Int. J. Radiation Oncology Biol. Phys., Vol. 61, No. 5, pp. 1523–1534, 2005

0360-3016/05/$–see front matter

doi:10.1016/j.ijrobp.2004.12.070

BIOLOGY CONTRIBUTION

ALTERATION OF CANCER PAIN–RELATED SIGNALS BY RADIATION: PROTEOMIC ANALYSIS IN AN ANIMAL MODEL WITH CANCER BONE INVASION

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Purpose: Although radiotherapy is highly effective in relieving bone pain due to cancer invasion, its mechanism remains unclear. The aim of this study was to explore this mechanism in an animal model system.

Methods and Materials: A hind paw model of cancer pain was developed by transplanting a murine hepatocarcinoma, HCa-1, into the periosteal membrane of the foot dorsum of C3H/HeJ mice. Bone invasion from HCa-1 was histopathologically confirmed from sequential tumor sampling. For three experimental groups, a control (N), tumor without radiation (T), and tumor with radiation (TR), the development and level of pain were objectively examined in mice with a growing tumor by assessing pain-associated behavior. The differential expression of pain-related signals in the spinal cord was analyzed by proteomic analysis using high-resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry, and those of proteins by Western blotting. The pain-mediating neurotransmitters in the spinal cord were also examined by immunohistochemical staining for calcitonin gene–related peptide (CGRP) and substance P.

Results: In the histopathologic examinations, bone invasion from HCa-1 was seen from Day 7 and was evident at Day 14 after transplantation, and measurable pain-associated behaviors were developed from Day 7. After 25 Gy of radiation to the tumors, the objective level of pain in the TR group decreased, with higher thresholds to mechanical and thermal stimulation than in the T group. From the 2-DE of spinal cord, 107 spots were identified; 12 proteins were changed more than fivefold because of tumor formation but then reversed after radiation in the tumor-bearing mice. The proteins involved included secretagogin, syntenin, P2X purinoreceptor 6 (P2X6), and Ca^{2+}/Calmodulin-dependent protein kinase 1 (CaM kinase 1), the functions of which have been known to be involved in the Ca^{2+}-signaling cascade, ATP-mediated fast synaptic transmission, or control of vesicular trafficking. Validations using Western blotting were successful for the CaM kinase and P2X6. In immunohistochemical staining of the spinal cord, a significant decrease after irradiation was shown in the expression of CGRP, but not in substance P.

Conclusions: We developed a novel model for bone pain due to cancer invasion, which was confirmed by histopathologic examination and measurable pain-associated behaviors. Radiotherapy decreased the objective level of pain. The underlying mechanism seems to be related to the Ca^{2+}-signaling cascade or control of vesicular trafficking. © 2005 Elsevier Inc.

Radiotherapy, Nociceptor, Spinal cord, Proteome, Ca^{2+}/Calmodulin-dependent protein kinase.

INTRODUCTION

Bone pain caused by the invasion of cancer cells is the most serious, highly debilitating, and difficult to manage symptom of cancer (1–3). Radiotherapy is highly effective in relieving bone pain due to cancer invasion and can relieve the pain in 90% of patients, regardless of tumor response to radiation (2, 4). For example, patients with bone invasion from a hepatocellular carcinoma, a relatively radioresistant tumor, have presented substantial relief of pain after radiotherapy (5, 6).

It is speculated that radiation-induced analgesia is related to specific mechanisms other than tumor regression (5). However, because of the lack of an appropriate animal model system, the mechanism of radiation-induced analgesia remains to be well documented (2, 7).

In this study, a hind paw model of cancer pain was developed in C3H/HeJ mice. With this model, the mecha-

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Presented at the 90th scientific assembly and annual meeting of the Radiological Society of North America, Chicago, IL, 2004.

This work was supported by a grant from the South Korean Ministry of Health and Welfare (01-PJ8-PG1–01CN01–0034).

Received Oct 13, 2004, and in revised form Dec 19, 2004. Accepted for publication Dec 21, 2004.

nism of radiation-induced pain relief was investigated with a proteomic analysis that allowed for the analyses of a repertoire of regulating molecules (8).

METHODS AND MATERIALS

Animal model

Male 8–10-week-old C3H/HeJ mice were used to develop the animal model. The mice were maintained at the specific-pathogen–free colony of the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. The temperature and humidity were maintained at 22°C and 55%, respectively, with water and diet supplied *ad libitum*. The care and use of mice in this study were based on "Guidelines and Regulations for Use and Care of Animals at the Yonsei University".

Syngeneic murine hepatocarcinoma cells, HCa-1, were used with 5×10^5 cells injected into the periosteal membrane of the hind foot dorsum of the experimental mice. The mice were regularly examined for tumor formation and growth.

Mice were killed at regular intervals after the tumor cell injections. Tumor-bearing hind paws were fixed overnight in 4% zinc-buffered formalin in 0.1 M PBS at 4°C, decalcified in 10% EDTA (pH 7.4) for 2 weeks, and then embedded in paraffin. The paraffin blocks were sectioned in 7-μm thicknesses, stained with hematoxylin and eosin, and examined under light microscopy for invasion of cancer cells to the bone.

Analysis of the behavioral responses

To monitor the development of pain resulting from the invasion of cancer cells to the bone, the behavioral responses were measured at 1, 3, 5, 7, 14, 21, and 28 days after the tumor cell injection. Fifteen mice were used in each group. The behavioral responses were analyzed by assessing the threshold of limb withdrawal or nocifencive behavior in response to graded mechanical, radiant heat, or cold thermal stimuli. All the procedures were performed using a clear plastic observation chamber, with the mice kept in the chamber for at least 15 min to become habituated to the experimental conditions (9).

The sensitivity to mechanical stimulation was determined by measuring the limb withdrawal threshold in response to the von Frey monofilament probing of the plantar surface of the ipsilateral hind paw, which was determined by assessing the 50% withdrawal in response to 10 successive stimulations (0.2, 0.4, 0.6, 0.8, 1, 2 mN of bending force) at the 10 s interval.

To assess the sensitivity to cold thermal stimuli, a drop of acetone (80%) was delivered to the plantar surface of the ipsilateral hind paw, which was repeated 5 times with 5-min intervals, and the withdrawal frequency was assessed.

Hyperalgesia to radiant heat was measured by the time to withdrawal (seconds) after the application of radiant heat stimulation with a halogen lamp. To prevent injury from the radiant heat, the stimulation was discontinued if the mice did not respond within 1 min of the stimulation.

Radiotherapy

The experimental mice were divided into 3 groups: control mice without tumor transplant (group N), mice with a tumor but without radiation (group T), and mice with a tumor and administered radiation (group TR). Radiation was given to the tumor-bearing mice 15 days after tumor transplantation. Mice were immobilized in an acryl jig, and a single dose of 25 Gy was delivered only to the tumor-bearing area using a collimator system with 4 MV X-rays.

2-DE and image analysis

To determine the pain-related signals in the spinal cord, mice were killed 7 days after radiotherapy. The L1-L5 spinal cord segments were removed, and the tissues suspended in sample buffer containing 40 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS (Bio-Rad, Hercules, CA), 100 mM 1,4-dithioerythritol (DTT; Sigma, St. Louis, MO), 0.2% (v/v) Bio-Lytes (Bio-Rad) and endonuclease (Sigma). Suspensions were sonicated for approximately 30 s, and then centrifuged for 1 h at 100,000 g to remove DNA, RNA and any particulate materials. The supernatants contained the total spinal cord proteins solubilized in sample buffer. The protein concentrations in the supernatants were determined using the Bio-Rad assay system (Bio-Rad), according to the manufacturer's guidelines. All samples were stored at −70°C until used.

Two-dimensional gel electrophoresis was performed in a Bio-Rad Electrophoresis system with 1 mg of total spinal cord protein used for each electrophoretic run (10). Aliquots of spinal cord proteins in sample buffer were applied to immobilized pH 3–10 nonlinear gradient (IPG) strips (Bio-Rad). The first dimensional isoelectric focusing (IEF) was performed at 100,000 Vh after which the strips were equilibrated for 10 min in the equilibration buffer containing 6 M urea, 2.5% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) DTT, 5 mM tributylphosphine, 50 mM Tris-HCl (pH 6.8) and 20% (v/v) glycerol (Sigma). The second dimensions were analyzed on 9%–18% linear gradient polyacrylamide gels using the Protean XL system (Bio-Rad) at 20°C. Immediately after electrophoresis, the gels were fixed in 40% methanol and 5% phosphoric acid, and then stained with Coomassie blue G 250 (Bio-Rad) for 24 h.

The stained gels were scanned using a GS-800 calibrated densitometer (Bio-Rad) and the digitized gel images normalized and comparatively analyzed using the PDQUEST program (v6.2, Bio-Rad). The percentage spot volume representing a certain protein was determined in comparison with the protein present in the 2-dimensional gels.

Protein identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

For mass spectrometry fingerprinting, protein spots were directly cut out of the gels, destained with 50% acetonitrile in 25 mM ammonium bicarbonate and dried in a speed vacuum concentrator (Savant, Pleasanton, CA). Dried gel pieces were reswollen with 50 mM ammonium bicarbonate (pH 8.0) containing 100 ng/μL trypsin (Promega, Lyon, France) and incubated at 37°C for 17 h. Supernant peptide mixtures were extracted into 50% acetonitrile, with 5% trifluoroacetic acid (TFA) and dried in a speed vacuum concentrator. The peptide mixtures were then dissolved in 4 μL of 50% acetonitrile and 0.1% TFA. Aliquots of 0.5 μL were applied to a large disk and allowed to air-dry. A matrix of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, containing 0.1% TFA was used to obtain the spectra using a MALDI-TOF mass spectrometer (Micromass, Manchester, UK).

A protein database search was performed using MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm), employing the average molecular weight of monoisotropic peptide ions and the value