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 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 immunoconjugate was 79.4% as efficient as free daunomycin and, at 1 μg/ml concentration, 36.8% as efficient. Control nonspecific murine monoclonal antibody P3 immunconjugates were relatively ineffective.

The distribution of 14C-daunorubicin, 125I-labeled monoclonal antibody 19-24, and 125I-labeled 19-24 immunoconjugate was also evaluated over a 24-h 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 immunoconjugates, 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, 0.25 ± 0.01) for nonspecific monoclonal antibody P3 immunconjugates. Thus, it appears that, compared to free daunomycin, monoclonal antibody 19-24 immunoconjugates may be more effective 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 cytotoxic to normal tissues.

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 immunoconjugates 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 P3 (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 mEq phosphate-0.15 M NaCl-1 mEq MgCl₂-0.02% Na₂HPO₄, 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 immunoconjugation 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. DEXoxid was then dialyzed against water, lyophilized, and stored at 4°C. The DEXoxid was incubated with DAU in PBS at drug:DEXoxid weight ratios varying from 1:7 to 1:1. The DAU-DEXoxid conjugate was separated from free DAU by dialysis against PBS. Finally, the DAU-DEXoxid conjugate was incubated with MoAb (19-24 or P3), and any remaining polyaldehyde-avid active sites in the DAU-DEXoxid-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 doceyl sulfate-polyacrylamide gel electrophoresis (25).
In V'aro labeled)DAU-Dextran-IgG. Loading well; used in the immunoconjugate preparation with a molecular weight of ~150,000; w, plates with protein A-purified MoAbs 19-24 (10 μg/ml) or 23-26 (10 μg/ml) p102 epitope, direct radioimmunoassay was performed to determine the modulation and other.

The cells were washed and harvested with 0.05% trypsin-EDTA (GIBCO). The uptake was calculated by incubating HT-1080 cells (4 × 10^6 cells/well) with MoAb at 37°C or 4°C for 1 h. The cells were then washed and harvested for 24 h. After incubation, the cytotoxicity of DAU-DEX-MoAb immunoconjugates was determined using p102 antigen; therefore, no additional studies were performed.

In a different experiment, ~6 × 10^6 fibrosarcoma cells were grown in a flask and incubated with 125I-labeled MoAb 19-24 at 37°C in 5% CO_2 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).

In V'aro labeled)DAU-Dextran-IgG. Loading well; used in the immunoconjugate preparation with a molecular weight of ~150,000; w, plates with protein A-purified MoAbs 19-24 (10 μg/ml) or 23-26 (10 μg/ml) p102 epitope, direct radioimmunoassay was performed to determine the modulation and other.

The cells were washed and harvested with 0.05% trypsin-EDTA. The cells were resuspended in 0.4% trypsin 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 N 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 immunoconjugates 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) immunoconjugate. 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 immunoconjugates, the calculated DAU:total protein molar ratio ranged from 1.9 to 6.1. The presence of high molecular weight immunoconjugates 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 immunoconjugates; therefore, we used protein G to remove untreated (free) antibody from the reaction mixture. Furthermore, there was no difference in binding between immunoconjugates diluted up to 1:5000 (60 ng MoAb in immunoconjugate/ml) and free (unconjugated) MoAb 19-24 (60 ng/ml).

**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 (10⁴ 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% CO_2 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 MoAb at 37°C or 4°C for 1 h. The cells were then 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 internalization of anti-p102 MoAb 19-24 was examined using p102-antibody 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 immunoconjugate preparation with a molecular weight of ~150,000; w, loading well; t, top of resolving gel.

**Fig. 1.** Polyacrylamide gel electrophoresis (7% under nonreducing conditions) of 125I-labeled MoAb at 37°C or 4°C for 1 h. The supernatant was removed, and washed cells were lysed in 0.05 ml of 1% sodium dodecyl sulfate. A cotton swab was used to wipe out the lysed cells, and the radioactivity was measured. All assays for this experiment and other in vitro studies reported here were performed in triplicate.

In a different experiment, ~6 × 10⁶ fibrosarcoma cells were grown in a flask and incubated with 125I-labeled MoAb 19-24 at 37°C for 1 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% CO_2 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).

**Fig. 2.** Uptake of anti-p102 MoAb 19-24 by fibrosarcoma cells. Cells (10⁶ 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.
After 36 h, the cells were harvested, resuspended in trypan blue, and counted. Cytotoxicity binding of radiolabeled MoAb 23-26. The mean was similar (2.5 ± 0.1 ng/ml) for radiolabeled 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 × 10^4 cpm.

Table 1

<table>
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<tr>
<th>Cytotoxicity</th>
<th>10.0</th>
<th>1.0</th>
<th>0.1</th>
<th>0.001</th>
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<td></td>
<td>µg/ml DAU</td>
<td>DAU</td>
<td>DAU</td>
<td>DAU</td>
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<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>
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<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>
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<tr>
<td>DAU-DExMoAb 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-DExMoAb 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 × 10^4 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 × 10^4 cpm.

The viscera (liver, spleen, kidney, and lung) blood:radioactivity ratios of 125I-labeled MoAb 23-24 at 1 h ranged from 0.51 to 0.57, and, except for the lung, the organ:radioactivity ratios were lower at 6 and 24 h. The tumor:radio 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:radio 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:radio ratio similar to that of normal tissue tested for 125I-labeled MoAb 19-24. However, the tumor:radio 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 2 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 immunocojugate. A decrease in the tumor:radio 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 immunocojugate 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 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).

Table 2

| Tissue uptake of 14C-daunomycin in fibrosarcoma-bearing athymic mice |
|-----------------------------|---------|---------|---------|
|                             | 1 h     | 6 h     | 24 h    |
| Blood                       | 3.7*    | 4.4     | 3.2     |
| Liver                       | 140.5   | 247.5   | 60.6    |
| Kidney                      | 60.5    | 155.3   | 42.2    |
| Spleen                      | 123.2   | 240.7   | 151.5   |
| Lung                        | 33.6    | 70.8    | 27.9    |
| Heart                       | 37.3    | 78.6-83.0 | 21.2-25.3 |
| Muscle                      | 16.2    | 20.1    | 9.1     |
| Skin                        | 15.7-16.7 | 12.9-27.3 | 8.4-9.7 |
| Brain                       | 4.2     | 4.2     | 3.3     |
| Tumor                       | 15.3    | 13.2    | 6.1     |

*Mean value from two animals.
*Numbers in parentheses, range.

ADR 14C radioactive equivalents in cpm/ml blood or g wet tissue.
(p102) with pl 4.7 (11, 25). Radiolabeled MoAb 19-24 was shown to xenograft-bearing athymic mice (12, 14, 15) and sarcoma-bearing DAU was, on average, 2.3 times more effective than for, in this study we investigated the specificity and ability of MoAb possible p102 modulation. Internalization of 125I-labeled MoAb 19-24 radioactivity was measured in the cytoplasm and cell membranes. described by Uadia > (109 M -1)

Several methods of coupling anthracycline antineoplastic drugs to antibodies have been described (8–10). Conjugating DAU and antibody via a glutaraldehyde bridge produced immunoconjugates with moderate activity, whereas using carbodiimide yielded inactive con-

Kidney 0.36 ± 0.21 0.61 ± 0.08 1.62 ± 0.26 0.66 ± 0.05

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.4 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 immunoconjugate by tumor tissue is higher than by normal tissues, supporting our suggestion that MoAb 19-24 immunoconjugates may play an important role in managing soft tissue sarcomas.

ACKNOWLEDGMENTS

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8. Hruby, R., Levy, R., Maron, R., Wilchek, M., Arnon, R., and Selc, M. The covalent binding of daunomycin and Adriamycin to antibodies, with retention of both drug and inhibition pattern of ADR (33, 34). However, the loss of in vitro cytotoxicity of MoAb linked to ADR (33) and similar cytotoxicity patterns of immunoconjugates 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 immunoconjugate with control antibody (Table 1). In addition, DAU-DEX-MoAb 19-24 immunoconjugates 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 investi-
gate the ability of immunoconjugates 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 immunoconjugates 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 immunoconjugate 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 immunoconjugates.

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.4 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 immunoconjugate by tumor tissue is higher than by normal tissues, supporting our suggestion that MoAb 19-24 immunoconjugates may play an important role in managing soft tissue sarcomas.

Table 3 Tissue: blood ratio of 125I-labeled MoAb 19-24 and Daunomycin-dextran-MoAb 19-24 in fibrosarcoma-bearing mice (24 h)


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