Visualization of Immunotoxin-mediated Tumor Cell Death in Vivo

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Abstract

We present a novel methodology to visualize tumor cells directly in a whole mouse. This technique combines immunohistochemistry with whole mouse sectioning. It lets one see the exact distribution of tumor cells throughout an animal and how effectively these cells are eliminated by cancer therapeutics. We used this technique to assess the efficacy of a T cell-specific immunotoxin in a severe combined immunodeficient mouse model of human T-cell leukemia. Severe combined immunodeficient mice were injected with one of two human T-cell acute lymphoblastic leukemia cell lines (Molt 3 and Molt 13) and were either left untreated or were treated with DA7, an immunotoxin specific for the T cell-associated antigen CD7. Mice were sacrificed after tumor cell injection and immunotoxin therapy, whole mouse cross-sections were prepared, and tumor cells in the sections were visualized by immunohistochemistry. No tumor cells were detected in DA7-treated mice injected with Molt 3, consistent with the long-term survival of this group and the sensitivity of Molt 3 to DA7 in vitro. In contrast, DA7 treatment did not visibly eliminate tumor cells in mice challenged with Molt 13, nor did it result in their long-term survival. Furthermore, tumor cells were detected in areas that may have otherwise been overlooked, and their distribution differed from that of mice injected with Molt 13 alone. These analyses indicate that whole mouse sectioning will be a valuable tool for assessing residual disease in the preclinical evaluation of cancer therapeutics.

Introduction

Animal models are indispensable for the preclinical evaluation of cancer therapeutics because they provide requisite toxicity, pharmacokinetic, and efficacy data (1–3). Two common readouts for therapeutic efficacy in animal models are mean survival time and measurements of residual disease. Although both furnish biologically relevant data, each has its drawbacks. Enhanced mean survival time is only a gross measurement of efficacy and does not afford information about differential tumor cell killing because of, for example, antigen modulation or effects of the local microenvironment (4). Measurements of residual disease by the PCR or organ-specific histochemistry are restricted to the tissues sampled and are therefore subject to investigator-induced biases in tissue selection. Tumor cell accumulation in discrete areas could therefore be missed and the residual tumor burden correspondingly underestimated.

To overcome these obstacles in assessing therapeutic efficacy, tumor cells can be tracked throughout the entire animal. This is achieved in humans via nuclear imaging, such as immunoscintigraphy to detect the accumulation of radiolabeled anti-tumor antibodies, and by nonradioactive methods, such as computed tomography scans (5, 6). However, many of these techniques do not adequately discriminate tumor boundaries from normal tissue and are limited in their evaluation of heterogeneous lesions. They also are either too costly or impractical for routine preclinical evaluations of cancer therapeutics in the laboratory setting. For these reasons, we developed a whole mouse sectioning technique that uses standard immunohistochemistry. Whole mouse sectioning previously has been combined with in situ hybridization to analyze viral pathology, and more recently, with fluorochrome-labeled, anti-T-cell and anti-B-cell antibodies to track antigen-specific interactions during immune responses (7). Both applications allow for the reproducible and efficient screening of all tissues in the host for the cells of interest, but neither provides a permanent record of the primary data attributable to the transient nature of the labels (e.g., radioisotope decay and fluorescence quenching, respectively). In contrast, immunohistochemical detection provides a permanent record of cell distribution that can be reanalyzed as often as necessary.

We combined whole mouse sectioning with immunohistochemistry to assess the efficacy of a CD7-specific IT4 in a SCID mouse model for human T-ALL (1). ITs are a class of targeted therapeutics designed to kill cells specifically (8). These proteins consist of potent, catalytic toxins linked to targeting moieties such as antibodies. For cancer therapy, the targeting moieties are specific for tumor-associated or differentiation antigens expressed on malignant cells. CD7 is a differentiation antigen found on T cells, natural killer cells, and on a subset of committed bone marrow myeloid (TdT+, My9+) and lymphoid (subset of CD19+) progenitors (9–11). The function of CD7 is not clear, but its expression in disease has important clinical

References

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4. The abbreviations used are: IT, immunotoxin; SCID, severe combined immunodeficient; DAB, diaminobenzidine; FBS, fetal bovine serum; IC50, concentration required to achieve 50% inhibition; T-ALL, T-cell acute lymphoblastic leukemia.
Technologies, Inc.). The cultures were grown in a humidified NY) supplemented with 10% FBS (BioWhittaker, Walkersville, MD) and Molt 13 (a kind gift from Daniel Vallera, University of Minnesota) were main-

Materials and Methods

Cells. The human CD7+ T-ALL lines Molt 3 (American Type Culture Collection, Rockville, MD) and Molt 13 (a kind gift from Daniel Vallera, University of Minnesota) were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS (BioWhittaker, Walkersville, MD) and 50 units/ml penicillin and 50 μg/ml streptomycin (Life Technologies, Inc.). The cultures were grown in a humidified incubator with 5% CO2 at 37°C. Prior to injection into SCID mice, cells were washed twice in RPMI 1640 without FBS and incubated in RPMI 1640 without FBS to yield a final concentration of 0.1, 1, 10, or 100 μl. Each sample was performed in quadruplicate. After a 48-h incubation, 20 μl of the metabolic indicator alamarBlue (AccuMed) were added to each well for an additional 18 h. A redox indicator in the alamarBlue reagent changes from an oxidized, nonfluorescent form to a reduced, fluorescent form because of a chemical reduction in the growth medium caused by the metabolic activity of viable cells. Increases in fluorescence intensity are therefore directly related to metabolic activity. Fluorometric analyses were performed in a Cytofluor II plate reader (Biosearch, Bedford, MA) using 530 and 590 nm wavelengths for excitation and emission, respectively. Data were plotted as the percentage of viability (fluorescence emissions) relative to DA7-untreated control cells.

Whole Mouse Sectioning and Immunohistochemistry. Mice were sacrificed on the designated days or at the point of morbidity by CO2 asphyxiation. Ears, legs, tail, and upper and lower incisors were removed to avoid air bubbles and blade wear during sectioning. The mice were then suspended in OCT medium (Sakura Finetek, Torrance, CA) and quickly frozen in liquid nitrogen. Six- to ten-μm-thick sections of the embedded mice were cut at −20°C in a Leica CM1900 microtome (Deerfield, IL). Multiple sections were taken of each mouse to view all organs. The sections were either adhered to Probe-on glass slides (FisherBiotech, Itasca, IL) by thaw mounting or to adhesive-coated glass slides (Instrumented, Hackensack, NJ) by tape transfer. In the latter method, polymer-coated tape (Instrumedics) was adhered to the embedded mouse, and the section was sliced. The tape-adhered section was pressed onto an adhesive-coated slide and subjected to a flash of UV light to depolymerize the adhesive of the tape, allowing the section to stick only to the glass. The sections were then fixed in 100% acetone. All of the above steps were carried out at −20°C.

To detect T-ALL cells, fixed sections were first flooded with PBS containing 10% normal horse serum (Sigma Chemical Co., St. Louis, MO) and incubated for 20 min to block Fc receptors. Free biotin and avidin were blocked using a kit as described by the manufacturer (Vector, Burlingame, CA). Biotinylated mouse monoclonal antibodies specific for a monomorphic HLA class I epitope and human CD7, respectively (17). The DAB and ABC Elite kits were purchased from Vector (Burlingame, CA), whereas the TSA Indirect kit was obtained from NEN Life Sciences (Pittsburgh, PA). alamarBlue was purchased from AccuMed International, Inc. (Westlake, OH). All solutions were stored and used as recommended by the manufacturers.

Mice. C.B-17/ICR-Hsd-scid mice, 5–6 weeks of age, were purchased from Harlan (Indianapolis, IN). When the mice reached 8 weeks of age, they were injected i.v. in the tail vein with 5 × 106, 107, or 5 × 107 cells suspended in 0.5 ml of RPMI 1640. Cohorts of mice also received daily i.v. injections of 10 μg of DA7 in 100 μl of PBS for 5 days, beginning on the same day as tumor cell injection. Mice were examined every other day for signs of illness such as hunched back, rough fur, weight loss, and inactivity. The mice were maintained under specific pathogen-free conditions at the American Association of Laboratory Animal Care-accredited University of Minnesota Research Animal Resources facilities.

In Vitro Cytotoxicity Assay. For the metabolic activity studies, 2 × 106 Molt 3 or Molt 13 cells in 100 μl of RPMI 1640 supplemented with 10% FBS were added to wells in a 96-well, flat-bottomed plate (Costar, Cambridge, MA). This was followed by the addition of 100 μl of DA7 diluted in RPMI 1640 supplemented with 10% FBS to yield a final concentration of 0.1, 1, 10, or 100 μg/ml. Each sample was performed in quadruplicate. After a 48-h incubation, 20 μl of the metabolic indicator alamarBlue (AccuMed) were added to each well for an additional 18 h. A redox indicator in the alamarBlue reagent changes from an oxidized, nonfluorescent form to a reduced, fluorescent form because of a chemical reduction in the growth medium caused by the metabolic activity of viable cells. Increases in fluorescence intensity are therefore directly related to metabolic activity. Fluorometric analyses were performed in a Cytofluor II plate reader (Biosearch, Bedford, MA) using 530 and 590 nm wavelengths for excitation and emission, respectively. Data were plotted as the percentage of viability (fluorescence emissions) relative to DA7-untreated control cells.

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Injected with Molt 3 as they remained healthy and apparently disease-free for the duration of the experiment (200 days). In contrast, DA7 treatment did not cure mice challenged with Molt 13, although it did significantly (P < 0.005; Wilcoxon two sample ranked test) prolong their survival (69 days mean survival time versus 52 days for untreated controls).

Results

Molt 3 and Molt 13 Are Differentially Sensitive to DA7 in Vitro. Molt 3 and Molt 13 are human T-ALL cell lines that express comparable levels of CD7 (data not shown). To compare their sensitivities to the CD7-specific IT DA7, both cell lines were cultured in vitro with DA7, and cell viability was assessed with alamarBlue, a redox-sensitive indicator of metabolic activity (18). Viability of both cell lines decreased in a dose-dependent manner, but Molt 3 was more sensitive than Molt 13 to DA7 (IC_{50} of 1 and 7 pM, respectively; Fig. 1). A human T-cell leukemic line that does not express CD7 (HUT 102) was not significantly affected by DA7 at concentrations up to 10 nM (data not shown).

Molt 3 and Molt 13 Are Differentially Sensitive to DA7 in Vivo. The ability of DA7 to kill Molt 3 and Molt 13 cells was examined in a SCID mouse model for human T-ALL (1). In this model, SCID mice were injected i.v. with Molt 3 or Molt 13 and then were either left untreated (controls) or were treated with five daily injections of DA7 (10 μg daily for 5 days). Results are expressed as the percentage of mice surviving after T-ALL cell injection.

Visible Residual Tumor Burden Is Consistent with Survival Time. Tumor cells in DA7-treated and control mice were visualized by immunohistochemistry of whole mouse sections. Mice were typically sacrificed at the point of morbidity, and multiple sections from at least two mice/group were analyzed. Tumor cells were identified with a mix of two biotinylated monoclonal antibodies directed against human CD7 and HLA class I. Antibody-reactive cells were visualized with DAB, and the surrounding tissue architecture was revealed by a dilute hematoxylin counterstain. Positive cells were easily identified at ×400 by their brown coloration concentrated at the periphery. Controls included serial sections from the same mouse stained the same day with all solutions except the antibody mixture. Brown regions in the latter sections were regarded as background.

Immunohistochemical analyses of sections from a mouse injected with leukemic cells is shown in Fig. 3A. Immunohistochemical analyses of sections from a mouse injected with Molt 13 revealed that the tumor cells were widely disseminated (Fig. 3B). This mouse was sacrificed 50 days after injection because it looked diseased: humped back, rough fur, and cachetic. Also, one of its eyes was protruding. Human leukemic cells (CD7⁺, HLA class I⁺) were concentrated in the connective tissue and muscle tissue, especially in the hindquarters. These cells had also invaded the periphery of the brain, nerves of the spinal cord, and the Harderian gland and were detected in a thin film behind the protruding eye. They were also found in regions of the liver, intrathoracic cavity, and pelvic try. Images of the stained sections were captured with an Agfa Duoscan T2000 XL scanner using Agfa Fotolook PS 3.03 powered by a 7500/100 Power Macintosh computer.

![Fig. 1](image1.png)

DA7 kills Molt 3 and Molt 13 cells in vitro. Quadruplicate wells of 2 × 10⁴ cells were incubated with 0, 0.1, 1, 10, or 100 pm DA7 in RPMI 1640 for 48 h. alamarBlue (10% v/v) was added, and fluorescence was measured 18 h later. The means and SDs are plotted as percentages of untreated controls (in most cases, error bars are obscured by symbols). IC_{50} extrapolation lines are shown (---). This is one of two comparable experiments.

![Fig. 2](image2.png)

Survival curve of DA7-treated and nontreated mice in a T-ALL SCID mouse model. SCID mice were injected with 1 × 10⁷ Molt 3 or 5 × 10⁷ Molt 13 cells i.v. with or without i.v. administration of DA7 (10 μg daily for 5 days). Results are expressed as the percentage of mice surviving after T-ALL cell injection.
Whole sections from DA7-treated and untreated Molt 3- or Molt 13-injected mice. DA7-treated and untreated SCID mice challenged with tumor were sectioned, and immunohistochemistry was used to identify T-ALL cells. All sections have the same posterior to anterior orientation. H&E-stained section from a mouse not injected with leukemic cells (A) is shown as a reference: b, brain; li, liver; i, intestine; h, heart; lu, lung. Molt 13 cells infiltrated the skeletal muscle, brain, intrathoracic cavity, liver, and bone marrow in the untreated mouse (B). DA7-treated Molt 13-injected mice had tumor cell infiltration mostly in the brain (C). Molt 3 cells were localized mostly in the cerebral cortex of the brain of the untreated mouse shown here (D), and Molt 3 cells were not found in this DA7-treated long-term survivor (E). All images are approximately ×4. Areas of high background were identified using controls as described.

Bone marrow. Similar tumor distribution occurred in a second mouse injected with Molt 13 (data not shown).

DA7-treated mice challenged with Molt 13 displayed a different pattern of tumor cell localization. A representative mouse was sacrificed 67 days after tumor cell injection because it exhibited disease symptoms similar in severity to those described above. In this mouse, tumor cells were found mostly in the brain. They were seen in the central core, brain sulci, and meninges (Fig. 3C). Tumor cells were also present in the brain stem and spinal cord, and they had densely invaded the epineurem of a major nerve bundle in the submaxillary region. There was no significant muscle invasion seen in this DA7-treated mouse.

A mouse injected with Molt 3 cells displayed yet a different tumor cell localization pattern (Fig. 3D). This mouse had a high tumor burden in the cerebral cortex of the brain but nowhere else. The mouse was sacrificed at day 79 after tumor challenge because it was cachectic, had a humped back, and rough fur. At this time, the animal also exhibited frequent abrupt and jerky head movements, and it had difficulty maintaining its balance. These observations are consistent with neurological impairment.

DA7 treatment extended the life of all Molt 3-challenged mice to the experimental end point (200 days; Fig. 2). A DA7-treated, Molt 3 mouse was sacrificed at day 179 with no outward sign of illness. No tumor cells were detected by immunohistochemistry in any of the sections taken from this mouse (Fig. 3E).

Discussion

The major finding of our study is that the preclinical efficacy of cancer therapeutics can be determined directly by visualizing tumor cells in whole mouse sections. Visualization of tumor burdens revealed by whole mouse sectioning and immunohistochemistry were in agreement with survival data and observable disease symptoms. For example, DA7 treatment
cured mice injected with Molt 3 cells and in these long-term survivors, there were no visible tumor cells. In contrast, tumor cells were abundant throughout the brains of mice challenged with Molt 13 and treated with DA7, consistent with the inability of the IT to cure these mice. This technique allowed us to visualize infiltrating tumor cells in the Harderian gland, a thin film behind the eye, and in other tissues. Tumor cells in these locations would most likely have been missed using more common methods that rely on selective tissue sampling to assess residual tumor burden. In addition, different tumor infiltration patterns within organs of DA7-treated versus control mice were observed.

Mouse survival times were consistent with the in vitro IC50 of DA7-treated Molt 3 and Molt 13. Molt 3 is ~7-fold more sensitive to DA7 than Molt 13, and DA7 treatment resulted in the long-term survival of Molt 3 but not Molt 13 challenged mice. Because both cell lines express CD7 at comparably high levels, their differential sensitivity to DA7 in vitro most likely reflects variability in CD7 internalization rates or intracellular routing after internalization. Intertumor variability in DA7 intoxication, combined with differential homing in vivo, may account for the enhanced efficacy of DA7 in the Molt 3 versus the Molt 13 model.

The distinct distribution of Molt 13 cells in DA7-treated and untreated mice suggests that either IT treatment alters tumor cell migration or that tumor grows in sites less accessible to IT. Tumor cells were localized in the brain of the DA7-treated, Molt 13 mouse, suggesting that the blood-brain barrier prevented IT access to this site. Our data and that of Gunther et al. (4) indicate that Molt 3 cells also migrate to the brain in untreated SCID mice. However, treatment with CD7-specific ITs containing either ricin toxin A chain (this report) or pokeweed antiviral protein resulted in distinct organ-distribution pattern compared with untreated controls (4). We speculate in our case that the Molt 3 cells were eliminated by DA7 before they migrated to the privileged site of the brain. Additional studies are under way to test this hypothesis.

With the appropriate controls, whole mouse sectioning is a robust and powerful technique for the identification of residual tumor deposits posttreatment. Controls include: (a) comparing sections from several mice in the same treatment group to ensure reproducibility; (b) staining multiple sections from the same mouse to ensure that all organs are analyzed; and (c) including the appropriate negative controls to identify background staining. We anticipate that this technique, combined with image analysis software, will allow us to quantitate tumor cell number in the sections. The numbers can then be extrapolated to the whole mouse to quantitate tumor burden. These data will aid in the design of more effective therapeutics because drug limitations can be identified and addressed.

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References