 post-inoculation. The results are similar to those of our previous report (6). Submaximal allodynia was significantly suppressed by the ET\(_{\alpha}\)-receptor antagonist BQ-123 (0.3 – 3 nmol/site), suggesting that the ET\(_{\alpha}\) receptor is at least partly involved in allodynia of the melanoma-bearing paw. The dose-response curve of the BQ-123 effect was bell-shaped and the highest dose tested (10 nmol/site) was without effect. Similarly, in a mouse model of sarcoma-induced bone cancer pain, pain-like behaviors are partially inhibited by the ET\(_{\alpha}\)-receptor antagonist BQ-123. Thus, it is suggested that ET-1 acts on ET\(_{\alpha}\)-receptor (8 – 10). A subcutaneous injection of ET-1 alone elicited licking, allodynia (22). The present study demonstrated that an intraplantar injection of ET-1-induced mechanical allodynia was significantly suppressed by the ET\(_{\alpha}\)-receptor antagonist BQ-123; the dose-response curve is bell-shaped, and the effect peaks and reduces at 1.6 and 16 nmol/site, respectively (17).

ET\(_{\alpha}\)-receptor mRNA was contained in the DRG. The ET\(_{\alpha}\) receptor is expressed in small- and medium-sized DRG neurons (18), which have chiefly unmyelinated C fiber (20) and play an important role in pain (21). Thus, primary afferent terminals may be a primary site of the algesiogenic action of endothelin. ET\(_{\alpha}\) receptor has high affinity against ET-1, but not ET-2 and -3 (8 – 10). A subcutaneous injection of ET-1 induces mechanical allodynia (22). The present study demonstrated that an intraplantar injection of ET-1 alone elicited licking, which was inhibited by BQ-123. Thus, it is suggested that ET-1 acts on ET\(_{\alpha}\) receptors on primary afferent terminals to induce allodynia and spontaneous pain.

An intraplantar injection of extract of melanoma mass isolated at the late phase of cancer pain induced mechanical allodynia in naive mice, confirming a previous report (5). The allodynia was inhibited by BQ-123. ET was contained in cultured melanoma cells and markedly increased in the melanoma mass at the late phase. Therefore, it is suggested that ET, probably ET-1, produced by melanoma mass is involved in allodynia of the melanoma-bearing paw.

Mice with melanoma at the late phase of cancer pain showed higher sensitivity to intraplantar injection of ET-1. The expression of ET\(_{\alpha}\)-receptor mRNA was increased in the DRG on the melanoma-bearing side. This suggests the increase of ET\(_{\alpha}\) receptors in primary sensory neurons and may be a cause of the increased sensitivity to ET.

The mechanisms of the increase of ET\(_{\alpha}\)-receptor expression in the DRG are unclear. Melanoma cells contain nerve growth factor (23) and high affinity nerve growth factor receptor, Trk A, is expressed in small and medium diameter DRG neurons (24), which also express ET\(_{\alpha}\) receptor (18). Nerve growth factor elevates cAMP (25) and cAMP up-regulates ET\(_{\alpha}\)-receptor mRNA (26). Thus, nerve growth factor might be a cause of the increase in ET\(_{\alpha}\)-receptor mRNA in the DRG of melanoma-bearing mice.

The ET\(_{\beta}\)-receptor antagonist BQ-788 at a dose range of 1 – 3 nmol/site did not affect allodynia in the melanoma-bearing paw, suggesting that ET\(_{\beta}\) receptor does not play an important role in the melanoma allodynia. Bone cancer pain is slightly inhibited by BQ-788 at a dose of 16 nmol/site, but not lower (1.6 nmol/site) and higher dose (48 nmol/site) (17); rather, it is increased by another ET\(_{\beta}\)-receptor antagonist, A-192621 (18). Thus, the role of ET\(_{\beta}\) receptor in cancer pain is unclear. It may be due to the expression of ET\(_{\beta}\) receptors in the Schwann cells and satellite cells in the DRG rather than primary sensory neurons (18).

Although the ET\(_{\alpha}\)-receptor antagonist BQ-788 did not significantly affect allodynia of the melanoma-bearing paw, it inhibited alldodynia induced by an intraplantar injection of extract of melanoma mass. Although a cause of this apparent discrepancy is unclear, it should be noted that ET\(_{\beta}\) receptors are expressed in the epidermal keratinocytes (27) but not in the primary sensory neurons (18). The epidermal keratinocytes may be a site of action of the melanoma extract. Although the effect of melanoma growth on ET\(_{\beta}\) receptors in the keratinocytes is unclear, nerve fibers in the skin are markedly decreased in the skin at the middle of grown melanoma mass (6), which may be a cause of the lack of involvement of ET\(_{\beta}\) receptors in allodynia of the melanoma-bearing paw.

The concentration of ET was time-dependently increased in the melanoma mass. Since melanoma showed the high levels of ET-1 immunoreactivity, the tumor itself may be the major source of ET-1. It was reported that melanoma cells did not spontaneously release ET-1 in vivo and in vitro and that inoculation into the calcareous bone did not induce mechanical allodynia (17). In their study, B16-G3.26 melanoma cells were used. This cell line lacks the ability of lung metastasis (28). On the other hand, B16-BL6 melanoma cells that were used in this study have high ability of lung metastasis (29). Therefore, the production of ET-1 and the involvement in pain may depend on the types of melanoma cells. In cancer patients, inflammation is thought to be a critical component of tumor progression (30). Inflammatory cells infiltrate the tumor site and participate in the neoplastic process, fostering proliferation, survival, and migration. In addition, angiogenesis followed endo-
In summary, orthotopic melanoma cell-inoculation into the hind paw induced the expression of ET$_A$-receptor mRNA in the DRG and increased the concentration of ET mainly produced in melanoma. These results suggest that ET-1 and ET$_A$ receptor are at least partly involved in melanoma-associated pain.

References