Analytical Biochemistry 486 (2015) 70–74

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

An improved intrafemoral injection with minimized leakage as an orthotopic mouse model of osteosarcoma

Hiromi Sasaki [a], Swathi V. Iyer [a], Ken Sasaki [a], Ossama W. Tawfik [b], Tomoo Iwakuma [a,*]

[a] Department of Cancer Biology, The University of Kansas Medical Center, Kansas City, KS 66160, USA
[b] Department of Pathology, The University of Kansas Medical Center, Kansas City, KS 66160, USA

ARTICLE INFO

Article history:
Received 22 April 2015
Received in revised form 12 June 2015
Accepted 23 June 2015
Available online 30 June 2015

Keywords:
Osteosarcoma
Orthotopic
Intrafemoral
Mouse model
Leak
Metastasis

ABSTRACT

Osteosarcoma, the most common type of primary bone cancer, is the second highest cause of cancer-related death in pediatric patients. To understand the mechanisms behind osteosarcoma progression and to discover novel therapeutic strategies for this disease, a reliable and appropriate mouse model is essential. For this purpose, osteosarcoma cells need to be injected into the bone marrow. Previously, the intratibial and intrafemoral injection methods were reported; however, the major drawback of these methods is the potential leakage of tumor cells from the injection site during or after these procedures. To overcome this, we have established an improved method to minimize leakage in an orthotopic mouse model of osteosarcoma. By taking advantage of the anatomical benefits of the femur with less bowing and larger medullary cavity than those of the tibia, osteosarcoma cells are injected directly into the femoral cavity following reaming of its intramedullary space. To prevent potential leakage of tumor cells during and after the surgery, the injection site is sealed with bone wax. This method requires a minor surgery of approximately 15 min under anesthesia. Our established orthotopic osteosarcoma model could serve as a valuable and reliable tool for examining progression of various types of bone tumors.

Introduction

Osteosarcoma is the most common type of bone cancer in children and young adults. It generally occurs in the long bones, and 60% of the cases localize around the knee (40% in the distal femur and 20% in the proximal tibia). At the time of diagnosis, 15 to 20% of patients with osteosarcoma have clinically detectable metastases. More important, nearly all patients have pulmonary micrometastasis, making chemotherapy the first choice of treatment. Due to intensive neoadjuvant and adjuvant chemotherapy, the 5-year survival rate for osteosarcoma has improved to 50 to 80%; however, for metastatic osteosarcoma, it remains at 15 to 40% and has not changed over the past three decades [1–4]. This is mainly due to the poor understanding of mechanisms underlying the malignant properties, such as high tumor-initiating potential, metastasis, and drug resistance, in high-grade osteosarcoma.

To understand the mechanisms governing malignant properties of bone tumors and to develop novel therapeutic approaches, there is a need for mouse models that recapitulate the entire physiological process of cancer progression. Osteosarcoma originates from mesenchymal stem cells or their progenitor cells that reside in the bone marrow. Hence, it is ideal to transplant osteosarcoma cells into the bone marrow space. Such orthotopic transplantation of tumor cells allows them to grow in a proper microenvironment and, thereby, is most suited for testing the abilities of osteosarcoma cells to initiate tumor formation and metastasize to other organs. Toward this goal, the intratibial injection method was previously established [5]. Although it is simple and was used in several publications [6–8], it has several technical pitfalls. The major pitfall is leakage of cells from the injection site. This is because the tibia does not have sufficient space in the medullary cavity and the percutaneous injection method does not allow verifying the absence of leakage. Moreover, reaming the intramedullary space of the tibia is not done, likely due to the curved anatomical feature of this bone. Although the intrafemoral injection method also showed successful tumor establishment in the femur [9,10], no publication described details of the procedures to minimize the leakage. Leakage from the bone marrow cavity could alter the onset of tumor formation and result in experimental variation or inconsistency. Leakage can be mainly caused by confined intramedullary space and increased intramedullary pressure after tumor cell injections.

* Corresponding author. Fax: +1 913 588 4701.
E-mail address: tiwakuma@kumc.edu (T. Iwakuma).

http://dx.doi.org/10.1016/j.ab.2015.06.030
0003-2697/

To overcome these concerns and improve the orthotopic tumor model of osteosarcoma, we have established a novel method for orthotopic injections. We inject tumor cells into the femur because mouse femur is relatively straight with mild bowing and has a larger medullary cavity than tibia. Tumor cell suspension is injected into the femoral cavity from a hole generated at the intercondylar notch after reaming the intramedullary space. Afterward, the injection hole is sealed with bone wax. These procedures not only allow investigators to directly observe tumor cell suspension into the femur but also minimize potential leakage from the injection site during and after surgery. This minor surgery takes approximately 15 min. Our method demonstrates successful primary osteosarcoma establishment in the femur and metastases to the liver and lungs. This orthotopic tumor model could be a valuable tool for research of any types of bone sarcoma such as Ewing sarcoma and pleomorphic sarcoma.

Materials and methods

Animal maintenance

Non-obese diabetic/severe combined immunodeficiency (NOD–scidIL2Rγ^{null}) mice at 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were maintained under specific pathogen-free conditions, and experimental procedures were performed according to the protocol approved by the institutional animal care and use committee.

Cell lines and cell culture

Human osteosarcoma cell lines, including SJSA-1 and KHOS/NP, were used for intrafemoral injection studies. Cells were maintained in Dulbecco's modified Eagle's medium (Corning Cellgro) supplemented with 10% heat-inactivated fetal bovine serum and 50 IU/ml penicillin–0.5 mg/ml streptomycin (MP Biochemicals) at 37 °C in an atmosphere of 5% CO_2 in air.

For the intrafemoral injection studies, osteosarcoma cells were detached using Gibco Cell Dissociation Buffer (Life Technologies) and counted following trypan blue staining. Live cells (up to 1,000,000) were suspended in 15 µl of 4.5 mg/ml growth factor reduced Matrigel (BD Biosciences) in Hank's balanced salt solution (HBSS)[1] to allow cell suspension to solidify and stay in the femoral cavity, which helps to minimize the leakage.

X-ray imaging

X-ray images were taken with a Faxitron LX-60 (Tucson, AZ, USA) using the conditions of 28 kV, 0.3 mA, and 10 s of exposure time.

H&E staining

Tissues were fixed in 10% buffered formalin overnight and transferred to 70% ethanol. Tissues were then paraffin-embedded, sectioned (5 µm), and used for standard hematoxylin and eosin (H&E) staining [11].

[1] *Abbreviations used:* HBSS, Hank's balanced salt solution; H&E, hematoxylin and eosin; PDTX, patient-derived tumor xenografts.

Results

Description of intrafemoral injection procedure

All instruments were sterilized by autoclaving before surgery. Mice were anesthetized with 1 to 5% isoflurane. The hair around the knee joint of one leg was removed, and skin was then scrubbed with iodophor and 70% ethanol. The knee of the mouse was flexed beyond 90°, and a longitudinal skin incision was made across the front of the left knee (Fig. 1A). The patellar tendon was transected near the tendon attachment site of the tibia and reversed to the proximal side to expose condyles of the distal femur (femoral articular surface, Fig. 1B). The vastus lateralis muscle was separated by a scalpel to expose distal shaft of the femur. In this way, the surface of the femoral articular was exposed to make a hole for tumor cell injection. Using an electric drill (Fine Science Tools, Foster City, CA, USA), a small hole was made to reach the bone marrow space (Fig. 1C and D). Then, a small stick was inserted through the hole and was turned around to ream the bone marrow space (Fig. 1E). This procedure helps to reduce intramedullary pressure and retain sufficient space for tumor cell injections. To stop the bleeding, small gauzes were inserted into the bone marrow space. After confirming that bleeding had ceased, 15 µl of cell suspension in Matrigel in HBSS was injected into the intrafemoral space using a 27-gauge needle with a tuberculin syringe. Cell numbers can range from as few as 100 to as many as 1,000,000, depending on the experiments. Following tumor cell injection into the medullary space of the femur, the hole was immediately sealed with bone wax (World Precision Instrument, Sarasota, FL, USA) to prevent the potential leakage of cell suspension (Fig. 1F). Then, the patellar tendon was sutured near the tendon attachment site of the tibia (Fig. 1G). In case the suturing to the tendon attachment site was not feasible, the patellar tendon was sutured to the tibialis anterior muscle. This procedure did not cause any dysfunction of the mouse leg. Finally, the skin was closed using 5-0 Surgilon (Fig. 1H) and wiped with iodophor and 70% ethanol. The total operation time per one procedure was approximately 15 min.

Outcome of intrafemoral injections of osteosarcoma cells

To demonstrate tumor formation, X-ray images were taken 1 month after KHOS/N P cell injections (1,000,000 cells), showing a soft tissue mass and bone destruction with periosteal reaction and production of an osteoid matrix (Fig. 2A and B). Importantly, the distal end of the femur was nearly intact, indicating that the tumor had arisen from inside the bone but not from leakage. In addition, histopathological specimen sections were made. H&E stains revealed mitotically active spindle-shaped cells, highly pleomorphic malignant cells, osteoid formation with eosinophilic staining, and invading malignant cells into bone cortex (Fig. 3A and B). We also detected metastatic nodules to the liver and lungs (Fig. 3C).

Another set of experiment was also performed using SJSA-1 osteosarcoma cells. Approximately 2 or 3 months after tumor cell injection (100,000 cells), we detected a primary tumor formation in the femur (Fig. 4A) as well as metastases to the lungs (Fig. 4B) showing malignant mesenchymal cells in the lungs in H&E staining (Fig. 4C).

Discussion

Xenograft mouse models of cancer are crucial for elucidating physiological functions of human proteins in cancer and developing novel cancer therapies. During the past few decades, various xenograft animal models have been established, including ectopic