Penetration of anti-melanoma immunotoxin into multicellular tumor spheroids and cell kill effects

Takao Kikuchi[1], Takao Ohnuma[1], James F. Holland[1], and Lynn E. Spitler[2]*

[1] Department of Neoplastic Diseases and the Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, N.Y., USA
[2] Xoma Corporation, Berkeley, California, USA

Summary. In order to gain a better understanding of the interaction between immunotoxins and tumor cells at the level of three-dimensional tumor mass, we evaluated the cell kill effects of monoclonal antimelanoma-antibody/ricin-A-chain immunotoxin (ITN) on melanoma cells in multicellular tumor spheroids (MTS) as well as the penetration of ITN into MTS. For Minor melanoma cells in monolayer the ITN exerted cytotoxic effects after as little as 1 h of exposure. Increasing exposure time resulted in progressive increases in cytotoxic activity. In contrast, the cell kill effects of ITN were markedly delayed and reduced when Minor cells were in MTS. The ITN cytotoxic effects on the melanoma MTS were more than 100 fold less than those in monolayer. Patterns of ITN-induced cytotoxicities for Minor and for another melanoma cell line, DND-1A, were comparable. The native ricin A was more active against PC-10 squamous lung cancer cells than Minor cells, whereas the ITN was more cytotoxic against Minor cells than PC-10 cells, thus exhibiting selectivity. An auto-radiographic study revealed time-dependent penetration of radiolabeled ITN from the surface of Minor MTS into the core. Incubation for 1 h resulted in the penetration of ITN into only the two or three outer layers of the Minor MTS, and low grain counts. Prolonged exposure resulted in inhomogeneous penetration of ITN into almost the entire melanoma MTS. Penetration of ITN into PC-10 MTS was extremely poor. The reduced cytotoxicity of ITN on melanoma cells in MTS as compared to cells grown in monolayer appears to correlate with its inhomogeneous distribution in the MTS. The delayed cytotoxicity of ITN is also consistent with its slow penetration into the core of the MTS.

Key words: Immunotoxin – Melanoma – Multicellular tumor spheroids

*Present address: Biotherapeutics, 2220 Livingston, Oakland, Calif., USA

Correspondence to: T. Ohnuma, Department of Neoplastic Diseases, Box 1128, Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029, USA

Introduction

The development of hybridoma technology provided large quantities of monospecific antibody against a variety of cell-surface antigens and has accelerated interest in targeted therapy using monoclonal antibody as a specific carrier [1]. The cytotoxic materials could be either antineoplastic agents [9, 21], toxins [2, 6, 25] or radioactive isotopes [4]. Conjugates between monoclonal antibodies and toxins, immunotoxins (ITN), have been produced and clinical studies carried out with variable success [8, 26].

One major concern identified in the therapeutic use of ITN is its variation in biological activity [16]. Since ITN is a large molecule, we questioned whether it can penetrate into a three-dimensional tumor mass. For this purpose, we developed human melanoma multicellular tumor spheroids (MTS) and evaluated the effects an ITN specific for human melanoma had on them.

MTS have certain characteristics similar to de novo solid tumors [5, 17, 27, 29]. MTS are known to contain such extracellular matrix material as fibronectin, laminin and collagen [17], have a hypoxic area in the core [27], and show a heterogeneous distribution of cell-cycle time [5].

In the present study we questioned specifically (a) the cell kill pattern of a specific ITN when tumor cells are in MTS rather than in monolayer, and (b) whether the differences in cell kill pattern, if any, can correlate with the degree of ITN penetration into the MTS.

Materials and methods

Cell lines. The DND-1A human melanoma cell line was established in our laboratory from the pleural effusion of a 60-year-old man with malignant melanoma [19]. The minor human melanoma cell line [24] was developed in the laboratory of Dr. B. Giovanella of the Stehlin Foundation for Cancer Research, Houston, Texas. As a non-melanoma control, we used PC-10 human squamous lung cancer cells [11], which were provided by Dr. S. Inoue of Hokkaido University Medical School, Sapporo, Japan.

These cell lines were maintained as monolayer cultures in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) (complete medium) under

humidified 5% CO_2/air at 37°C. Minor and DNA-1A cells were fed twice a week with complete medium and subcultured weekly after being dispersed with 0.05% trypsin (Gibco). PC-10 cells were subcultured weekly using 0.05% trypsin and 0.02% EDTA (Gibco). *Mycoplasma* contamination was routinely tested by Mycotrim TC (Hana Biologicals, Berkeley, Calif.) and the lines were shown to be negative.

Drugs and radiomaterials. ITN (Xomazyme-Mel, lot no. 60127 and X101530), consisting of human-melanoma-specific monoclonal antibody conjugated to ricin A chain, was produced by Xoma Corporation (Berkeley, Calif.). The conjugation technique has been described [24].

The monoclonal antimelanoma antibody was produced using cultured M21 human melanoma cells as the immunogen and was of the IgG2a subclass [24]. In vitro studies for the binding specificity determined by enzyme-linked immunosorbent assay and radioimmunoassay showed that eight of ten human melanoma cell lines tested reacted with the antibody. Two remaining melanoma cell lines had no reactivity, most likely because of loss of antigen secondary to serial tissue-culture passage. The Minor cell line was among those with a high level of reactivity. The DND-1A cell line was not tested in this system. None of the eight human tumor cell lines of non-melanoma origin reacted in this assay. The conjugate of the antibody and ricin A showed essentially identical binding specificity towards the melanoma cells [24].

^{125}I-labeled ITN (specific activity 1 µCi/2 µg ITN) was also provided by Xoma Corporation. Native ricin A chain was obtained from Sigma (St. Louis, Mo.).

Development of MTS. MTS were produced by the method described by Yuhas et al. [29] as followed in our laboratory [12, 13]. Cells in monolayer culture were used to initiate spheroid growth. Cells were removed from the floor of the flask with trypsin, washed once and resuspended in the complete medium. Samples of 10 ml medium, containing 1×10^5 cells, were placed in 100-mm plastic Petri dishes (Falcon, Cockysville, Md.) which had previously been coated with 0.5% agar (Noble, Difco, Detroit, Mich.). They were incubated in humidified 5% CO_2/air at 37°C. MTS so developed were transferred to new agar-coated petri dishes and fed with fresh medium once a week. MTS of DND-1A and PC-10 cells could reach approximately 500–600 µm in diameter in 3–4 weeks. The growth of Minor MTS was slow and it took 5–6 weeks to grow to the same size. At this size MTS tended to contain a small necrotic core and we used such "mature" MTS in these studies.

Measurement of cytotoxic effects. Cytotoxic effects were measured by a "modified" colony-inhibition assay. Three MTS, 500–600 µm in diameter, were preincubated in 0.9 ml fresh medium placed in the agar-coated 12-well culture plate (Flow Laboratories, McLean, Va.) for 24 h. A 0.1-ml sample of graded concentrations of ITN or ricin A was added to each well and further incubated for various lengths of time, up to 72 h. After the exposure, MTS were transferred in a 15-ml snap-cap plastic tube (Falcon, Cocksville, Md.) and gently washed by repeated aspiration with PBS. MTS were then trypsinized for 10 min at 37°C and single cells in suspension so obtained were washed once more with the medium by centrifugation. Samples containing 10^3 viable cells in 2.5 ml complete medium were seeded into 60-mm petri dishes (Corning Glass Works, Corning, N.Y.). After the seeding, each dish was examined under an inverted microscope for even distribution of well-dispersed single cells and the lack of cell aggregates. After 7–10 days of incubation, the number of colonies was counted under an inverted microscope and expressed as the surviving fraction. Colonies were defined as an aggregate of more than 50 cells. This corresponds to a cell aggregate of more than 8 cells in a row and this was easily verifiable for PC-10 cells. In the cases of Minor and DND-1A colonies, cells were often fibroblastic or dendritic and the aggregates contained many cells with spreading pseudopods. In these cases it was often difficult to count individual cells. A total cell aggregate with a size corresponding to that of PC-10 colonies was arbitrarily chosen as a colony. In this setting, cell aggregates smaller than colonies were minimal for all three cell lines.

As a control, cytotoxic effects of ITN or ricin A were also tested against cells in monolayer. Single-cell suspensions from a monolayer culture were adjusted to 1×10^5 cells/ml and preincubated for 24 h in
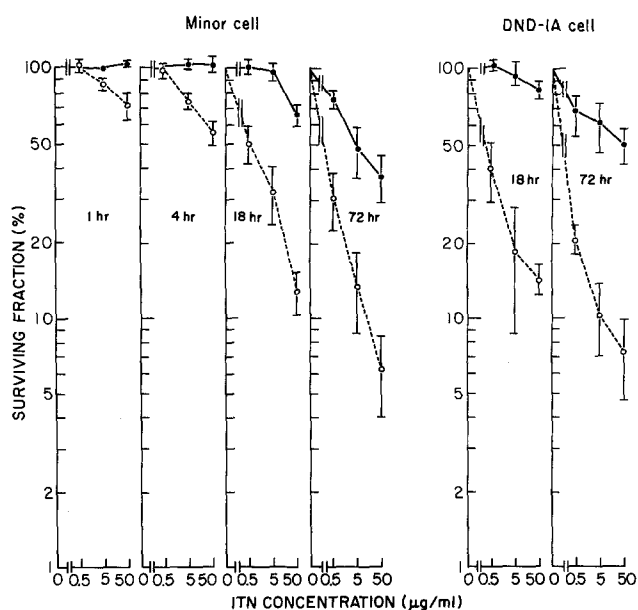

Fig. 1. Effects of immunotoxin (*ITN*) concentration and exposure time on cytotoxicity of Minor and DND-1A cells in monolayer (○) and multicellular tumor spheroids (MTS) (●). Each data point represents a mean of at least two experiments in triplicate. *Bar*, SD

60-mm petri dishes. After confirmation that cells were attached to the floor of the dishes as a monolayer, the cells were exposed to graded concentrations of ITN or ricin A for various lengths of time. The dishes were then washed twice with PBS and trypsinized. Single cells in suspension so obtained were washed once more with the medium and subjected to the modified colony-inhibition assay as described above.

This method is different from the colony-inhibition assay reported by others [2] in that, after exposure to ITN, viable cells were counted and the seeding for colony development was done after adjustment of viable-cell density. This modification was necessary to adapt the colony-inhibition assay for the determination of cytotoxicity in MTS. Pilot studies showed that both original and modified methods produced dose- and time-dependent cell kill effects after exposure to ITN.

We found other assay methods, such as a protein synthesis inhibition assay [18, 22, 23], DNA synthesis inhibition assay [25] and Kuroki's clonogenic assay [14], awkward to use for MTS and/or not more sensitive than the method we employed.

Autoradiographic study. Two MTS approximately 500–600 µm in diameter were placed in 12-well culture plates (Flow Laboratories) in 1.0 ml complete medium containing 5 µCi/well (equivalent to 10 µg ITN) radiolabeled ITN. The culture plates containing MTS were incubated for 1, 4, 18 or 72 h. The incubation was terminated by aspiration of the medium and by gently washing the MTS with Dulbecco's phosphate-buffered saline (PBS) (Gibco). MTS were then fixed in 2.5% glutaraldehyde for 30 min. Fixed spheroids were embedded in epon 812, sectioned at 1 µm thickness and dipped into NTB-2 (Kodak) nuclear tract emulsion (diluted 1:1 with distilled water) in complete darkness. Emulsion-covered slides were dried and exposed at 4°C in a light-sealed desiccator for 14 days. These slides were then developed with full-strength Kodak D19-B (2 min) and fixed with full-strength Kodak rapid fixer (2 min). Finally, the slides were stained with methylene blue.

Results

The effects of ITN concentration and exposure time on cytotoxicity to Minor cells are shown in Fig. 1. For Minor cells in monolayer, ITN was active after an exposure as short as 1 h. ITNs cytotoxic effects became more pro-
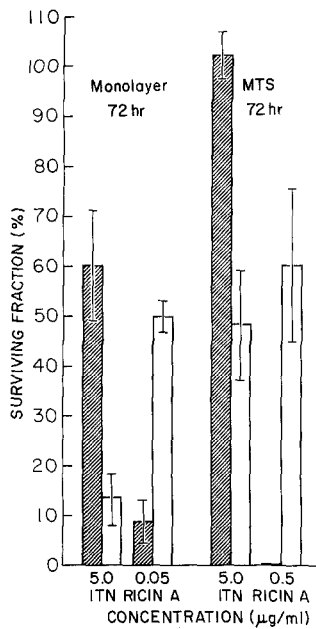
Fig. 2. Cytotoxic effects of ITN or native ricin A on Minor (white columns) and PC-10 cells, (shaded columns) in monolayer and MTS after 72 h of exposure. On a weight basis, ricin A is about one-third of ITN. Each data point represents a mean of at least two experiments in triplicate. *Bar*, SD

The cytotoxic effects of native ricin A were evaluated similarly using Minor cells and PC-10 cells and the results are shown next to the ITN data in Fig. 2. In contrast to ITN, the intact ricin A was found to be more active against PC-10 cells than against the Minor cells irrespective of whether cells were in monolayer or MTS. These data clearly show that ITN is selective to melanoma cells.

To evaluate the penetration of ITN into MTS, autoradiographic studies were performed. ^{125}I-labeled ITN did penetrate the surface of melanoma MTS into almost the entire core in a time-dependent manner. Thus, after 1 h exposure, the labeling was seen only in the two or three outer layers of MTS (Fig. 3). At this time of exposure the grain counts were low, indicative that the concentration of ITN in MTS was probably too low to produce cell lethality (Fig. 1).

After 72 h of exposure, the labeling was observed in almost the entire core of Minor MTS. The distribution of the grains in the MTS was, however, inhomogeneous and extremely strong labeling was sporadically seen in a small number of cells per specimen (Figs. 4, 5). Virtually identical penetration and distribution patterns of the ITN label were observed in DND-1A MTS after 72 h of exposure (figure not shown). We interpret the inhomogeneous distribution of ITN in the MTS and the fact that only a small number of cells accounted for a high concentration of labeling to be the factors responsible for its limited cytotoxic effects, as compared to cells in monolayer. For PC-10 MTS, the penetration of ITN was slow and only after 72 h of exposure had it penetrated four to five outer layers of MTS; the grain counts were extremely low (figure not shown).

We conclude that the lesser cytotoxic effect of ITN on melanoma MTS as compared to that on cells in monolayer correlates with the inhomogeneous penetration of ITN into the entire three-dimensional tumor mass. In addition, the delay in the start of this cytotoxic effect is consistent with the time required for the penetration of ITN into the MTS core and for equilibrium to occur.
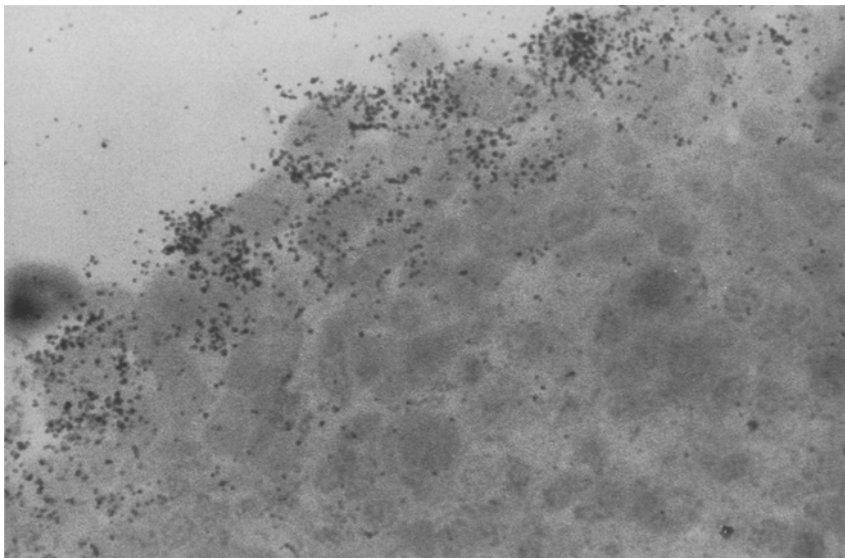
nounced with increases in its concentration and exposure time. ITNs cytotoxic effects were markedly delayed and reduced when Minor cells were in MTS. Thus, the cytotoxic effects were recognized only after 18 h or more of exposure. After 72 h of exposure, ITN produced substantial cytotoxic effects at all dose levels studied. In DND-1A cells in monolayer and MTS, the ITN produced degrees of cytotoxicity comparable to those of Minor cells after 18–72 h of exposure.

In order to test the specificity of ITN, its cytotoxic effects on Minor cells were compared with those on PC-10 cells (Fig. 2). ITN was shown to be more active against Minor cells than PC-10 cells, both in monolayer and spheroid forms. Identical results were obtained with DND-1A cells (data not shown).

Fig. 3. Autoradiographic section from a Minor spheroid after 1 h exposure to ^{125}I-labeled ITN (× 600). Grains are visible only on the two or three outer cell layers

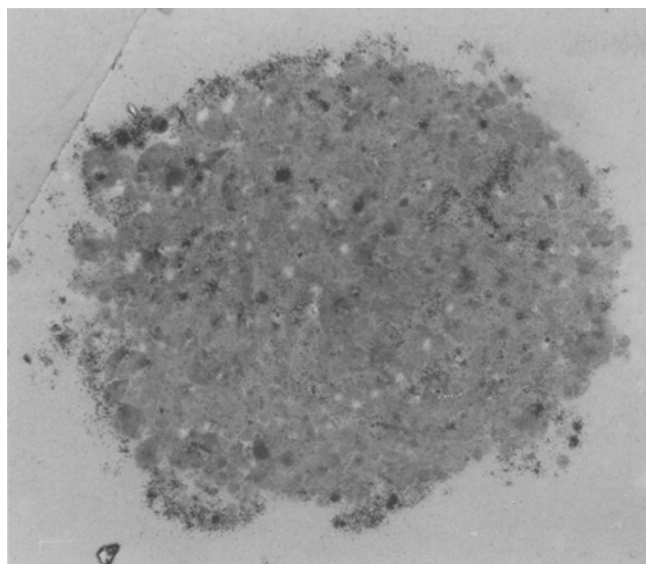Fig. 4. Autoradiographic section from a Minor spheroid after 72 h exposure to ^{125}I-labeled ITN (×150) Grains are visible as dark spots irregularly distributed in the entire MTS. These spots are grains accumulated in certain cells (see Fig. 5 for higher magnification)

Discussion

In this study, we investigated the relationship between the penetration of ITN into MTS and its cytotoxic effects. For this study we selected Xomazyme-Mel, which had been well characterized [24], and the biological effects of which had been tested both in animals and man [7, 26]. We observed that the ITN penetration into the core of melanoma MTS was time-dependent and the distribution of ITN in MTS inhomogeneous. These penetration and distribution patterns correlated with the cytotoxic effects of ITN.

To evaluate the immunotoxins's selectivity to melanoma cells, we compared its penetration patterns into melanoma MTS with those produced by PC-10 cells. We found that there was virtually no penetration of the melanoma

ITN into the squamous cell carcinoma MTS and there was little cell killing. For further evaluation of selectivity we also compared cell kill effects induced by ITN and by native ricin A. Native ricin A was found to be more effective against PC-10 cells than Minor cells, whereas ITN was more active in melanoma cells than in squamous carcinoma cells, both in monolayer and MTS. Only after prolonged exposure (72 h), was ITN shown to be cytotoxic against PC-10 cells, probably reflecting the non-specific killing of the cells by ricin A [25].

The patterns of the penetration of intact monoclonal antibody [15, 28], as well as monoclonal antibody conjugated to doxorubicin [9] into MTS have been reported by several investigators but no side-by-side comparison with the cell kill effects has been made. Kwok et al. [15] and Inoue et al. [9] reported that multiple prolonged exposures of the cells to antibody resulted in diffuse and homogeneous penetration of the antibody into the entire MTS. In contrast, Sutherland et al. [28] reported that whole anti-CEA antibody penetrated only the outer layers of MTS of colon adenocarcinoma cells. When antibody fragment was used, penetration was better but was found to be inhomogeneous. Our observation of the inhomogeneous distribution of ITN in melanoma MTS is comparable with the reports by Sutherland et al. [28]. The reasons for inhomogeneous distribution of labeling are yet to be determined. It is known that the individual cells composing MTS are in different biological states with respect to cellular pH, nutrition, composition, oxygen tension, and cell-cycle status. It is likely, therefore, that the amount and expression of melanoma antigen are also different among cells in MTS, which in turn might have caused inhomogeneous distribution of labeling. Studies are in progress in our laboratory to test this hypothesis.

The MTS and physiological tumors differ in that the former are devoid of vasculature, of host cell infiltration and of stroma cells. Tumor vasculature develops when tumor mass reaches a certain size. In this context, MTS in vivo may correspond to microscopic tumor nodules. Whether intratumoral lymphocyte infiltration or the pres-
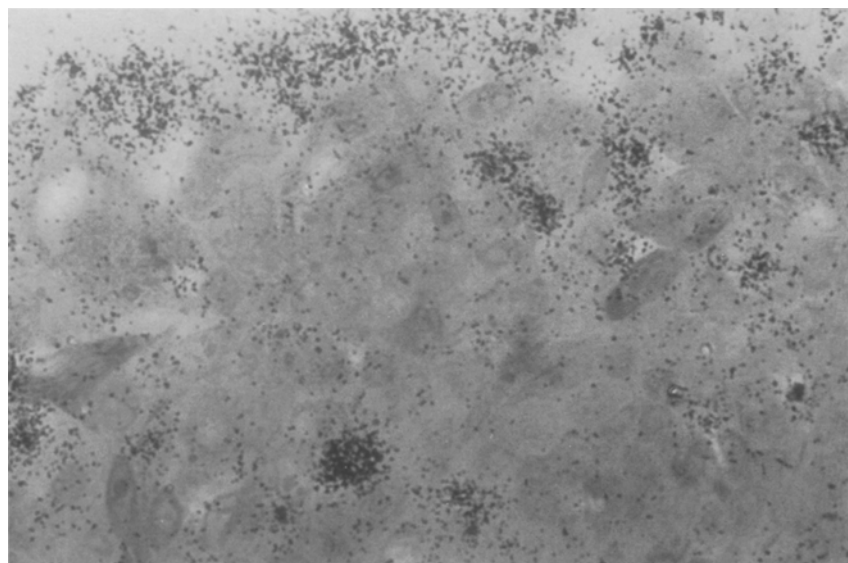

Fig. 5. Higher magnification (×600) of Fig. 4 (left upper portion). Grains are distributed inhomogeneously throughout the preparation. In addition, grains have accumulated in certain cells. These cells are recognized as dark spots in Fig. 4

ence of stroma cells influences the ITN penetration into a tumor mass is unknown. In vivo studies with animal tumors, human tumor xenografts and clinical studies have identified maldistribution of antibodies to heterogeneity of tumor-associated antigen expression [3, 16], spatially heterogeneous filtration of fluid from blood vessels and elevated interstitial pressure in the inner core of a tumor mass [27]. With the exception of the influence of vasculature on the ITN penetration, which can not be duplicated in our in vitro model, these reports from in vivo work corroborate our in vitro observations. Thus, the prolonged time required for the ITN penetration into the MTS and its concurrent delay in producing cytotoxic activity are consistent with in vivo data showing an antibody's slow penetration into the inner core against the gradient of interstitial pressure [10] and the time required for equilibrium to occur [20].

Our data provide information about ITN penetration into human melanoma tumor mass and may serve as an in vitro model for the development of appropriate strategies for improved targeted therapy.

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