Evaluation of a Preclinical Model of Bone Metastasis for the Study of Adoptive Immunotherapy

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Abstract
The development of new therapeutic strategies for the treatment of bone metastases strongly depends on the availability of valid animal models. In this paper, we evaluate a preclinical model of bone metastases using a technique of tumor cell injection into the left heart ventricle of mice to study the efficacy of adoptive immunotherapy. Using flow cytometric analysis and histopathological and radiological examination, we investigated whether this experimental model of bony metastases using two murine cell lines of melanoma and breast cancer would be suitable for the study of adoptive immunotherapy for these diseases. We further report that anti-CD3-activated and IL-2-expanded tumor vaccine draining lymph node cells cause regression of tumor metastases, including bone metastases, following adoptive transfer to mice bearing 3-day metastases from the D5 melanoma cell line. These promising early results lead us to conclude the following: (1) this model of experimental bone metastases is suitable for the study of immunotherapy, and (2) further studies are warranted to extend these promising early findings of the therapeutic effects of adoptive immunotherapy in this animal model.

Introduction
Any malignant neoplasm has the capacity to metastasize to the musculoskeletal system, although some do so more frequently than others. The incidence of bone metastases in breast cancer patients is estimated to be 65–75% [1]. Even in melanoma, the skeleton is a target organ for distant metastases in 14–45% of the patients [1]. The clinical consequences are devastating: pathologic fractures, spinal cord/nerve root compression, hypercalcemia, and severe pain. At present, bisphosphonates, chemotherapy, local radiation, palliative surgery, and analgesics are considered to provide supportive benefit [2]. However, no life-prolonging or curative treatment is known. Despite the availability of various animal models of bone metastases [3–8], many questions about the metastatic process to bone and potential strategies to eradicate them remain unanswered. Thus far, no attempts have been made to
explore the potential of adoptive immunotherapy specifically for the treatment of metastases to bone. Adoptive transfer of effector T lymphocytes has, however, demonstrated therapeutic efficacy in different animal tumor models [9–11]. It remains unknown if a preclinical model of bone metastasis exists that is suitable for the study of adoptive immunotherapy using effector T cells. In this study, we address this question, also demonstrating a first set of experiments. We anticipated that adoptively transferred effector T cells would be able to migrate to bone/bone marrow after intravenous application and be capable to exhibit their antitumor activity in the unique microenvironment of the bone.

**Materials and Methods**

**Mice and Tumor Cell Lines**

Female C57BL/6J (B6) and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Me., USA) and Charles River Laboratories (Wilmington, Mass., USA), respectively. Recognized principles of laboratory animal care were followed [Guide for the Care and Use of Laboratory Animals, National Research Council; Washington, National Academy Press, 1996], and all animal protocols were approved by the Earle A. Chiles Research Institute animal care and use committee.

D5 is a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma. D5-G6 is a stable clone of D5 that was originally transduced with a murine GM-CSF retroviral MFG vector [12]. D5-G6 cells secrete ~ 200 ng GM-CSF/ml/10⁶ cells/24 h. D5-eGFP describes the D5 cell line after retroviral transduction with cDNA for enhanced green fluorescent protein (eGFP), a noninvasive marker widely used as a reporter molecule for gene expression, protein localization, intracellular protein targeting, and cell trafficking. This cell line, which was recently generated in our laboratory, exhibits similar tumorigenicity when compared to the original D5 population. 4T1 is a subline of breast cancer cells, derived from a spontaneous carcinoma in a BALB/cF3H mouse [13]. To determine whether a subpopulation of 4T1 that selectively metastasizes to bone could be selected, the bone marrow cavities of femurs from tumor-bearing mice were flushed and the resulting cell suspension cultured and cultured for 5 days. The cells were then reinjected into the left heart ventricle of mice, and the described procedure was repeated. The final cell population was named 4T1-P2.

All tumor cells were cultured as described previously [14]. In brief, we used complete medium which consisted of RPMI 1640 (BioWhittaker, Walkersville, Md., USA) supplemented with β-ME (Aldrich, Milwaukee, Wisc., USA), 10% fetal bovine serum (Life Technologies, Grand Island, N.Y., USA), NEAA, sodium pyruvate, and L-glutamine. Cell lines were maintained in T-75 or T-150 culture flasks in a 5% CO₂ incubator at 37°C.

**Systemic Intravascular Tumor Cell Injection (SIA)**

To reproduce the reproductibility of metastases to the skeleton, we used a modification of the technique first described by Arguello et al. [4] in 1988. After brief trypsinization in the culture flasks, 10⁴ tumor cells were washed and resuspended in 0.1 ml Hanks’ balanced salt solution (BioWhittaker). The animals were anesthetized with pentobarbital (Nembutal) 0.05 mg/g i.p. Without shaving, the left anterior chest wall was disinfected with 70% alcohol. Tumor cells were inoculated into the left cardiac ventricle of the animal. For this, a tuberculin syringe with an integrated 27-gauge needle was inserted from a left-cranial direction into the second intercostal space aiming centrally. The animals were sacrificed 14–18 days after tumor cell injection, and a necropsy was performed. For the purpose of tumor cell tracking and assessment of tumor burden at the tumor site, the bone marrow cavity of the femur was flushed and the resulting cell suspension cultured. This procedure allowed for FACS analysis of eGFP-expressing cells in the cultured cell population.

**Radiographs**

The animals were anesthetized deeply, laid in prone position against the films (X-OMAT AR; Eastman Kodak, Rochester, N.Y., USA), and exposed to an X-ray at 20 kV for 22 s using a Faxitron X-Ray (Wheeling, Ill., USA) radiographic inspection unit.

**Histological Examination**

In brief, forelimbs or hind limbs from animals were fixed in 10% phosphate-buffered formalin (pH 7.2) and decalcified in 14% EDTA solution. Standard techniques were used for paraffin embedding. For detection of bone metastases, the embedded samples were cut into 5-μm sections and stained using hematoxylin and eosin.

**Flow Cytometric Analysis (FACS)**

Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, Calif., USA), and data were analyzed using CellQuest software (Becton Dickinson). No dye-conjugated antibodies had to be applied with D5 cells transfected with eGFP, as described above.

**Adoptive Immunotherapy (AIT)**

For adoptive T cell transfer, we used a standardized protocol as described earlier [10, 14]. Briefly, one million D5-G6 tumor cells were injected subcutaneously into both the hind limbs and foreflanks of wt B6 mice. Eight days following vaccination, the tumor vaccine draining superficial axillary and inguinal lymph nodes were harvested. These were resuspended and cultured in complete medium with anti-CD3 antibody 2C11 (5 μg/ml). After 2 days of activation, the T cells were harvested and expanded in complete medium containing IL-2 (60 IU/ml) for 3 additional days to generate effector T cells. Effector T cells were then harvested, washed twice in Hanks’ balanced salt solution, and used for adoptive transfer. For the first set of experiments, 100 x 10⁶ effector T cells were injected intravenously into mice 3 days after SIA of 10⁴ D5-eGFP cells. Starting on the day of T cell infusion, one group of animals received IL-2 (90,000 IU i.p.) once daily for 3 days, and another group underwent AIT without injection of IL-2. One of two control groups was injected with IL-2. Each of the four groups consisted of at least 5 mice. The animals were sacrificed 16–17 days later, and a necropsy was performed on each animal.

To confirm that skeletal metastases were present 3 days following SIA of tumor cells, another group of animals was sacrificed on day 3 after tumor cell inoculation. The long bones were flushed, and the resulting cell suspension was cultured as described above.
Results

Distribution of Organ Metastases following SIA

Using SIA, we found a mortality directly associated with the procedure of <5%. After 17 days, SIA of $10^4$ D5-eGFP cells resulted in melanotic colonization of ovaries and adrenal glands in all animals (Table 1). Metastases to other organs, particularly the lungs, liver, or mesentery, turned out to be a less frequent event. All animals developed macroscopically visible melanotic nodules in the skeletal system, principally to distal femur and proximal tibia. When long bones were flushed 3 days after SIA of tumor cells and the resulting cell suspension cultured for 10 days, D5-eGFP cells were detectable using FACS analysis.

Following intracardiac injection of $10^4$ 4T1 murine mammary cancer cells, metastatic colonization of the bone was also observed; however, only 70% of the animals had bone metastases detectable either by radiological examination or by direct culture of cells from the bone marrow. To determine whether a subpopulation of 4T1 cells that selectively metastasized to bone could be selected, bone marrow from the femur was flushed and the resulting cells cultured for 8 days. This cell population, called 4T1-P2, produced skeletal metastases in 100% of the animals following SIA.

Radiological Examination

There were no obvious radiological signs of bone metastases following SIA with $10^4$ D5-eGFP cells. Although osteolytic lesions, with or without adjacent soft-tissue masses, are observed in patients with skeletal metastases from a malignant melanoma, no areas of bone resorption or reactive osteoblastic activity were seen in mice (Fig. 1A). In contrast, SIA of the same number of cells of the mammary carcinoma cell line 4T1-P2 resulted in osteolytic bone metastases, especially to the proximal humerus, the distal femur, and the proximal tibia (Fig. 1B).

Histological Examination

Histological examination, performed 17 days following SIA of $10^4$ D5-eGFP cells, revealed that the bone marrow cavity of long bones was almost completely replaced by tumor cells (Fig. 2A). This pattern of tumor growth may also explain the absence of radiological findings in this group of mice. Seventeen days after SIA of 4T1-P2 ($10^4$ cells), the bone marrow cavity of long bones was also

Table 1. Distribution of organ metastases following intracardiac tumor cell inoculation

| Percentage of mice with tumor colonies (n = 10) |
|-----------------|-------|-----|
|                 | D5-eGFP | 4T1 | 4T1-P2 |
| Bone/bone marrow | 100    | 70  | 100    |
| Ovaries         | 100    | 100 | 100    |
| Adrenal glands  | 100    | 100 | 100    |
| Lungs           | 30     | 50  | 40     |
| Liver           | 10     | 20  | 20     |
| Spleen          | 0      | 20  | 20     |
| Kidney          | 0      | 70  | 60     |
| Others1         | 100    | 100 | 100    |

1 Peritoneum, myocardium, mesentery, thymus, lymph nodes.
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Fig. 2. Histological examination 17 days following SIA of 10^4 tumor cells. ×19. HE. A D5-eGFP control. The transverse section through the distal femur shows the bone marrow cavity almost completely occupied by tumor cells. B D5-eGFP with AIT (100 × 10^6 T cells) plus systemic IL-2 treatment (90,000 IU i.p. for 3 days). No tumor cells are detectable, and no signs of bone destruction/resorption are visible. Note the normal-appearing bone marrow.

Fig. 3. FACS analysis of bone marrow cell suspension 3 days following SIA of 10^4 D5-eGFP tumor cells. A D5 control. B D5-eGFP control. C Cell suspension from the bone marrow cavity of long bones cultured for 10 days. Note the clearly detectable cell population expressing eGFP, indicating the presence of melanoma cells in the bone/bone marrow at 3 days after SIA.

almost entirely occupied by tumor cells. However, strong osteoclastic activity, consistent with active bone resorption, was evident along the endosteal surface. The quality of all slides would have allowed for quantitative assessment of tumor burden using histomorphometric analysis as described by others [15].

Flow Cytometry (FACS)

After transduction with a retrovirus containing the cDNA encoding eGFP, 99% of D5 melanoma cells expressed green fluorescent protein. Seventeen days after SIA of D5-eGFP, the bone marrow cavity of long bones was flushed and the resulting cell suspension washed and cultured for 6 days. When analyzed by flow cytometry, this cell population exhibited identical high levels of expression for eGFP, just as it did before injection, indicating that there had been no loss of green fluorescent protein expression during the metastatic process in vivo. Like histomorphometric analysis, flow cytometry would allow for accurate assessment of tumor at the level of the bone. Three days after SIA, FACS analysis detected D5-eGFP cells in the cell suspension cultured from the marrow cavity, indicating the onset of skeletal metastases at this time point. Figure 3 illustrates the high percentage of eGFP-positive cells, i.e., D5 melanoma cells, after culturing the cell suspension from the bone marrow cavity for 10 days.
AIT for 3-Day Bone Metastases

Three-day-tumor-bearing control mice that received saline colonization or IL-2 therapy only developed clearly visible colonization of the skeleton, primarily the distal femur and the proximal tibia, with melanotic nodules. In addition, SIA of D5-eGFP cells resulted in metastatic disease to ovaries and adrenal glands. Individual animals had metastases to other organs (table 1). In contrast, the adoptive transfer of $10^6$ effector T cells clearly mediated tumor regression whether or not systemic IL-2 treatment was administered. Macroscopically, no tumor nodules were detectable on any part of the skeleton. In addition, metastases to other organs were either completely abolished, or their growth was effectively suppressed. The absence of metastatic disease to the skeleton in mice following AIT was also confirmed by histological examination (fig. 2B). Clinical observation of the treated animals also confirmed the efficacy of adoptive T cell transfer. None of the mice that received AIT showed signs of distress, whereas control animals showed a significantly decreased motor activity. The control animals also developed early paralysis of the hind limbs, known to be the result of tumor metastases to thoracic and lumbar vertebral bodies [4]. In contrast, none of the mice treated with AIT showed any signs of paralysis or other disturbances in motor activity.

Discussion

SIA into the left heart ventricle represents a preclinical model that meets many of the necessary requirements to study the efficacy of AIT in animals with skeletal metastases: (1) high reproducibility of metastatic bone lesions; (2) immunocompetent mice can be used with murine tumor cell lines, and (3) FACS and histomorphometric analysis allow for accurate assessment of tumor burden at the bone site.

For the first time, in a preliminary set of experiments, we were able to demonstrate the therapeutic value of AIT in a 3-day model of metastatic disease to the skeleton. Since the prevalence of skeletal disease in humans is greatest in breast carcinoma [1], we used the murine mammary cancer cell line 4T1 and its subpopulation 4T1-P2, selected from a bone marrow metastasis. Yoneda et al. [16] suggested the use of the human estrogen-independent breast cancer cell line MDA-MB-231 in SIA technique, which reliably results in the formation of osteolytic bone lesions, to mimic the clinical situation in humans. This model, however, requires immunocompromised animals and might, therefore, not be applicable for the study of T cell trafficking, memory T cell function, and T cell effector mechanisms in metastatic disease of the skeleton. The previously established value of AIT in models using the murine melanoma cell line D5 [10, 14] and the fact that melanotic tumor deposits are easily detected in most organs led to the selection of D5 for this study.

Next to SIA, which has been studied by Arguello et al. [4, 17–19], four other routes of tumor cell administration have been used to establish skeletal metastases: local injection adjacent to a bone surface or into a marrow cavity [5, 6], intravenous application of tumor cells [8], direct injection of tumor cells into the abdominal aorta [7], and orthotopic administration, e.g., into the mammary fat pad with secondary spontaneous metastases to bone [3]. Whereas intraosseous injection fails to mimic the metastatic process, orthotopic administration resembles the clinical situation in human metastatic disease most closely. However, the formation of bone metastases takes up to 4 weeks as compared with 2 weeks using the SIA technique. When tumor colonization is detectable at the bone site, the primary tumor may have reached a considerable size ($\geq 1$ g) [3] which makes excision necessary for long-term experiments and causes animals additional distress. The simplest model, intravenous injection of tumor cells, was established with administration of a human lung cancer cell line in natural killer cell depleted SCID mice [8]. The use of congenitally immunodeficient mice to study human tumor metastases and immunotherapy may, however, not be suitable for the study of AIT with T cells.

The major disadvantages of SIA for tumor cell inoculation are its technical difficulty and the large bolus of tumor cells that is injected at one time. This is not a completely accurate reflection of the metastatic process in vivo, where only a few cells from the primary tumor site enter the systemic circulation gradually over time from the primary tumor site. However, early studies by Arguello et al. [4] showed that SIA with as few as 100 tumor cells resulted in metastatic tumor deposits in bone. Although SIA appears technically difficult, the associated lethality, which was previously reported to be 10% [17], turned out to be less than 5% in our study.

The adoptive transfer of effector T cells to animals with systemic metastases has demonstrated therapeutic efficacy in disease models as different as melanoma [10, 14] and colorectal [9] and brain [11] cancer. The metastases in these models have generally involved lung, liver, and central nervous system, respectively. To date, no attempt has been made to assess the therapeutic value of AIT in a preclinical model of bone metastases. We con-
clude from our studies that SIA represents a valuable model to assess the efficacy of AIT for experimental bone metastases. Future studies will focus on determining the optimal/minimal dose of T cells necessary to mediate tumor regression in this model. Adoptive transfer of effector T cells will be performed in the late time course following SIA, although our studies revealed colonization of the skeleton as early as 3 days following SIA. The latter observation is in good accordance with the findings by Arguello et al. [4] who reported tumor cell arrest in the first capillary bed they reach.

The role of systemic IL-2 in AIT for bone metastases will also be the subject of future experiments, especially since the efficacy of IL-2 therapy has been demonstrated in tumor models of metastases to the bone from neuroblastoma and prostate carcinoma [20, 21]. Finally, to investigate the involvement of CD4+ and CD8+ T cell subsets in the antitumor response, in vivo depletion with monoclonal antibodies will help to define the mechanisms for successful AIT of metastatic disease to the skeleton.

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