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-associating 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 IT 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<sup>+</sup>, My<sup>9</sup><sup>+</sup>) and lymphoid (subset of CD19<sup>+</sup>) progenitors (9–11). The function of CD7 is not clear, but its expression in disease has important clinical implications.

1 This work was supported in part by grants from the University of Minnesota Cancer Center, Minnesota Medical Foundation, and Graduate School (to C. A. P.).

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4 The abbreviations used are: IT, immunotoxin; SCID, severe combined immunodeficient; DAB, diaminobenzidine; FBS, fetal bovine serum; IC<sub>50</sub>, concentration required to achieve 50% inhibition; T-ALL, T-cell acute lymphoblastic leukemia.

R. L. Reinhardt and M. Jenkins, personal communication.
ramifications. It is one of the most useful markers for T-ALL, because >95% of T-ALL cells are CD7+ in >95% of cases (12, 13). CD7 is expressed at high densities (>60,000 molecules/cell) on T cells and is rapidly internalized, even when bound by monovalent antibody fragments (14). It therefore makes an ideal target antigen for IT-mediated therapy of T-cell disease. DA7 is an IT constructed by chemically conjugating deglycosylated ricin toxin A chain to 3A1e, a mouse monoclonal antibody specific for human CD7 (15). We chose to study DA7 because it was well characterized preclinically and clinically. DA7 cured SCID mice challenged with a CD7+ human leukemia, and in a Phase I clinical trial, DA7 achieved objective responses at its maximal tolerated dose (1, 16). As proof of principle, we used immunohistochemical analyses of whole mouse sections to evaluate the ability of DA7 to eliminate tumor cells in two SCID mouse models for human T-ALL. We found that the visible tumor burden was consistent with survival times and disease symptoms. Our data indicate that this system should prove to be a valuable tool in evaluating the preclinical efficacy of cancer therapeutics such as ITs.

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.). Cells were cultured in a humidified animal resources facilities.

Reagents. The CD7-specific IT DA7 was a generous gift from Dr. Daniel Vallera (University of Minnesota) and was synthesized as described (15). The mouse monoclonal antibodies W6/32 (a gift from Dr. Tucker LeBien, University of Minnesota) and 3A1e (a gift from Dr. Barton Haynes, Duke University, Durham, NC) are 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/Scid-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 x 10^6, 10^7, or 5 x 10^7 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 x 10^5 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 pM. 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 (Biosource, 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 (Saikura 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 (FisherBiotec, Itasca, IL) by thaw mounting or to adhesive-coated glass slides (Intrumedics, 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 (W6/32) or human CD7 (3A1e) were combined and diluted in PBS with 10% normal horse serum. This antibody mixture was applied to the sections for at least 30 min at room temperature. Endogenous peroxidase activity was then quenched by incubating the slides in 0.3% H2O2/methanol. The antibody signal was amplified by deposition of biotinyl tyramide (TSA Indirect kit; NEN Life Sciences), followed by application of an avidin/biotin-coupled horseradish peroxidase complex (ABC kit; Vector). DAB (Vector) was used to visualize the antibody-bound cells, and sections were counterstained in dilute (50%) hematoxylin. Other sections were fixed in 100% acetone and stained in H&E. Slides were examined at ×100 and ×400 for antibody-reactive cells (identified by brown staining on the cell periphery). Control serial sections from each mouse were treated identically as described above, except the antibody mixture was omitted. Multiple sections from at least two mice/group were analyzed by immunohistochemis-
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.

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 DA7 dose-dependent manner, but Molt 3 was more sensitive than Molt 13 to DA7 (IC50 of 1 and 7 pM, respectively; Fig. 1). A human T-cell leukemia 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) beginning the day of tumor cell challenge. Because preliminary experiments revealed that Molt 3 was more aggressive than Molt 13 in this model, five times fewer Molt 3 than Molt 13 cells were injected per mouse. Mice receiving 107 Molt 3 cells had a mean survival time of 75 days, whereas mice receiving 5 × 107 Molt 13 cells had a mean survival time of 52 days (Fig. 2). DA7 treatment cured mice 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).

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. In addition, nonspecific staining was detected in sections from mice not injected with tumor by treating these sections with all solutions, including the antibody mixture. Background staining was mainly limited to the gut, glands, fat, and kidney, and the pattern of background staining was more diffuse than that seen with tumor cells (data not shown). Serial sections from each mouse were also stained with H&E to aid in tissue identification. Whole mouse section images were equivalent to images of individually removed and sectioned organs with no loss of cellular detail. An example from a mouse not 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 cachectic. 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
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.

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 IC<sub>50</sub> 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.

Acknowledgments

We thank Dr. Daniel Vallera for giving us DA7 and Gerald Sedgewick, from the University of Minnesota Biomedical Image Processing Laboratory, for expert assistance and technical advice.

References

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Clin Cancer Res 2001;7:890s-894s.

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