Influence of Sex Differences on the Progression of Cancer-induced Bone Pain

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Abstract. Background: Pain caused by bone metastases has a severe impact on the quality of life for many patients with cancer. Good translational in vivo models are required to understand the molecular mechanism and develop better treatment. In the current study we evaluated the influence of sex differences on the progression of cancer-induced bone pain. Materials and Methods: 4T1-luc2 mammary cancer cells were introduced into the femoral cavity of female and male BALB/cJ mice. Bioluminescence tumor signal, pain-related behavior and bone degradation were monitored for 14 days. Results: Female mice demonstrated a significantly greater bioluminescence signal on day 2 compared to male mice and, in addition, a significant earlier onset of pain-related behavior was observed in the females. No sex difference was observed for bone degradation. Finally, a strong correlation between pain-related behavior and bone degradation was observed for both sexes. Conclusion: Although differences were observed between the sexes, these were minor and did not affect the overall progression of the pain state.

Pain is a common complication for many patients with cancer (1). Metastasis to the bone is the most common source of severe cancer-induced pain and is closely-related to the advanced stage of many common types of cancer, such as breast, melanoma, lung and prostate cancer (2). Although cancer-induced bone pain is a common problem in the clinic, little is known about its underlying biology. The current treatment options are insufficient leaving patients without adequate pain relief, and are often associated with dose-limiting side-effects (3, 4), thereby significantly compromising the quality of life for many of these patients (1, 5). In order to optimize treatment, good pre-clinical models accurately mimicking human conditions are required to offer a better understanding of the molecular mechanisms involved.

During the past decade it has become increasingly clear that a wide variety of biological functions and molecular interactions are affected by sex differences, which should be considered when developing good translational in vivo models. However, in the field of pain, most biomedical research has relied heavily on models in male animals. In 2005, Mogil et al. demonstrated that in 79% of the studies published in PAIN® from 1996 to 2005, only male animals were used, whereas only 4% were set up to test for differences between the sexes (6). This is paradoxical since an increasing body of evidence suggests significant gender-specific differences in pain thresholds, tolerance and response to pain treatment in both clinical and in vivo models. Furthermore, there is an over-representation of females in many common chronic pain states (7, 8), and as a result of this, in 2007, the Sex, Gender and Pain Special Interest Group of the International Association for the Study of Pain recommended that “all pain researchers consider testing their hypotheses in both sexes, or if restricted by practical considerations, only in females” (8). Although all in vivo pain models should be tested for potential sex influences, it is especially important for those mimicking human diseases predominating in a specific sex or induced by tumor cell lines with a sex-specific origin, such as models of breast or prostate cancer. In addition, studies have demonstrated that some tumor cell lines display different metastatic properties depending on the sex of the host, clearly demonstrating the importance of considering sex differences when using in vivo models of various types of cancer (9, 10).

In cancer-induced pain research, a common in vivo model of metastatic bone cancer is based on the direct inoculation of tumor cells, such as 4T1 mammary carcinoma cells or osteosarcoma cells, into the intramedullary space of long
bone. Despite the fact that no previous study has tested the actual influence of sex differences on the progression of the cancer-induced bone pain in the 4T1 mammary carcinoma cell model, both female and male mice are used to model the pain state. We hypothesize that the microenvironment of the female bone marrow might be more advantageous for the settlement and survival of the tumor cells compared to the male bone marrow, due to the female origin of the tumor cells (11). Therefore, in the present study, we evaluated the potential influence of sex differences on the overall progression of cancer-induced bone pain in a murine model of metastatic mammary cancer.

Materials and Methods

Animals. Six-week-old male and female BALB/cJ mice were purchased from Taconic, Tornbjerg, DK. Mice were housed in groups of seven with a 12-hour light/dark cycle and allowed free access to water and standard diet. All animals where acclimatized for 1-2 weeks prior to experiments. All experiments were approved by the Danish Committee for Experiments on Animals, 2009-561-1622C4, Copenhagen, DK, and conducted according to the guidelines of the International Association for the Study of Pain (12).

Cell line. 4T1-Luc2 mammary carcinoma cells were kindly provided by Caliper, Teralfene, Belgium, and cultured in RPMI medium (Invitrogen, Nærum, DK), according to the manufacturer’s instructions. Cancer cells were split two days prior to surgery and on the day of surgery harvested with 0.25% trypsin-EDTA (Invitrogen, Nærum, DK) and resuspended in RPMI medium to a final density of 10^6 cells/ml. All cancer cells were kept on ice until use.

Bone cancer surgery. Bone cancer was induced as previously described (13) with a few modifications. Briefly, 7 to 8-week-old mice were anesthetized with a mixture of hypnorm/dormicum (VetaPharma, Leeds, UK and Roche, Hvidovre, DK; 1.25 mg/ml midazolam, 2.5 mg/ml fluanisone and 0.079 mg/ml fentanyl citrate; 10-12 ml/kg). An incision was made in the skin overlying the patella, and the lateral site of the patella tendon and lateral retinaculum tendon loosened and the patella pushed aside to expose the distal femoral epiphysis. A 30-gauge needle (Mediq, Brøndby, DK) was used to drill a hole into the medullary cavity through which 10^4 4T1-Luc2 mammary carcinoma cells in 10 μl RPMI medium were inoculated with a 0.3 ml insulin syringe (Terumo Medical Corporation, Therumo, Herlev, DK). The hole was closed with surgical Ethicon bone wax (Mediq, Brøndby, DK) and the open wound thoroughly irrigated with sterile saline. The skin was sutured with 4-0 Ethicon vicryl rapid suture (Mediq, Brøndby, DK), and Xylocaine gel (2% w/v; Hamlets Pharmacy, Copenhagen, DK) applied to the wound. Sham-operated control mice underwent the same operation, but were inoculated with RPMI medium alone.

Pain-related behavior test. Limb use: The mouse was allowed to move freely around in a transparent standard cage without bedding (125 mm x 266 mm x 185 mm; Tecniplast polycarbonate, Scanbur A/S, Karlslund, DK). Following 10-15 min of acclimation, the animal was observed for 3 min and a limb use score from 4 to 0 was assigned to the gait of the operated hind limb as follows: 4: normal use of hind limb, 3: insignificant limping, 2: significant limping, 1: significant limping and partial lack of limb use and 0: total lack of limb use.

Weight-bearing: Weight-bearing deficit was measured using a TSE Powermeter (TSE Systems GmbH, Homburg, Germany). The mouse was placed with the hind legs on two separate scales and the individual load of each hind limb was measured for 10 s. The test was performed in triplicate, and the mouse forced to change position before each measurement. An average weight-bearing ratio was calculated as the weight placed on the right hind limb divided by total weight on hind limbs and this average ratio was subjected to data analysis.

Bioluminescence imaging. D-Luciferin (Caliper Life Sciences, Teralfene, Belgium) was dissolved in PBS and administered by intraperitoneal injection (150 mg/kg), 9 min prior to bioluminescence imaging. Animals were anesthetized in an induction chamber with 3% isoflurane (ISOBAR Vet; 100%, Nomeco, Copenhagen, DK) at 2 l/min mixed with purified oxygen (Conoxia, AGA, Copenhagen, DK) for 3 or 4 min, males and females, respectively. Following induction of anesthesia the animals were placed on their back in a nose cone in a Lumina XR instrument (Caliper Life Sciences, Teralfene, Belgium) and anesthesia was maintained with a 0.5 l/min 3% isoflurane/oxygen mix. Image capture was performed with binning: M(4), F(stop: 1 and exposure time from 10 s to 5 min according to signal power. The position of the animals was standardized by fixing the hind legs in the same position according to an outlined template placed beneath the animal. For each animal, an average of three images were used for analysis. Between each capture, the animal was moved inside the Lumina XR to minimize bias caused by placement of the animals in the machine. Bioluminescence images were analyzed using IVIS Imaging Software (Living Image®, version 4.0.9.9801; Caliper Life Sciences, Teralfene, Belgium). For each image, the threshold was manually adjusted to fit the outline of the signal, and the readout was measured in total flux, photos/s/cm².

X-ray analysis. X-Ray images were captured subsequent to the bioluminescence images. The severity of bone degradation was analyzed using bone densitometry software from VisioPharm (VisioPharm, Hørsholm, DK). Each X-Ray image was calibrated to a standard aluminum wedge. The mean grayscale value of a standard region of interest within the trabecular bone of the distal femur was measured and the average of two corresponding background regions in the soft tissue proximate to the distal femur was subtracted. The grayscale value was translated into millimeter aluminum equivalents (mmAl) according to the standard wedge and used as estimate of the relative bone density of the distal femur. 

Microcomputed tomographic analysis. Mice were euthanized by cervical dislocation and tissue was collected at day 4. The femur and the proximal part of the tibia were removed, drop fixed in 4 % paraformaldehyde (PFA) for seven days and subsequently stored in phosphate buffered saline (PBS) with 0.1% PFA and 0.1% NaN3 at 4°C until scanned. Distal femoral bones were scanned with a high-resolution microcomputed tomographic (μCT) system (vivaCT 40; Scanco Medical AG, Brüttisellen, Switzerland). The scan resulted in a 3-dimensional (3D) reconstruction of cubic voxel sizes of 10.5×10.5×10.5 μm³. Each 3D image dataset consisted of approximately 210 μCT slice images, of which 100 (1050 μm) were
used for analysis of bone tissue (2048×2048 pixels) with 16-bit gray levels. The 10 mm length volume of interest was defined as starting 0.5 mm proximally to the subchondral bone plate of the distal femoral condyles and extending 10 mm proximal along the femur. From accurately segmented μCT image datasets, all microarchitectural properties of the distal femur were calculated using true, unbiased and assumption-free 3D methods. Based on the defined volume of interest, the total volume (TV) was calculated as the total specimen volume including bone and marrow within the volume of interest. Bone volume fraction (BV/TV) and bone surface to total volume ratio (BS/BV) were computed (14).

Estrous cycle determination. Vaginal smears were obtained at day 0, 2 and 4. The samples were collected immediately following the imaging procedure and evaluated under a light microscope.

Blinding of experiments. All experiments and subsequent analyses were blinded for the researchers. Determination of estrous cycle phases was, in addition, evaluated by two independent experimenters.

Statistical analysis. All data are presented as the mean±standard error of mean (S.E.M.). Statistical analysis was performed with GraphPad Prism (v. 4.03 for Windows; GraphPad Software, San Diego, CA, USA). Bioluminescence data was analyzed using one-tailed Mann-Whitney test. Limb use score was evaluated at the specific test days with Kruskal–Wallis test followed by Dunn’s multiple comparison tests, or with one-tailed Mann–Whitney U-test. Weight-bearing deficit was evaluated by two-way ANOVA followed by Bonferroni comparison test. Subanalysis at specific time points was performed with one-way ANOVA followed by Newman–Keuls post-test or with Student’s t-test. X-Ray data was analyzed by a two-way ANOVA followed by Bonferroni comparison test. Subanalysis at specific time points was performed with one-way ANOVA followed by Bonferroni comparison test. μCT data was analyzed by two-sided paired Wilcoxon matched-pairs signed rank test or two-sided paired Student’s t-test for male and females respectively. Correlation analysis of bioluminescence signal, bone degradation rate and pain-related behavior was evaluated by Spearman r or Pearson r correlation test. For all statistical analyses, a probability value of 0.05 was considered significant.

Results

Female mice had greater bioluminescence signals in the early phase. To evaluate if sex influenced the progression of the tumor cells in the bone marrow, the bioluminescence signal from the tumor cells was followed over time. The bioluminescence signal from the tumor cells increased exponentially from day 1 throughout the first week, reaching a plateau beyond day 8 (Figure 1A). In several experiments female mice tended to have a greater bioluminescence signal compared to the male mice (n=5-6, p>0.05) (Figure 1A). As the tendency was more pronounced in the first days, the early phase was investigated further. On day 2, the bioluminescence signal was significantly greater in the females compared to the males (p=0.026), indicating a faster progression and/or a higher survival rate of the tumor cells in the females during the first days following inoculation of the cells (Figure 1B). No difference was detected on day 4 (p>0.05).

Female mice had an earlier onset of pain-related behavior. To investigate whether the difference in early-phase tumor signal would translate into a sex-specific difference in the onset or severity of pain-related behavior, male and female mice were tested for limb use and weight-bearing at baseline and day 3, 6, 9, 12 and 14. Female mice had a significantly lower limb use score on day 9 compared to male mice (p=0.039), demonstrating a significant earlier onset of pain-related behavior in the females (Figure 2A). In contrast, evaluation of weight-bearing ratio showed no significant
difference between the sexes; however, a slight tendency for more pronounced pain-related behavior was observed in the females compared to the males (Figure 2B).

No sex differences were observed in bone degradation. The bone density was analyzed by bone densitometry to evaluate whether the differences observed in bioluminescence signal and pain-related behavior were related to the degree of bone degradation. Using x-ray images with a reference aluminum wedge, the relative bone density was quantified at day 0, 1, 6, 9 and 12 by translating the grayscale intensity of the distal femur into mmAl equivalents (Figure 3B). All groups had similar relative bone density on day 0, 1 and 6 (Figure 3A). On day 9 and 12, the relative bone density in both male and female mice was significantly reduced compared to their sham groups ($p=0.0002$ and $p<0.0001$) (Figure 3A). No difference in the time of onset or degree of bone degradation was observed between the sexes. In addition, it was found that the relative bone density of both female and male cancer groups was significantly increased from day 1 to day 6 ($p=0.044$ and $p=0.048$) (Figure 3A). The increase in relative bone density during the first week was supported by μCT analysis of the trabecular bone tissue from day 4. Micro-architectural analysis showed a significant increase in the apparent density in the cancer-bearing femur compared to contralateral control leg in both females and males ($p=0.018$ and $p=0.02$) and a significant decrease in tissue material density ($p=0.0002$ and $p=0.026$) (data not shown). In addition, a significantly increased bone volume fraction (BV/TV) ($p=0.014$ and $p=0.024$) (Figure 3C), and a significant decrease in the bone surface-to-volume ratio was observed in the cancer-bearing femur of both female and male animals compared to the contralateral leg ($p=0.003$ and $p=0.002$) indicating an overall increase in bone mass at day 4 (Figure 3D). This was likely caused by callus formation following the inoculation of the tumor cells.

Both sexes demonstrated a tight correlation between bone degradation and pain-related behavior. Next, the correlation of tumor growth, bone degradation and development of pain-related behavior were examined. In both female and male mice bone degradation and pain-related behavior were highly-correlated ($p<0.0001$ for both sexes) (Figure 4A and B). In contrast, the bioluminescence signal from the tumor cells did not correlate with bone degradation or pain-related behavior neither in the early phase, day 4 (females: $p=0.54$ and $p=0.51$, males: $p=0.25$ and $p=0.49$) nor in the late phase, day 9 (females: $p=0.68$ and $p=0.15$, males: $p=0.21$ and $p=0.56$) (Data not shown).

The estrous phase did not influence the bioluminescence signal. As the cycling of the female estrous phases has been shown to affect the growth rate and risk incidence of some types of cancer (15), the influence of the four estrous phases on the bioluminescence signal was investigated. No correlation between signal intensity and estrous phase was observed on either day 2 or 4 ($p>0.05$) (Figure 5A). In addition, no synchronization of cycles was observed between the animals and on each measuring day, animals in each of the four phases were represented (Figure 5B).
As a consequence of the increasing need to develop and optimize *in vivo* models to more accurately mimic human diseases, we tested the potential influence of sex differences on the progression of bone pain in a murine model of metastatic mammary cancer. A significantly greater bioluminescence signal was detected in female mice compared to male mice in the early phase following inoculation of the tumor cells. The observed difference supports the hypothesis that the female bone marrow provides an advantageous microenvironment for tumor cells when it comes to settlement and survival of the cells. On the other hand, the minor lead seen in the females is quickly lost and male and female mice demonstrate equally fast progression of the tumor signal during the remaining part of the exponential growth phase, reaching a plateau at the same time.

Figure 3. A: Bone densitometric analysis of bone degradation. All groups showed an increased relative bone density of the affected femur until day 6. On day 9 and 12, both female and male cancer-bearing animals displayed a significant decrease in the relative bone density compared to sham-operated animals (*n*=3-5). B: X-ray image demonstrating severe degradation in the distal femur, arrow, and to the left, the standard aluminum wedge used for calibration in quantification of bone density. C and D: Day 4 microcomputed tomographic analysis of cancer-bearing femur and contralateral femur as control. Both sexes showed a significant increase in the bone volume fraction (C) and decrease in the bone surface to bone volume ratio (D), indicating an increase in overall bone density at day 4 (*n*=9-10). *p*<0.05, **p*<0.01, ***p*<0.0001, ****p*<0.00001.
time point. The plateau should not be interpreted as stagnation of tumor growth, but more likely a physiological artifact caused by the beginning of a necrotic center in the tumor inducing loss of signal from the inactive and dying cells in the middle at about the same rate as the signal is increasing by tumor growth at the periphery of the tumor.

The difference observed in bioluminescence signal in the early phase of tumor growth was reflected in the onset of pain-related behavior. A significantly earlier onset was observed in females compared to males with limb use scoring, but not with weight-bearing ratio assessment. However, this is not necessarily conflicting, as the difference in the tumor signal, although significant, was minor, and limb use assessment tends to be a more sensitive method compared to the assessment of weight-bearing ratio, as limb use evaluation reflects a combination of both spontaneous and movement-evoked pain whereas weight-bearing is mainly evaluating the spontaneous pain intensity. Despite the differences detected in both tumor signal and pain-related behavior, these were not reflected in the degree of bone degradation. It is possible that minor differences in the onset or severity of bone degradation also occur but are below the detection limit of the methods employed in the study, as very strong correlation was found between pain-related behavior and bone degradation. However, in general, it should be emphasized that the differences observed were...
minor and quite large sample sizes were required to detect them. It can therefore be questioned whether these differences are of physiological importance in a model of cancer as aggressive as the 4T1 mammary carcinoma.

The inconsistency when it comes to preference for male or female animals for in vivo models of cancer-induced bone pain is likely due to a crossover of various disciplines working with the models. Whereas there is a long tradition for working with male animals in pain research, female animals are, not surprisingly, preferably used for breast cancer research. However, clinical data support the notion that breast cancer can be as aggressive in men, who have even been reported to have an inferior outcome, compared to women suffering from breast cancer (16, 17). Following this study, we can conclude that in the 4T1 mammary carcinoma model, some sex differences do affect the model, however, the differences are minor and therefore do not affect the overall progression of the pain state. However, one should be aware that therapeutic intervention could still be affected by various sex differences, and females and males might, therefore, respond differently to treatment.

Acknowledgements

The Authors would like to thank Anna Mathilde Caldera, Faculty of Health and Medical Sciences, Copenhagen University, for excellent technical assistance, and Michael Grunkin, Visiopharm, for his advice concerning bone densitometry. The research was supported by Fonden til lægevidenskabens fremme v/A.P. Møller og Hustru Chastine Mc-Kinney Møllers Fond til almene Formaal.

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