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Effect of Peripheral Endothelin-1 Concentration on Carcinoma-Induced Pain in Mice

Victoria Pickering¹, R. Jay Gupta¹, Phuong Quang¹, Richard C. Jordan, DDS, PhD^{2,3}, and Brian L. Schmidt, DDS, MD, PhD¹

¹ Department of Oral and Maxillofacial Surgery, University of California San Francisco

² Department of Orofacial Sciences, University of California San Francisco

³ Department of Pathology, University of California San Francisco

Abstract

In this study we investigated the role of the peripheral endothelin-1 (ET-1) concentration in a cancer pain model. To test the hypothesis that the concentration of ET-1 in the tumor microenvironment is important in determining the level of cancer pain we used two cancer pain mouse models that differed significantly in production of ET-1. The two mouse cancer models were produced by injection of cells derived from a human oral squamous cell carcinoma (SCC) and melanoma into the hind paw of female mice. Pain, as indicated by reduction in withdrawal thresholds in response to mechanical stimulation, was significantly greater in the SCC group than the melanoma group. The peripheral concentration of ET-1 within the cancer microenvironment was significantly greater in the SCC group. Intra-tumor expression of both ET-1 mRNA and ET-1 protein were significantly higher in the SCC model compared to the melanoma model. ET receptor antagonism was effective as an analgesic for cancer pain in the SCC model only. To address the potential confounding factor of tumor volume we evaluated the contribution of tumor volume to cancer pain in the two models. The mean volumes of the tumors in the melanoma group were significantly greater than the tumors in the SCC group. In both groups, the pain level correlated with tumor volume, but the correlation was stronger in the melanoma group. We conclude that ET-1 concentration is a determinant of the level of pain in a cancer pain mouse model and it is a more important factor than tumor volume in producing cancer pain. These results suggest that future treatment regimens for cancer pain directed at ET-1 receptor antagonism show promise and may be tumor type specific.

Keywords

cancer pain; endothelin-1; mouse model; oral cancer; oral squamous cell carcinoma; melanoma; tumor volume

Introduction

The etiology of cancer pain remains unknown (Mantyh et al. 2002). Nociceptive (pain-producing) mediators secreted by the tumor and subsequent sensitization of pain sensory fibers

Address correspondence to: Brian L. Schmidt, DDS, MD, PhD, Department of Oral and Maxillofacial Surgery, C-522, University of California, San Francisco, San Francisco, CA 94143-0440, (415) 502-3297, Fax (415) 476-6305, Email: brian.schmidt@ucsf.edu.

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in the tumor microenvironment have been proposed to produce cancer pain. Endothelin (ET-1), a vasoactive peptide that is produced by a number of different malignancies, sensitizes peripheral afferent sensory nerve fibers and contributes to both soft tissue and bone cancer pain (Davar et al. 1998; Peters et al. 2004; Yuyama et al. 2004; Schmidt et al. 2007). We recently demonstrated that the site of action for ET-1 in producing cancer pain is in the periphery within the tumor microenvironment (Schmidt et al. 2007) where ET-1 activates both ET-A and ET-B receptors (ETAR and ETBR, respectively). We found that peripheral antagonism of ET-1 activation of ETAR produces the same level of antinociception produced by high dose, systemic morphine in a cancer pain model (Schmidt et al. 2007). However, malignancies of different histologic type vary widely in the concentration of ET-1 produced and the effect of ET-1 concentration in the tumor microenvironment on the magnitude of cancer pain has not been analyzed. Understanding the relationship between the peripheral ET-1 concentration and the magnitude of cancer pain will be critical in designing an appropriate antagonist. We hypothesize that the magnitude of cancer pain depends directly on the peripheral concentration of ET-1 produced by a particular malignancy. In this current study, we evaluated the effect of peripheral ET-1 concentration in the tumor microenvironment on cancer pain using two histologically distinct cancer mouse models.

Materials and Methods

Cell Culture

Two malignant cell lines, oral squamous cell carcinoma (SCC) and melanoma, were utilized to produce the mouse cancer models. The human malignant oral SCC cell line, HSC-3, and the malignant melanoma cell line, WM164, were cultured at 37°C with 5% CO₂. The HSC-3 cell line was cultivated in Dulbecco's modified Eagle's medium (DMEM H-21) supplemented with 10% fetal bovine serum (10×), fungizone (0.5×), penicillin-streptomycin (1×), non-essential amino acids (1×), and sodium pyruvate (1×). The WM164 cell line was cultivated in MEM Eagle's with Earle's BSS medium supplemented with 10% fetal bovine serum (10×), fungizone (0.5×), and penicillin-streptomycin (1×). The mediums for each cell line were then vacuum filtered through a disposable tissue culture filter (Nalge Nunc International, Rochester, New York, USA) prior to storage at 4°C.

Oral SCC, Melanoma and Control Paw Models

Three separate groups of mice were used in this study: oral SCC, melanoma and control (sham injection). Experiments were performed on adult female *Foxn1^{nu}*, athymic, immunocompromised mice with ages ranging from 4 to 5 weeks old at the time of oral SCC, melanoma, and sham injections. The mice were housed in a temperature-controlled room on a 12:12 h light cycle with lights on from 06:00 to 18:00, with unrestricted access to food and water. Estrous cycles were not monitored. All procedures were approved by the UCSF Committee on Animal Research, and researchers were trained under the Animal Welfare Assurance Program.

For the SCC paw group (5 animals) and melanoma paw group (5 animals), 1.5×10^6 tumor cells in 25 μ L of DMEM and 25 μ L of Matrigel™ (Becton Dickinson & Co., Franklin Lakes, NJ) were deposited into the right hindpaw of each mouse using a 25-gauge, 5/8 inch long needle (Becton Dickinson & Co., Franklin Lakes, NJ). The mixture of 25 μ L of DMEM and 25 μ L of Matrigel™ was injected into the right paw of the sham group (3 animals). All three groups of mice were administered general anesthesia with Isoflurane (Summit Medical Equipment Company, Bend, Oregon) for the inoculation.

Behavioral Testing of Paw Withdrawal Threshold

Testing was performed for all three groups during the afternoon portion of the circadian cycle only (06:00 – 18:00 h) between 14:00 and 16:00. Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. The cage consists of 6 cubicles and allows for a maximum of 6 mice to be tested consecutively. Quantitative assay guidelines were similar to Chaplan *et al* (Chaplan et al. 1994) and as previously described (Schmidt et al. 2007). Mice were placed into the cage starting at the left-most cubicle and proceeding to the right. Fifteen minutes were allowed for cage exploration and grooming activities prior to testing. The mice were then tested in the sequence that they were loaded into the cage. The area tested was the mid-plantar right hind paw, or the tumor-front towards the later stages of tumor development. The paw was touched with 1 of a series of 14 von Frey fibers with logarithmically incremental stiffness (1.65, 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31, 4.56, 4.74, 4.93, 5.07) (TouchTest®, Stoelting Co., Wood Dale, IL). The von Frey fibers were held perpendicular to the testing surface with sufficient force to cause buckling. A positive response was noted if the paw was sharply withdrawn and if there was an immediate flinching upon removal of fiber. The fibers were presented at least 1 minute apart to allow resolution of previous stimuli, and held for at most 3 seconds. Once testing for a mouse was complete the mouse in the adjacent cubicle was tested. The 50% paw withdrawal threshold in grams of force was calculated with a formula based on the up-down method of Dixon (Dixon 1980; Chaplan et al. 1994; Schmidt et al. 2007).

$$50\%g \text{ threshold} = (10^l X_f + k\delta^l) / 10,000$$

X_f = value (in log units) of the final von Frey fiber used; k = statistical constant that is calculated based on the pattern of positive/negative responses according to the Up and Down Method originally described by Dixon (Dixon 1965); see Appendix in (Chaplan et al. 1994), and δ = mean difference (in log units) between von Frey fiber stimuli (0.2264333).

The oral SCC, melanoma, and sham groups were tested under the above paradigm on three separate days prior to inoculation. The baseline paw withdrawal threshold for each animal was defined as the mean of the three readings taken prior to inoculation. The animals were then tested using the same paradigm on days 4, 9, 11, 14, 16, 18, 21, 23, 25, 28, and 30 post-inoculation.

The investigator performing the behavioral testing was blind to the group being tested and blind to drug/control administration.

Measurement of Tumor Volume

The tumor volumes in all mice were measured on post injection days 14, 16, 18, 21, 23, 25, 28 and 30 by measuring three dimensions, length, width, and height, using a metric caliper. The tumors were measured through the skin. The site of the tumor front relative to the normal surrounding skin was used as the site of the tumor border. The length was the greatest anterior-posterior dimension. The width was the greatest medial-lateral dimension. The height was the distance from the unaffected midplantar surface to the highest point of the tumor.

Intra-tumor administration of endothelin A receptor (ETAR) antagonist followed by nociceptive behavioral testing

To evaluate the role of ET-1 in the SCC and melanoma pain mouse models, a selective ETAR antagonist was administered prior to paw withdrawal testing. Testing was performed for each group of animals at 32 days following inoculation with either oral SCC or melanoma. The

control experiments consisting of intra-tumor administration of saline was performed four to five days following testing with ETAR antagonist. 50 μ L of 0.1 nmol/ μ L of BQ-123 (American Peptide Company, Sunnyvale, CA) or saline (vehicle) were injected directly into the mid-plantar hind paw at the site of greatest tumor development with a 30 gauge beveled needle. The pre-drug administration baseline paw withdrawal was measured 60 minutes prior to administration of either BQ-123 or saline. Paw withdrawal testing was then performed at 30, 60, 90 and 120 minutes following administration of either BQ-123 or saline.

Tissue collection and histology

All mice were sacrificed within 2 days of completion of the behavioral tests. Euthanasia was performed with inhalation of CO₂ followed by cervical dislocation. The right hind paws injected with SCC and melanoma were harvested. One portion of each tumor was fixed in 10% buffered formaldehyde and embedded in paraffin. Five μ m sections were microtome cut and stained with hematoxylin and eosin to confirm SCC or melanoma growth. The remainder of the tissue was frozen at -80°C.

Real-time quantitative RT-PCR measurement of ET-1 and ETAR

ET-1 and ETAR mRNA expression were measured in the SCC and melanoma tumors as well as in the associated HSC-3 and WM164 cell lines. mRNA expression was assessed using real-time quantitative RT-PCR as previously described (Macabeo-Ong et al. 2002; Connelly et al. 2005). Total RNA was extracted from frozen tissues using RNeasy®Mini Kit (Qiagen, Inc., Valencia, CA). 15-25 mg of tissue and 3×10^6 cells of each cell line were homogenized in the lysis buffer provided in the kit. The lysate was then applied to an RNeasy mini spin column, and total RNA was eluted according to the manufacturer's instructions. Reverse transcription was performed using Gibco BRL Reverse Transcriptase kit (Life Technologies, Carlsbad, CA). We purchased Assays-on-Demand Expression assays from Applied Biosystems (Foster City, CA). Samples were run on an ABI 7700 Prism (PE Biosystems, Foster City, CA). Relative expression of ET-1 and ETAR mRNA were calculated using the comparative Ct method (Connelly et al. 2005). This method of analysis was selected because the slopes of the dilution standard curves for ET-1, ETAR, and the reference gene β -N-acetyl-glucosaminidase (β -GUS) were comparably similar across a range of input RNA. Thus differences in relative abundance for RNA species expressed at low levels would not distort the analysis. Moreover, absolute threshold cycle (Ct) values for each sample were found to lie within the range of RNA quantities used for standard curve generation. Analysis was carried out using the software supplied with the ABI 7700 Prism (PE Biosystems). ET-1 and ETAR mRNA overexpression was defined as expression > 1.0 relative to β -GUS expression levels.

ELISA measurement of ET-1 in paw tumors and cell medium

ET-1 levels were quantified in the SCC and melanoma tumors as well as in the conditioned medium from the cultured HSC-3 and WM164 cells by ELISA. The samples were prepared as follows. 20-40 mg of frozen tissue was homogenized in the T-PER Reagent (Pierce Biotechnology, Inc., Rockford, IL). The lysates were centrifuged at 13,000 rpm for 5 min. The supernatants were removed, aliquoted and stored at -80°C. The extraction of ET-1 was performed using a BondElut® C₁₈, 200 mg column (Varian, Inc., Lake Forest, CA). The column was activated by addition of 1 ml of methanol followed by 1ml of water, and 1 ml of 10% methanol. 500 μ L of tissue lysates was acidified with an equal volume of 20% acidic acid and centrifuged at 14,000 rpm for 10 min. The supernatants were removed and applied to a column. The cartridge was washed with 2 ml of ethyl acetate. The peptides were eluted with 1 ml of methanol/0.5M ammonium bicarbonate (80/20 v/v). The eluants were evaporated to dryness using a centrifugal concentrator under vacuum (Eppendorf, Westbury, NY), reconstituted with assay buffer, and assayed immediately. 1×10^6 cells of HSC-3 and WM164

cell lines were cultured for 48 hours. The conditioned medium was then removed, centrifuged, and stored at -80°C. The concentration of ET-1 was measured by ELISA using a TiterZyme® Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI). The concentrations were calculated from the standard curve using a 4-parameter logistics data reduction software (Bio-Rad Laboratories, Inc., Hercules, CA). The standard curve was generated using the following concentrations: 100, 50, 25, 12.5, 6.25, 3.1, 1.56, and 0.78 pg/ml. The optical density of the standards and samples was read at 450 nm wavelength using a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA). Results are reported as mean $\pm$ standard error.

Immunohistochemistry

Immunohistochemistry for ET-1 was performed on 5 μ m tissue sections as we have described (Connelly et al. 2005). Briefly, the sections were deparaffinized and incubated with trypsin for 20 min at 37°C for antigen retrieval. The endogenous peroxidase was quenched by immersing the sections into the freshly made 3% hydrogen peroxide solution for 5 min. The non-specific binding was blocked with normal goat serum for 20 min following by the incubation with ET-1 primary antibodies (Mouse Monoclonal antibody, Affinity Bioreagents, Golden, CO) 1:250 overnight at 4°C. Incubation with the secondary antibody was performed using a Vectastain®Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The bound complexes were visualized by the application of AEC substrate (Vector Laboratories, Burlingame, CA). Counterstaining of the tissues was performed with Mayer's hematoxylin (Sigma Aldrich, St. Louis, MO). Immunohistochemical controls consisting of omission of the primary antibody were performed in parallel.

Statistical Tests

The Mann-Whitney U test was used to compare mean % difference of 50% paw withdrawal threshold across groups (SCC, melanoma, sham), since threshold values computed do not yield a mathematical continuum (Chaplan et al. 1994). It was also used to compare mean tumor volumes and mean % change of 50% paw withdrawal threshold across groups (SCC, melanoma, sham) that were treated with ETAR Antagonist. The paired student's *t*-test was used to analyze paw withdrawal threshold following administration of BQ123 within each group. A linear (Pearson) correlation test was performed to evaluate the correlation between the tumor volume and mean 50% paw withdrawal threshold within each group and the correlation coefficients were calculated and compared. The unpaired *t*-test was used to compare the ET-1 levels in tumors measured by ELISA. The unpaired *t*-test was also used to compare mRNA expression levels. All tests were two tailed and a *p* value of less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Instat software (GraphPad Software, Inc., San Diego, CA).

Results

Behavioral testing for the SCC, melanoma, and sham mouse model

The mean percent difference in 50% paw withdrawal threshold for the SCC group was significantly lower than both the melanoma group and the sham group on all post-inoculation testing days (Figure 1). The mean percent difference in 50% paw withdrawal threshold for the melanoma groups was significantly lower than the sham group on all post-inoculation testing days except for day 4.

Tumor volume in SCC and melanoma injected mice

The mean tumor volumes for the SCC and melanoma mouse models were calculated starting post-inoculation day 14. The mean volumes of the tumors in the melanoma group were

significantly greater than the tumors in the SCC group from post inoculation day 23 forward (Figure 1).

Correlation between tumor volume and pain level

The correlation between tumor volume and 50% paw withdrawal threshold was assessed within the SCC and melanoma groups (Figures 2A and 2B, respectively). A linear (Pearson) correlation test demonstrated a negative correlation between tumor size and pain threshold in all experimental cancer groups. However, the correlation was stronger with $r = -0.726$ in the melanoma group ($p < 0.0001$) than in the SCC group with $r = -0.351$ ($p = 0.0474$).

ETAR antagonist intra-tumor administration and nociceptive behavioral testing

The antinociceptive effect of ETAR antagonism was evaluated in the SCC, melanoma, and sham mouse groups. The pre-drug administration baseline paw withdrawal was measured 60 minutes prior to administration of BQ-123. Mean 50% paw withdrawal threshold was calculated following intra-tumor administration of BQ-123 and compared to the pre-drug administration baseline measurement. There was no significant difference between the pre-drug administration baseline measurement and 30 minutes following intra-tumor administration of BQ-123 in the SCC group. However, BQ-123 increased paw withdrawal thresholds, significantly from the pre-drug administration baseline measurement at 60, 90, and 120 minutes after intra-tumor administration in SCC injected animals. No significant difference in paw withdrawal threshold was observed at any time points in the melanoma or sham group (Figure 3). Saline intra-tumor administration was performed in all three groups as a control. Paw withdrawal thresholds of the SCC group, melanoma group and sham group receiving intra-tumor injection of saline were not significantly different from the pre-drug administration baseline measurement at any of the time points.

Quantitative real time RT-PCR analysis of ET-1 and ETAR mRNA level

ET-1 mRNA and ETAR mRNA levels in the SCC paws ($n=5$), melanoma paws ($n=5$), SCC cell line and melanoma cell line were measured using quantitative real time RT-PCR. ET-1 and ETAR mRNA expression levels were calculated relative to the respective β -GUS expression levels. There was significantly more ET-1 mRNA in SCC tumors and SCC cell line (HSC-3) compared to the ET-1 mRNA in the melanoma tumors and the melanoma cell line (WM164), respectively ($p < 0.0001$). There was also significantly more ETAR mRNA in SCC tumors and SCC cell line compared to the ET-1 mRNA in the melanoma tumors and the melanoma cell line, respectively ($p < 0.0001$) (Table 1).

ELISA measurement of ET-1 in SCC and melanoma tumors and conditioned medium

ET-1 levels in the SCC paws ($n=5$), melanoma paws ($n=5$), and conditioned medium from the HSC-3 and WM164 cultured cells were measured by ELISA. The concentration was calculated from the standard curve using a 4-parameter logistics curve-fitting program (Bio-Rad Laboratories, Inc., Hercules, CA) recommended by the manufacturer of the ELISA kit (Assay Designs, Inc., Ann Arbor, MI). The sensitivity of the assay was 0.14pg/ml. The unpaired t-test revealed a significantly higher concentration of ET-1 in SCC tumors than in melanoma ($p=0.0014$) (Table 1). The ET-1 level in the cell culture medium for SCC was 34.02 pg/ml while ET-1 in the medium from the melanoma cell line, WM164, was not detectable (Table 1).

Immunohistochemistry

ET-1 in the SCC and melanoma paw tumors were localized using immunohistochemistry. All of the SCC sections (5/5 mice) displayed a strong homogenous cytoplasmic staining for ET-1. No significant staining for ET-1 was observed in any of the melanoma sections (5/5 mice) (Figure 4).

Discussion

This study demonstrates that the concentration of ET-1 produced by the malignancy in the tumor microenvironment is a determinant of the magnitude of pain in a mouse cancer model. ETAR antagonism is effective as an analgesic for cancer pain when ET-1 is produced by the malignancy. We also found that cancer pain increases with tumor volume; however, ET-1 concentration is a more important factor than tumor volume in producing cancer pain. The evidence for this conclusion is that SCC tumors which had lower volume and higher ET-1 concentration produce significantly higher levels of pain. We have previously demonstrated that SCC produces pain behavior in mice (Schmidt et al. 2007). In the current study we found that melanoma also produces pain behavior in the mouse model; however, the level was significantly lower than the SCC model. Melanoma can be painful in humans both at the primary site and at metastatic sites (Pandey et al. 1998; Jones et al. 2006; Noorda et al. 2007). Melanoma also produces mechanical hyperalgesia in an orthotopic mouse model similar to the one we have used in the current study (Sasamura et al. 2002). The SCC carcinoma mouse model demonstrated pain behavior at the earliest time point that pain behavior was measured (day 4) when tumor growth was not clinically evident. These results provide further evidence that a secreted nociceptive mediator within the tumor microenvironment is the primary etiology of carcinoma pain.

Our finding that a molecular factor such as ET-1 is a more important determinant of cancer pain than tumor volume is in accordance with a bone cancer pain model in mice which demonstrated that the magnitude of cancer pain depends on peripheral or central nervous system molecular factors rather than tumor volume (Vit et al. 2006). While tumor volume is not the primary factor, it clearly contributes to cancer pain. Investigators using a mouse model of bone cancer pain have shown that a reduction in tumor size with radiation or chemotherapy reduces the magnitude of cancer pain (Sabino et al. 2002; Goblirsch et al. 2004). In the only other study that has evaluated tumor volume and cancer pain in a soft tissue cancer pain model in a mouse, both tumor volume and pain behavior (mechanical hyperalgesia) were shown to increase with time; however, the authors did not perform a correlational analysis (Asai et al. 2005). Interestingly, these authors also used an oral SCC inoculation into the paw to create the model (Asai et al. 2005). While our correlational analysis supports the finding that increasing tumor volume produces worsening pain the mechanism is unclear. Potentially an increase in tumor volume reflects an increase in the cellularity of the tumor and an increase in the production and local concentration of a nociceptive mediator, such as ET-1, in the tumor microenvironment.

In this study we demonstrated that the magnitude of cancer pain depends upon both tumor type, as well as the local concentration of ET-1. ET-1 has been shown to have a role in a metastatic sarcoma (Peters et al. 2004) and a prostate cancer pain mouse models (Yuyama et al. 2004); moreover, we previously demonstrated the role of ET-1 in pain behavior in the oral SCC mouse model (Schmidt et al. 2007). These three studies utilized different cancer models and analyzed pain with different behavioral tests. Furthermore, in the studies using the metastatic sarcoma and prostate cancer model the concentration of ET-1 in the tumor microenvironment was not measured and the ET receptor antagonist differed in both concentration and route of administration. These discrepancies make interpretation of the role of the cell of origin of the malignancy, as well as the roles of ET-1 and the ETAR in cancer pain difficult. In the present study we attempted to clarify the role of ET-1 by directly comparing the pain behavior, ET-1 concentration and the antinociceptive response to a peripherally administered endothelin receptor antagonist in two different cancer pain mouse models each analyzed in the same manner with the same investigator performing the behavior analysis.

We found that the levels of ET-1 in the SCC tumors were 10 times higher than levels in melanoma. We measured levels of ET-1 in the cultured media to provide evidence that the source of ET-1 in the tumor microenvironment was the tumor. With the oral SCC model the finding of very high levels within the culture media strongly suggest that the source of ET-1 in the tumor microenvironment is the malignancy rather than the host inflammatory response for these tumor types. These results were supported by immunohistochemical localization of ET-1 within the malignant epithelium of the SCC tumors. For malignant melanoma the cultured media showed no ET-1; however, the tumors showed low levels of ET-1 mRNA transcript and protein. The low levels of ET-1 present in the malignant melanoma tumors, despite no levels measured in the cultured media, suggests that the low levels of ET-1 might be due to malignant melanoma-induced ET-1 production by the host tissue. The malignant melanoma mouse model showed no response to the ETAR antagonist. Therefore, for certain cancers other local tumor mediators may be responsible for the resultant pain behavior. The clinical implication of this finding is that ETAR antagonism might be significantly more effective in treating specific types of human cancer pain.

Sensitivity to pain and morphine attenuation of pain-like behavior have been shown to depend on gender in rodents and humans (Sternberg et al. 1995; Mogil et al. 2000). Men with head and neck cancer have been shown to have significantly greater difficulty maintaining pain relief with opiates (Mercadante et al. 1997). To avoid the possible confounding effects of gender we limited the study to female mice. Although estrous cycles were not monitored, and changes in gonadal hormones can affect pain-like behavior and opioid antinociception (Mogil et al. 2000; Terner et al. 2005), it is unlikely that the effects of estrous cycle could explain the observed differences between the experimental groups to which mice were randomly assigned.

In summary we have demonstrated that the severity of cancer pain depends on the cell of origin of the malignancy. Local production and secretion of ET-1 in the tumor microenvironment followed by activation of the ETAR is a clear determinant of certain forms of cancer pain. Future treatment regimens for cancer pain directed at ET-1 receptor antagonism show promise; however, this therapeutic approach will need to be tailored to the type of malignancy.

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Figure 1.

Mean percent difference of 50% paw withdrawal threshold and mean tumor volumes for the oral SCC, melanoma and sham groups. For each group the right hindpaws were injected with either oral SCC (n=5), melanoma (n=5), or cell media (sham, n=3) groups. The line graph represents the mean percent difference of 50% paw withdrawal threshold $\pm$ S.E.M for the oral SCC, melanoma and sham groups. There was a significant difference in mean percent difference of 50% paw withdrawal threshold between the oral SCC group and melanoma, as well as the SCC group and sham on all days ($p<0.01$). There was a significant difference in mean percent difference of 50% paw withdrawal threshold between the melanoma and sham group from on all days except day 4 ($p<0.01$). The vertical bar graph represents mean tumor volume (mm^3) of the right hindpaws inoculated with either oral SCC or melanoma. Each bar represents the mean tumor volume $\pm$ S.E.M of the designated group. The melanoma tumors were significantly larger than the oral SCC tumors on post-inoculation days 23, 25, 28 and 30 ($p<0.05$). The sham groups showed no evidence of tumor growth on any day.

FIGURE 2A

FIGURE 2B

Figure 2. Correlation between tumor volume and 50% paw withdrawal threshold in (A) oral SCC ($r = -0.351$, $p = 0.0474$), (B) melanoma ($r = -0.726$, $p < 0.0001$).

Figure 3.

Effect of BQ123 on paw withdrawal threshold. In oral SCC group, the intra-tumor administration of BQ-123 produced a statistically significant increase in the withdrawal threshold at 60 ($p=0.0075$), 90 ($p=0.0006$), and 120 ($p=0.0114$) minutes following the injection of the drug. No significant difference in paw withdrawal threshold was observed at any time points in the melanoma or sham group.

Figure 4A

Figure 4B

Figure 4. Immunohistochemical staining of ET-1 in (A) oral SCC showing a strong homogenous cytoplasmic staining within the malignant epithelium (brown stain) (B) melanoma showing no staining.

Table 1
ET-1 and ETAR mRNA Expression Levels in the Paw Cancers and Associated Cell Lines.
ET-1 Concentration in Paw Cancers and Conditioned Medium

Cancer Type	ET-1 mRNA expression level	ETAR mRNA expression level	ET-1 level in tissue or cell culture medium (pg/ml)
SCC	257.8 ± 72.1 [*]	13.00 ± 3.01 [*]	63.5 ± 21.9 [*]
Melanoma	0.1 ± 0.1	0.06 ± 0.02	6.6 ± 2.9
Cell Line			
HSC-3 (SCC)	1110.5 [†]	16.3 [†]	34.02
WM164 (melanoma)	1.22	0.29	Not detectable

^{*} significantly more in SCC relative to melanoma

[†] significantly more in HSC-3 relative to WM164