The Use of Daunomycin-Antibody Immunoconjugates in Managing Soft Tissue Sarcomas: Nude Mouse Xenograft Model

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ABSTRACT

Analysis of human fibrosarcoma cells exposed to radiolabeled monoclonal antibody 19-24, which recognizes sarcoma-associated antigen p102, revealed that over 54% of the cell surface-bound radioactivity was internalized. No modulation of cell surface p102 antigen by monoclonal antibody 19-24 was observed in human fibrosarcoma cells. Monoclonal antibody 19-24 coupled to daunomycin via a dextran bridge was found to be most effective. In different preparations, the daunomycin:total protein body molar ratio ranged from 1.9 to 6.1. In vitro cytotoxicity studies using human fibrosarcoma cells showed that, at 10 μg/ml concentration, this immunconjugate was 79.4% as efficient as free daunomycin and, at 1 μg/ml concentration, 36.8% as efficient. Control nonspecific murine monoclonal antibody F3 immunconjugates were relatively ineffective.

The distribution of 14C-daunomycin, 125I-labeled monoclonal antibody 19-24, and 125I-labeled 19-24 immunconjugate was also evaluated over a 24-hour period in tumor and normal tissues of athymic mice bearing a human fibrosarcoma xenograft. Poor uptake of radiolabeled Adriamycin by the tumor tissue was observed. The level of 14C radioactivity in the tumor tissue never exceeded 1% of the total injected dose and was 24.8-fold lower than the radioactivity found in the spleen tissue. Tumor tissue uptake of radiolabeled monoclonal antibody 19-24 was characterized by the high tumor tissue:blood ratio of 1.62 ± 0.28 (SD). However, for monoclonal antibody 19-24 immunconjugates, this ratio decreased to 0.66 ± 0.05, which was still higher than normal (liver; 0.48 ± 0.02; lung, 0.48 ± 0.07; spleen, 0.23 ± 0.01). Thus, it appears that, compared to free daunomycin, monoclonal antibody 19-24 immunconjugates may be more efficient and less cytotoxic to normal tissues.

INTRODUCTION

MoAbs to human tumor-associated antigens have been intensely investigated for use as carriers of toxins (1, 2) or drugs (3-7). Previous studies have shown that immunconjugates prepared by different methods varied in usefulness in targeting antineoplastic drug to tumor cells (8-10). Antisarcoma highly specific MoAb 19-24 (11) was selected as a carrier of antineoplastic agents, based on our previous observations that this MoAb selectively localizes in tumors in vivo (12-16). Recently, we described a method in which ADR (doxorubicin) was coupled to MoAb 19-24 using a biotin-avidin-biotin bridge (17). In vitro cytotoxicity studies using human fibrosarcoma cells, this ADR-MoAb immunconjugate maintained 40% of the efficacy of free ADR. Immunconjugates using a biotin-avidin-biotin bridge may be less effective in in vivo immunotherapy because egg white glycoprotein avidin M, 67,000 may induce an immune response after administration of immunconjugates into the bloodstream. Therefore, we decided to investigate other methods of coupling antinecyclic drugs to MoAb.

Below, we report the in vitro cytotoxicity results of conjugation of DAU (daunorubicin) to MoAb 19-24 through a dextran bridge. In vivo distribution of MoAb 19-24 immunconjugates in fibrosarcoma-bearing athymic mice was also examined.

METHODS AND MATERIALS

Tissues and Tumor Cells

Human fibrosarcoma HT-1080 cells (18), purchased from the American Type Culture Collection (Rockville, MD) were grown in adherent monolayer culture with MEM-E, supplemented with l-glutamine and 15% heat-activated fetal bovine serum (GIBCO, Grand Island, NY).

Tumor Xenografts

HT-1080 cells (2 × 10⁶ cells/mouse) were injected s.c. into the right flanks of male athymic (nude) mice (NCI Frederick Cancer Research Facility, Frederick, MD) to initiate a tumor xenograft (12). When the tumors had grown to ~1 cm in diameter (after 10-14 days of growth), animals were entered into the study.

Monoclonal Antibodies

Antisarcoma mouse MoAbs 19-24 and 23-26 and nonspecific mouse myeloma MoAb F3 (all of isotype IgG1) were generated as described previously (11, 19). Purified MoAbs were radioiodinated using Na125I (Amersham, Arlington Heights, IL) and chloramine-T (20).

Preparation of Immunconjugates

Method A. Glutaraldehyde was used to cross-link the amino groups of DAU (Wyeth-Ayerst Laboratories, Inc., Philadelphia, PA) and the free amino groups of the MoAb (8, 21) as follows. To 1 ml of 0.1 M phosphate buffer (pH 6.8) containing 1 mg of MoAb and 0.17 mg of DAU, we added 0.05 ml 1% (w/v) aqueous solution of glutaraldehyde and incubated it for 15 min at room temperature in the dark. The excess glutaraldehyde and DAU were removed by extensive dialysis against PBS (10 mM phosphate-0.15 mM NaCl-1 mM MgCl₂-0.02% Na₃VO₄, pH 7.3).

Method B. A MoAb was covalently coupled to DAU with EDC using a conjugation kit supplied by Pierce Chemical Co. (Rockford, IL) (8, 22). Briefly, a mix of 0.5 mg MoAb, 0.1 mg DAU, and 2.5 mg EDC, dissolved in conjugation buffer, was incubated for 1 h at room temperature in the dark. After Sephadex G-25 gel filtration, DAU-EDC-MoAb immunconjugate fractions were pooled and analyzed.

Method C. Conjugation of DAU to MoAb or 125I-labeled MoAb was effected through a dextran bridge (8, 23, 24). DEX (M, 40,000; Sigma Chemical Co., St. Louis, MO) was oxidized to polyaldehyde-dextran with sodium periodate. DEX oxid was then dialyzed against water, lyophilized, and stored at 4°C. The DEX oxid was incubated with DAU in PBS at drug:DEX oxid weight ratios varying from 1:7 to 1:1. The DAU-DEX oxid conjugate was separated from free DAU by dialysis against PBS. Finally, the DAU-DEX oxid conjugate was incubated with MoAb (19-24 or F3), and any remaining polyaldehyde-active sites in the DAU-DEX oxid-MoAb immunconjugate were reduced by a 30-min incubation with sodium cyanoborohydride. Excess sodium cyanoborohydride was removed by dialysis against PBS.

The total protein and DAU concentrations in the immunconjugates were determined spectrophotometrically (24), and immunconjugates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25).
In Vitro Experiments

Using two anti-p102 MoAbs (19-24 and 23-36), each recognizing a different p102 epitope, direct radioimmunoassay was performed to determine the modulation of the cell surface sarcoma-associated antigen p102 (11, 19). Fibrosarcoma HT-1080 cells (10⁶ cells/well) were incubated in sterile 96-well culture plates with protein A-purified MoAbs 19-24 (10 μg/ml) or 23-26 (10 μg/ml) at 37°C in 5% CO₂ for 24 h. The cells were washed and incubated in fresh MEM-E media at 0, 1, 5, 24, or 72 h; the p102 antigen on the cell surface was measured by direct radioimmunoassay as follows. The cells were incubated with 125I-labeled mouse IgG or DAU-DEX-MoAb diluted in culture medium for 24 h. After incubation, the cells were washed and harvested with 0.05% trypsin-EDTA. The cells were resuspended in 0.4% trypan blue (GIBCO), and live cells were counted.

In Vitro Experiments

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The internalization of anti-p102 MoAb 19-24 was examined using p102-positive fibrosarcoma HT-1080 cells incubated with 125I-labeled anti-p102 MoAb at 37°C or 4°C for 1 h. The cells were then washed and incubated for 1, 6, or 24 h at 37°C or 4°C. Finally, radioactivity was measured in cells harvested with 0.05% trypsin-EDTA (GIBCO). The uptake was calculated by subtracting the radioactivity binding at 4°C from the total radioactivity at 37°C (26).

In a different experiment, ~6 × 10⁶ fibrosarcoma cells were grown in a flask and incubated with 125I-labeled MoAb 19-24 at 37°C in 5% CO₂ for 24 h. After incubation, the cells were washed or incubated in fresh media for 24 h and harvested with 0.05% trypsin-EDTA. The cells were homogenized (4°C), and the radioactivity was measured for cytoplasm and crude plasma membrane fraction (27).

The cytotoxicity of DAU-DEX-MoAb immunconjugates was determined by incubating HT-1080 cells with the immunconjugates. The cells (4 × 10⁴ cells/well) were grown in sterile 96-well culture plates at 37°C in 5% CO₂ for 24 h, then washed with culture and MEM-E medium, and incubated with DAU, DEXxid, DEXxred, DAU-DEXxid, DAU-DEXxred, DAU-DEXxid-MoAb, or DAU-DEXxred-MoAb diluted in culture medium for 24 h. After incubation, cells were washed and harvested with 0.05% trypsin-EDTA. The cells were resuspended in 0.4% trypan blue (GIBCO), and live cells were counted.

Tissue Distribution Studies

14C-ADR (Amersham; specific activity, 96 μCi/μg) was injected i.p. at a dose of 0.02 μCi/μg into a group of six fibrosarcoma-bearing athymic mice. At 1, 6, and 24 h after the ADR administration, 2 mice from each of the 3 time intervals were sacrificed; blood, tumor, skin, muscle, brain, and the rest of the viscera were analyzed for the presence of 14C-ADR and its radioactive metabolites. Briefly, plasma (200 μl) or weighed tissue samples (~100 μg) were transformed to scintillation vials and solubilized in 0.5 ml 1 M KOH (28). Decolorization of the samples was performed by treatment with hydrogen peroxide at room temperature. Because this assay reflects 14C-ADR and 14C-labeled ADR metabolites, the data are expressed as cpm of 14C-ADR radioactivity equivalents/g of organ, wet weight (29). Recovery studies were performed on set plasma and tissue samples containing known amounts of 14C-ADR.

To investigate the penetration of MoAb or DAU-DEX-MoAb immunconjugates into solid tumor tissue, fibrosarcoma-bearing athymic mice were given a single i.p. injection of 125I-labeled MoAb or DAU-DEX-MoAb (125I-labeled) immunconjugate. At the stated times after injection, radioactivity was measured in blood, tumor, skin, muscle, brain, and the rest of the viscera. The results are expressed as cpm/ml(g) of blood (wet tissue) or in tissue:blood ratios.

RESULTS

Anti-p102 MoAb 19-24 conjugated to DAU using glutaraldehyde (Method A) or carbodiimide bridge (Method B) lost all binding activity to p102 antigen; therefore, no additional studies were performed.

Based on spectrophotometric analysis of DAU-DEX-MoAb 19-24 immunconjugates, the calculated DAU:total protein molar ratio ranged from 1.9 to 6.1. The presence of high molecular weight immunconjugates was clearly evident after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (Fig. 1, Lane 3). Protein G (Fig. 1, Lane 4) did not reorganize the immunconjugates; therefore, we used protein G to remove untreated (free) antibody from the reaction mixture. Furthermore, there was no difference in binding between immunconjugates diluted up to 1:5000 (60 ng MoAb in immunconjugate/ml) and free (unconjugated) MoAb 19-24 (60 ng/ml).

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Fig. 1. Polyacrylamide gel electrophoresis (7% under nonreducing conditions) of 125I-labeled/DAU-Dextran-IgG. Lane 3, molecular weight of over 200,000 at the top of the resolving gel; Lane 4, supernatant after incubation with agarose-bound protein G; Lane 1, 125I-labeled albumin with a molecular weight of 66,000; Lane 2, 125I-labeled mouse IgG used in the immunconjugate preparation with a molecular weight of ~150,000; w, loading well; t, top of resolving gel.

Fig. 2. Uptake of anti-p102 MoAb 19-24 by fibrosarcoma cells. Cells (10⁴ cells/ml) were incubated with 125I-labeled MoAb at 4°C (○) or 37°C (□) in MEM-E. At the times indicated, cells were washed and harvested, and radioactivity was measured. Points, mean of 3 determinants.
Table 1 Inhibition of fibrosarcoma cells growth

<table>
<thead>
<tr>
<th>Cytotoxicity</th>
<th>10.0 µg/ml</th>
<th>1.0 µg/ml</th>
<th>0.1 µg/ml</th>
<th>0.001 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunomycin</td>
<td>97.8 ± 0.5</td>
<td>68.9 ± 1.7</td>
<td>56.8 ± 2.5</td>
<td>25.4 ± 4.6</td>
</tr>
<tr>
<td>DAU-DEXoxid</td>
<td>96.1 ± 1.2</td>
<td>58.8 ± 5.7</td>
<td>35.8 ± 4.7</td>
<td>4.4 ± 7.6</td>
</tr>
<tr>
<td>DAU-DEXred</td>
<td>92.6 ± 0.9</td>
<td>64.8 ± 1.0</td>
<td>43.8 ± 2.2</td>
<td>8.2 ± 9.4</td>
</tr>
<tr>
<td>DAU-DEX-MoAb 19-24</td>
<td>77.7 ± 1.6</td>
<td>25.4 ± 5.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>DAU-DEX-MoAb P3</td>
<td>14.1 ± 4.8</td>
<td>3.2 ± 2.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Treating fibrosarcoma cells with MoAb 19-24 did not affect the binding of radiolabeled MoAb 23-26. The mean was similar (2.5 × 10⁴ cpm) for radiolabeled MoAb 23-26 binding to control cells (cells grown in MEM-E) and cells exposed to MoAb 19-24. However, when cells were exposed for 24 h to unlabeled MoAb 23-26 and then incubated in fresh media for up to 72 h, the binding of radiolabeled MoAb 23-26 was significantly lower (due to competition) with respective values for times 0, 1, 5, 24, and 72 h of 0.3, 0.72, 1.22, 1.93, and 1.91 × 10⁴ cpm.

Binding of 125I-labeled anti-p102 MoAb to human fibrosarcoma HT-1080 cells was examined at 37°C and 4°C. The data are shown in Fig. 2. The amount of cell-bound radioactivity after 24 h of incubation at 37°C was 3.6-fold higher than at 4°C. A progressive increase was observed in the uptake of MoAb during the 24-h period: 0.05, 0.09, and 0.21 ng at 1, 6, and 24 h, respectively.

In order to examine how much MoAb was internalized by human fibrosarcoma cells, the radioactivity of plasma cell membranes and cytoplasm was measured. Of the total radioactivity, 54% was locatedariolabeled MoAb, 94.6% of radioactivity was located in the cell membranes and only 3.9% in the cytoplasm.

Cytotoxicity assays revealed significantly higher specificity of the DAU-DEX-MoAb 19-24 immunoconjugate than nonspecific immunoconjugate DAU-DEX-MoAb P3; also, it showed a greater ability to reduce the viability of in vitro fibrosarcoma cells [expressed as a percentage of dead cells (Table 1)]. The highest concentration of noconjugate DAU-DEX-MoAb P3; also, it showed a greater ability to reduce the viability of in vitro fibrosarcoma cells, which was about 36.8% of the efficacy of free DAU. While free DAU at a concentration of 0.1 µg/ml was able to kill 56.8% of the fibrosarcoma cells, DAU at the same concentration in the immunoconjugate revealed no cytotoxicity to fibrosarcoma cells. The cytotoxicity of DEXoxid and DEXred was tested at concentrations up to 230 µg/ml; neither showed any cytotoxic effect on the growth of fibrosarcoma cells.

To examine the time course of ADR uptake into the fibrosarcoma xenograft, sarcoma-bearing mice received injections of 14C-ADR. Two mice were sacrificed at 1, 6, and 24 h after receiving i.p. injections of 14C-ADR. Radioactivity of 14C was measured in blood, tumor, skin, muscle, brain, and the rest of the viscera (Table 2). The mean total tumor tissue weight in six mice used in this experiment was 5742 ± 5742 g. The levels of ADR 14C-radioactive equivalents in blood and brain tissue were low, corresponding to only 0.3% of the injected radioactivity. The radioactivity of the tumor tissue was slightly higher than that of the blood; however, it never exceeded 1% of the total injected radioactivity (Table 2). At 6 h, radioactivity was 19-fold higher in the liver and spleen and 12-fold higher in the kidney than the tumor xenograft. Similarly, all other normal tissues (lung, heart, muscle, and skin) showed higher levels of radioactivity than the tumor.

In order to examine the uptake of anti-p102 MoAb by tumor tissue, fibrosarcoma-bearing athymic mice received injections of 125I-labeled MoAb 19-24. The %ID/ml of blood was markedly higher at 6 h (15.89 ± 0.39) than at 1 h (9.13 ± 0.39) or 24 h (5.16 ± 0.34). The viscera (liver, spleen, kidney, and lung); blood ratios of 125I-labeled MoAb 19-24 at 1 h ranged from 0.51 to 0.57, and, except for the lung, the organ: blood ratios were lower at 6 and 24 h. The tumor tissue: blood ratio was 0.36 ± 0.21 (0.95 %ID) at 1 h and 0.61 ± 0.08 (9.47 %ID) at 6 h. At 24 h, the tumor tissue: blood ratio reached the highest value, 1.62 ± 0.28 (7.86 %ID), which was 3.7-fold higher than the uptake by the liver and about 5.6-fold higher than the uptake by the spleen. The uptake of nonspecific MoAb P3, measured at 24 h after i.p. injection of 125I-labeled MoAb P3, showed a tissue: blood ratio similar to that of normal tissue tested for 125I-labeled MoAb 19-24. However, the tumor tissue: blood ratio was lower (0.34 ± 0.01).

The uptake of 125I-labeled DAU-DEX-MoAb by tumor and normal tissues of fibrosarcoma-bearing athymic mice at 24 h after i.p. injection was examined in the same fashion as described above. Data listed in Table 3 clearly indicate similar blood ratios in normal tissue and in a group of mice that received free 125I-labeled MoAb 19-24 or radiolabeled MoAb linked to the immunoconjugate. A decrease in the tumor tissue: blood ratio from 1.62 ± 0.28 to 0.66 ± 0.05 was observed in the group of mice after injection of 125I-labeled DAU-DEX-MoAb 19-24. However, the uptake of the immunoconjugate with nonspecific 125I-labeled MoAb P3 was much lower (0.22 ± 0.03).

**DISCUSSION**

This paper reports on our continuing effort to use MoAbs to human sarcoma-associated antigens as a tool in diagnosing and treating soft tissue sarcoma (11-17). To date, we reported the production of several MoAbs against sarcoma-associated antigens p102 (11), p160, and p200 (29). Previous studies have shown that MoAb 19-24 generated against a malignant fibrous histiocytoma reacted with a cell surface mannos containing a glycoprotein with a molecular weight 102,000.

Table 2 Tissue uptake of 14C-adriamycin in fibrosarcoma-bearing athymic mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.7</td>
<td>4.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Liver</td>
<td>140.5</td>
<td>247.1</td>
<td>60.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>65.5</td>
<td>155.3</td>
<td>42.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>123.2</td>
<td>249.7</td>
<td>151.5</td>
</tr>
<tr>
<td>Lung</td>
<td>33.6</td>
<td>70.8</td>
<td>27.9</td>
</tr>
<tr>
<td>Heart</td>
<td>37.4</td>
<td>73.1</td>
<td>17.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>16.2</td>
<td>20.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Skin</td>
<td>24.8</td>
<td>27.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Brain</td>
<td>4.2</td>
<td>4.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Tumor</td>
<td>15.8</td>
<td>26.5</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*Mean value from two animals.

Numbers in parentheses, range.
Radiolabeled MoAb 19-24 was shown to xenograft-bearing athymic mice (12, 14, 15) and sarcoma-bearing humans, with negligible binding to normal tissue (13, 16). MoAb Analysis of DAU cytotoxicity to the fibrosarcoma cells revealed that tested for DAU was, on average, 2.3 times more effective than possible p102 modulation. Internalization of 125I-labeled MoAb 19-24 issues needed to be addressed: internalization of MoAb 19-24; and described by Uadia (> 10^9 M^-1)

Data obtained by both methods indicate that MoAb 19-24 is internalized via a glutaraldehyde bridge produced immunoconjugates with bodies via a glutaraldehyde bridge produced immunoconjugates with antibodies have been described (8-10). Conjugating DAU and anti-human T-cell MoAb T 101 bound in ADR-DEX-MoAb T 101 dextran bridge to a solid-phase p102 antigen, the immunoreactivity of immunoconjugate is not caused by the difference in molecular structure between ADR and DAU.

In vitro experiments, based on inhibition pattern of ADR (33, 34). However, the loss of in vitro cytotoxicity of MoAb linked to ADR (33) and similar cytotoxicity patterns of immunconjugates with test and control antibody (34), were evident. In our study, the in vitro cytotoxicity of DAU-DEX-MoAb 19-24 was found to be lower than the efficacy of free DAU, but it was much higher than the cytotoxicity of immunconjugate with control antibody (Table 1). In addition, DAU-DEX-MoAb 19-24 immunconjugates retain in vivo specificity to tumor tissue (Table 3). The molar ratios of DAU to MoAb 19-24 (1.6 to 6.1) were similar to those reported previously (33, 34).

A number of in vivo experiments have been performed to investigate the ability of immunconjugates to kill tumor cells (3–5, 7, 23). Most of these studies based in vivo cytotoxicity evaluation on survival time of experimental animals and retardation of tumor growth under variable conditions: single or multiple doses; and administration of free drug mixed with immunconjugates and/or free MoAb. Thus, it is difficult to compare data obtained by different investigators. We assume that the therapeutic or toxic effect of a drug or immunconjugate depends mostly on its tissue concentration and its prolonged action time in a given tumor. Therefore, we decided first to examine the tissue uptake and clearance of an antineoplastic drug and of MoAb 19-24 immunconjugates.

There was low uptake of i.p.-injected ADR by tumor tissue [<1.1 %ID Table 2]. Normal tissue uptake of ADR by athymic mice differed from that described previously for rats (29) and CD1 mice (36). The normal liver:heart tissue ratio in athymic mice was 6.7:1, in CD1 mice it was 1.7:1, and in rats it was ~0.3:1.

Our previous studies investigated dehalogenation of 125I-labeled MoAb 19-24. The labeled material mixed with mouse serum was stable to storage, yielding over 87% trichloroacetic acid-precipitable radioactivity after 4 days at 37°C. *In vivo dehalogenation with rapid clearance of the tracer from tumor and normal tissues is a major problem when using antibodies labeled with iodine isotopes and when expressing data in %ID (15). Based on excretion of free radiiodine, ~18 %ID is dehalogenated over the first 24 h (3).

In summary, the results of our studies show that the uptake of the MoAb 19-24 immunconjugate by tumor tissue is higher than by normal tissues, supporting our suggestion that MoAb 19-24 immunconjugates may play an important role in managing soft tissue sarcomas.

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