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Inhibition of breast cancer-cell glutamate release with sulfasalazine limits cancer-induced bone pain

Robert G. Ungard, Eric P. Seidlitz, Gurmit Singh *

Department of Pathology & Molecular Medicine, McMaster University, Hamilton, ON L8N 3Z5, Canada

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ABSTRACT

Cancer in bone is frequently a result of metastases from distant sites, particularly from the breast, lung, and prostate. Pain is a common and often severe pathological feature of cancers in bone, and is a significant impediment to the maintenance of quality of life of patients living with bone metastases. Cancer cell lines have been demonstrated to release significant amounts of the neurotransmitter and cell-signalling molecule L-glutamate via the system x_C^- cystine/glutamate antiporter. We have developed a novel mouse model of breast cancer bone metastases to investigate the impact of inhibiting cancer cell glutamate transporters on nociceptive behaviour. Immunodeficient mice were inoculated intrafemorally with the human breast adenocarcinoma cell line MDA-MB-231, then treated 14 days later via mini-osmotic pumps inserted intraperitoneally with sulfasalazine, (S)-4-carboxyphenylglycine, or vehicle. Both sulfasalazine and (S)-4-carboxyphenylglycine attenuated in vitro cancer cell glutamate release in a dose-dependent manner via the system x_C^- transporter. Animals treated with sulfasalazine displayed reduced nociceptive behaviours and an extended time until the onset of behavioural evidence of pain. Animals treated with a lower dose of (S)-4-carboxyphenylglycine did not display this reduction in nociceptive behaviour. These results suggest that a reduction in glutamate secretion from cancers in bone with the system x_C^- inhibitor sulfasalazine may provide some benefit for treating the often severe and intractable pain associated with bone metastases.

1. Introduction

Cancer pain is reported to be experienced by 75%–90% of late-stage metastatic cancer patients [59]. The most common type of cancer pain is cancer-induced bone pain (CIBP), which is often debilitating and intractable and is a major factor in reduced quality of life and functional status in cancer patients [16,43]. Current analgesic management practices for CIBP often come at the cost of impairing the patient's quality of life through severe dose-limiting side effects [2,7,45,76]. Despite increases in incidence and prevalence, improved diagnosis and treatment have allowed an increase in breast cancer patient survival in recent years [22,24,25]. This finding supports investigation into treatments that focus on symptom management while preserving quality of life for patients living with breast cancer.

Cancers in the bone occur more frequently as the result of a metastasis than as a primary cancer of bone, and these metastases are often a product of cancers of the lung, prostate, kidney, thyroid, and most commonly, the breast [16]. Through animal models, CIBP has been revealed to be a pain state distinct from neuropathic and inflammatory pain both peripherally [4,73] and centrally [33,60,75]. There are multiple potential mechanisms for the generation and maintenance of CIBP, including direct effects of the tumour cells as well as the results of disruption of host bone cell processes. CIBP in animal models has yet to be successfully alleviated by individually targeting inflammation [19], acidosis [27], and neurotrophin-induced neurogenesis [4,36,40].

It has recently been demonstrated that cancer cells of types that readily metastasize to bone secrete the signalling molecule glutamate through the cystine/glutamate antiporter called system x_C^- [61,64]. Exogenous glutamate is a neurotransmitter capable of directly initiating a pain response in peripheral tissues [10,11], and a mediator of painful inflammation [50]. Furthermore, glutamate can directly activate and sensitize primary afferent nociceptor activity in musculoskeletal tissues [9]. Increased extracellular glutamate is also a prominent feature of several painful human conditions including chronic myalgia [57], arthritis [42],

* Corresponding author. Address: Department of Pathology & Molecular Medicine, McMaster University, 1280 Main Street West, Hamilton, ON L8N 3Z5, Canada. Tel.: +1 905 525 9140x28144.
E-mail address: singhg@mcmaster.ca (G. Singh).
URL: http://www.singhlab.ca (G. Singh).

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and herniated intervertebral discs [31]. Glutamate is recognized as a critical intercellular signalling molecule in bone and is used extensively by osteoblasts, osteoclasts, and osteocytes for their normal functions [62,66,69]. Disruption of glutamatergic signalling in bone cells has been shown to influence cell differentiation [44,52] and functions [35,62,70], although a definitive model of these interactions has yet to be produced. Disrupted bone cell metabolism and signalling is a feature of bone metastases, often resulting in the development of pathological and painful alterations of bone resorption and apposition [51].

We hypothesize that pharmacological inhibition of the system x_C^- transporter will reduce the amount of glutamate released from cancer cells and that this effect will result in a decrease in pain-related behaviours shown by mice with developing bone tumours. To investigate the role of the system x_C^- transporter in nociception, a mouse model of metastatic breast CIBP will be used to monitor the progression of nociceptive behaviours during bone tumour development. After bone tumours have been established, the animals will be treated with the system x_C^- inhibitors sulfasalazine (SSZ) or (s)-4-carboxyphenylglycine [(S)-4-CPG] [15,65].

2. Materials and methods

2.1. Cell culture

Mycoplasma free MDA-MB-231 human breast adenocarcinoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained at sub-confluent densities in a humidified incubator with 5% CO_2 in room air at 37°C using Dulbecco's Modified Eagle Medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin sodium and 100 μg/mL streptomycin sulfate) (Invitrogen). For all glutamate release experiments, 10,000 cells per well were plated in their normal growth medium in 96-well plates and incubated for 4 hours to allow cell adherence. The medium was aspirated and replaced with glutamate-free Dulbecco's Modified Eagle Medium without FBS or antibiotics for the duration of the 48-hour experiment to control for the variable amount of glutamate found in FBS and the potential influence of β-lactam antibiotics on glutamate transporter expression [58].

2.2. Treatments

Both system x_C^- inhibitors SSZ (Sigma-Aldrich, St. Louis, MO, USA) and (S)-4-CPG (Tocris Bioscience, Minneapolis, MN, USA) were prepared in accordance with manufacturer's recommendations in 1 M NH_4OH and 1 mM NaOH, respectively.

2.3. Cell growth and glutamate measurements

For all in vitro experiments, SSZ and (S)-4-CPG were added directly to culture media at the desired concentrations. Glutamate release was quantified in the growth media with the AMPLEX Red glutamic acid assay kit (Invitrogen/Molecular Probes, Eugene, OR, USA) and analyzed on a CytoFluor Series 4000 Fluorescence Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA, USA). The AMPLEX Red glutamic acid assay kit quantifies L-glutamate via the measurement of a fluorescent reaction product, resorufin, which is produced proportionately to the amount of glutamate in the test sample. In order to optimize the assay for measurement of glutamate concentrations above 0.5 μM, L-alanine and L-glutamate pyruvate transaminase were omitted from the reaction mixture, thereby eliminating the repeated cycling of glutamate and α-ketoglutarate [61]. Cell numbers were quantified in 96-well plates using a crystal violet assay that measures absorbance at λ = 570 nm with an optical plate reader (BioTek, Winooski, VT, USA).

2.4. Mice

Female athymic Balb/c nu/nu homozygous nude mice (Charles River, St. Constant, QC, Canada) at 4–6 weeks of age were used. The mice were sterile housed and maintained at 24°C with a 12-hour light/dark cycle and were provided autoclaved food and water ad libitum. All procedures were conducted according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [77], and guidelines established by the Canadian Council on Animal Care with ethical approval from the McMaster University Animal Research Ethics Board.

2.5. Behavioural analyses

All animals were acclimated to the behavioural testing room and equipment 1 week prior to commencing data recording and were tested 3 times per week for the duration of the experiment. Any experimental manipulations of the animals (e.g. pellet/pump insertion or cancer cell injection) that coincided with a testing day were performed after completion of the behavioural tests. To establish a stable baseline for the animal's normal behaviour, 5 behavioural tests were performed prior to experiment day 0. Results are expressed for each animal as a percentage of these baseline scores.

Two tests for nociceptive behaviours were used: the Dynamic Weight Bearing system (DWB; BioSeb, Vitrolles, France), a test for spontaneous pain-related discomfort and postural equilibrium, and the Dynamic Plantar Aesthesiometer (DPA; Ugo Basile, Comerio, Italy), a measure of mechanical allodynia and hyperalgesia. The DWB is related to an incapacitance test in that it measures postural disequilibrium [56,71], although it also records weight and time distribution of all points of body contact with a floor sensor pad and uses freely moving animals. To reduce experimenter bias, a video camera simultaneously recorded the animal's position in the chamber to provide user-independent spatial and temporal references for the weight recordings. The sensor pad in the testing chamber was connected to a computer for direct capture of the data at a sampling rate of 10 Hz, and the animal position on the sensor was manually validated using the associated software (DWB v.1.3.4.36). Postural disequilibrium using the DWB was defined as a favouring of the tumour-bearing limb and the resultant shift of weight-bearing to other body parts and was considered as indirect evidence of nociception. The DPA test, a semi-automated version of the classical von Frey test [49], was used to assess mechanical allodynia and hyperalgesia. A normally nonpainful mechanical stimulus was presented individually to the plantar surface of the hind paws and the threshold force and time at which the paw was withdrawn were recorded on a computer. The tactile stimulus was provided by a metal filament raised by an electrical actuator with variable force and acceleration.

On each testing day, animals were placed in the DPA testing apparatus for an acclimation period of at least 5 minutes prior to stimulation. DPA tests were performed 6 times for each hind paw of all animals on every testing day. Animals were then placed in the DWB testing chamber for 7 minutes – the first 2 minutes constituted an unrecorded acclimation period, and the final 5 minutes were recorded and saved for later analysis.

2.6. Tumour cell xenografts

The MDA-MB-231 human breast adenocarcinoma cell line reliably produces osteolytic lesions when grown in bone and was found to have the highest rate of glutamate secretion of a panel of cancer cell lines. This glutamate is released primarily through the system x_C^- transporter [64]. MDA-MB-231 cells also retain