Intratumoral Injection of an Adriamycin Immunoconjugate against Human Pancreatic Cancer Xenografts

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We have evaluated the effect of an adriamycin conjugate of monoclonal antibody Nd2 (ADM-Nd2) on the growth rate of SW1990 xenografts grown subcutaneously in athymic nude mice. Intravenous or intraperitoneal administration of radiolabeled Nd2 resulted in a maximum tumor accumulation of approximately 45% of the initial dose/g of tumor 3–7 days after administration. However, administration into the tumor produced retention of 1200%ID/g 1 day after, with 50% of this high value remaining even at 7 days after administration. In contrast, intratumoral administration of a non-specific immunoglobulin showed a lower initial retention and rapid loss of label. Both intravenously and intratumorally administered ADM-Nd2 reduced the growth rate of SW1990 xenografts. While a single intravenous administration arrested growth for about two weeks, a single intratumoral injection prevented any increase in tumor size even 45 days after administration. Xenografts treated with ADM-Nd2 showed degenerative changes at the histological level. Neither Nd2 alone nor Adriamycin alone inhibited growth when administered at the same dose as the conjugate.

Key words: Pancreatic cancer — Adriamycin — Immunochemotherapy — Immunoconjugate — Intratumoral administration

Conventional cancer therapeutic approaches have not been successful with pancreatic tumors. The five-year survival is presently less than 5%.[1] Radiation therapy is complicated by the fact that the pancreas is surrounded by radiation-sensitive organs. Moreover, irradiation alone appears to have little effect on overall survival, although there are short-term palliative benefits.[2] Chemotherapy with a single drug or a combination of drugs has little effect on survival.[3, 4] At this point, surgical intervention provides the best improvement in overall survival rates. The benefit is mainly to the patients with localized cancer.[5] However, at the time of diagnosis only a small percentage of patients have tumors sufficiently localized to allow surgical resection.[6] Currently, in addition to noninvasive external-beam photon radiation, treatment for unresectable cancer includes interstitial therapy with an implanted radioactive source (usually ^{125}I) or intraoperative radiotherapy. Widespread metastases frequently occur after treatment.[6] Recently, intratumoral administration of ^{125}I-labeled monoclonal antibodies (MAb) directed against colon tumors has been tried.[7] Intratumoral administration could also be applied to pancreatic cancer and might be an alternative to radiation after laparotomy. Physiological factors such as a poor blood supply[8] have prevented the delivery of a large portion of the initial dose of immunoconjugates to the tumor after systemic administration.[9, 10] Intratumoral administration of a cytotoxic immunoconjugate would circumvent the problem of poor vascularization of pancreatic tumors. Moreover, intratumoral administration would reduce the systemic toxicity of radiation or chemotherapy. Patients with pancreatic cancer are often so severely weakened by the disease that they cannot tolerate high doses of drugs or radiation.

In this study we examined the antitumor effect of both intratumorally and intravenously injected Adriamycin (ADM) conjugated to a monoclonal antibody directed against pancreatic tumor, Nd2.[11] Nakata et al. have shown that ADM has good cytotoxicity against pancreatic cancer cells in vitro.[12] The Nd2 antibody was developed against mucins purified from xenografts of the human pancreatic cancer cell line SW1990.[11] It did not react with fixed tissues of normal pancreas or chronic pancreatitis but did react with 84% of the pancreatic tumors examined.[13] It also successfully discriminated between normal and cancerous gastric and colonic tissues.[11] Nd2 antigen is not detectable in sera of pancreatic cancer patients.[11, 13] ^{111}In-labeled Nd2 produced good radioimages of pancreatic cancer xenografts in athymic nude mice[13] and was 67% positive in radioimaging pancreatic ductal cell carcinomas in humans.[14]

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MATERIALS AND METHODS

Radiolabeling of antibody Nd2 is a murine MAb of the IgG1 isotype directed against mucins purified from xenografts of the human pancreatic cancer cell line SW1990.[11] Ascitic fluid was produced in nude mice intraperitoneally implanted with 5×10^6 hybridomas. The ascitic fluid was centrifuged (3,000 rpm, 15 min), filtered through a 0.22 μm STERIVEX-GS filter and purified with an AffiGel Protein A MAPS II Kit (Bio-Rad Lab., Hercules, CA). The resultant solution was concentrated by ultrafiltration (THK24, Millipore, Bedford, MA) and passed through a PD-10 column of Sephadex G-25M (Pharmacia, Uppsala, Sweden). Protein was estimated with the BCA Protein Assay Reagent (Pierce, Rockford, IL).

The chloramine-T method[15] was used to iodinate Nd2 with ^{125}I. Free ^{125}I was separated from bound iodine by gel filtration on Sephadex G-25M. The radioactivity of labeled Nd2 was adjusted to approximately 40–60 MBq-^{125}I/mg-protein.

Conjugation of Nd2 to ADM Antibody was conjugated to ADM according to the reported method.[16] An acid-labile bond was introduced via *cis*-aconitic anhydride (cAA).[16–18] The carboxyl group of cAA is joined to amino groups on the antibody by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).[19] N-Hydroxysulfosuccinimide (sulfo-NHS) was used to increase the likelihood of EDC conjugation.[20] ADM hydrochloride (Kyowa Hakko, Tokyo) was dissolved in distilled water and cAA (Sigma Chemical Co., St. Louis, MO) was added at 4°C and pH 9.0. The pH was then adjusted to 2.0. The ADM-cAA precipitate was collected by centrifugation (3,000 rpm, 10 min). ADM-cAA and sulfo-NHS (Pierce) were dissolved in dimethylformamide, and EDC (Pierce) was added at 4°C. A mixture of this solution and a solution of Nd2 antibody was stirred for 2 h at 4°C. The ADM-Nd2 conjugate was isolated by gel filtration on Sephadex G-50 (0.1 M phosphate buffer). ADM and protein were quantitated by determining the absorbance at 490 nm and 280 nm without references, respectively, compared to standard curves previously constructed with known quantities of ADM and bovine serum albumin, respectively.

HPLC of ADM-Nd2 conjugate The ADM-Nd2 conjugate was eluted from a TSK G-3000SW column (Tosoh, Kanagawa) with 10 mM phosphate-buffered saline (pH 7.4) at 0.5 ml/min. Absorbances at 280 and 490 nm were monitored simultaneously.

ELISA of ADM-Nd2 conjugate Purified SW1990 mucins (3.1–0.019 ng/50 μl/well)[21] were adsorbed on 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ). Peroxidase-conjugated second antibody and ABTS were used in the secondary reactions.[22] Absorbances of the wells were determined at 405 nm.

Cytotoxic effect of ADM-Nd2 SW1990 cells were cultured in 24-well plates (Falcon Plastics, Becton Dickinson Labware) in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum in a 5% CO_2 atmosphere; 1×10^4 cells/well were cultured for 48 h at 37°C. The plates were washed with phosphate-buffered saline, ADM-Nd2, ADM or Nd2 was added to the wells (ADM concentration was 0.05–1.6 μg/well), and the cells were cultured for an additional 72 h. The cells were washed with buffered saline, harvested and counted with a Coulter Counter (Coulter Electronics Ltd., Luton, UK). The viability of counted cells was confirmed with trypan blue.

Production of xenografts Confluent SW1990 cells were harvested and resuspended in culture medium. SW1990 cells (5×10^6) were inoculated subcutaneously into the left flank of athymic Balb/c nude mice. Mice with tumors of approximately 0.5 cm in diameter (usually 2 weeks after inoculation) were used in the study. Tumor volume was estimated using the following formula:

$$V = 4/3 \times Pi \times a/2 \times b/2 \times h/2$$

Administration of antibody *in vivo* 1) Intravenous administration. ^{125}I-labeled Nd2 (7.5 μCi/5.0 μg) or ADM-Nd2 (100 μg Nd2 and 15 μg ADM) was administered via the caudal vein in a volume of 50 μl. 2) Intraperitoneal administration. A 27G needle was used. Antibody solution volume was 50 μl. 3) Intratumoral administration. A 27G needle was used. Antibody solution volume was 50 μl. ^{125}I-labeled non-specific mouse IgG1 was used as a control in the intratumoral studies.

Four mice were used in each group in the studies with radiolabeled Nd2. At 12 h, 1 day, 3 days and 7 days after the administration of radiolabeled antibody, mice were killed and the weight, volume, and radioactivity of tumor, blood, urine and other organs were determined. ^{125}I radioactivity was measured with a gamma counter (Auto-Gamma 5550 Counting System, Packard Japan, Tokyo).

RESULTS

Biodistribution of radiolabeled Nd2
Intravenous administration: Intravenously injected ^{125}I-Nd2 accumulated in SW1990 xenografts as shown in Fig. 1A: the percentage of initial dose/g of tissue (%ID/g) in the tumor was 6.5% at 12 h, 16.8% at 1 day, 45.0% at 3 days and 37.8% at 7 days. Thus, the tumor levels of Nd2 increased with time until 3 days after administration. Over the next four days (between 3 and 7 days after administration) there was a slight decrease in tumor levels. On the other hand, ^{125}I radioactivity in blood (Fig. 1A and 2) and other organs decreased continuously from their levels at the earliest time point (12 h) until 7 days
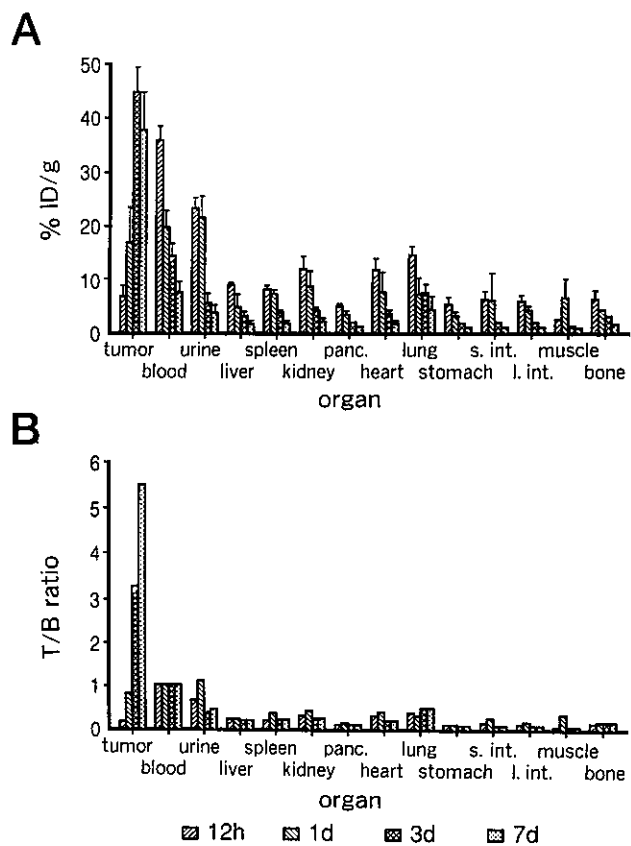
A

B

Fig. 1. Biodistribution of ^{125}I-Nd2 after intravenous and intraperitoneal administration. A. %ID/g after i.v. administration. B. Tissue-to-blood ratios of radioactivity. Values are from the same mice as in A. The bars are the means±SD (n=4).

Fig. 2. Change in %ID/g in tumor, blood and urine with time following intravenous, intraperitoneal, and intratumoral administration of ^{125}I-Nd2. The values are the means±SD (n=4).

after administration. The %ID/g in urine was 23.1% at 12 h, 21.5% at 1 day, 5.4% at 3 days and 3.47% at 7 days. Thus, most of the urinary excretion of ^{125}I-Nd2 administered i.v. had occurred by 1 day after administration. After the blood and urine, lung, heart and kidney had the highest %ID/g among the other organs. The tumor-to-blood (T/B) ratio of the tumor was 0.18 at 12 h, 0.83 at 1 day, 3.24 at 3 days and 5.53 at 7 days after administration (Fig. 1B). The T/B ratios at 3 days and 7 days were thus especially high. The T/B ratios of organs other than the tumor were never more than one.

Intraperitoneal administration: The %ID/g in the tumor increased over seven days: 7.3% at 12 h, 13.4% at 1 day, 40.8% at 3 days and 44.9% at 7 days (Fig. 2). Blood %ID/g reached its maximum 1 day after i.p. administration. At 12 h urinary %ID/g was almost twice that of blood. This differed from the i.v. group which showed a lower urinary %ID/g. Pancreas and lung showed the
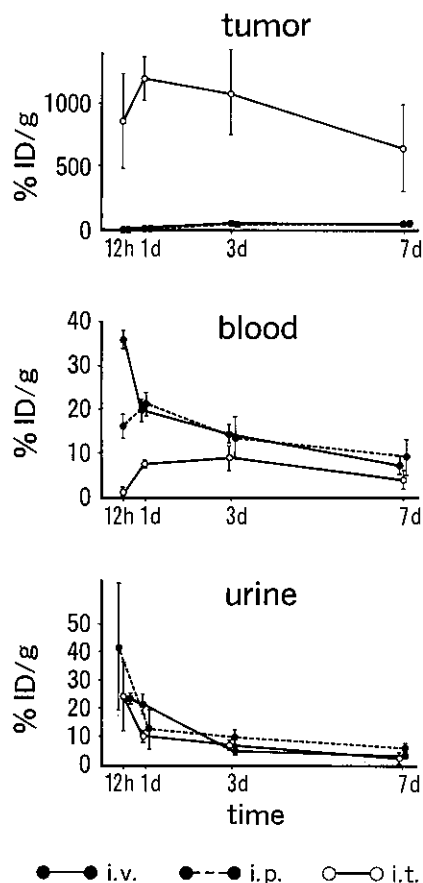
next highest %ID/g after blood and urine. The i.p. group had T/B ratios (not shown) similar to those of the i.v. group (Fig. 1B). There were no significant differences between the i.v. and i.p. groups in either the %ID/g in the tumor or the tumor-to-blood ratio (Student's t test). Intratumoral administration: The %ID/g in the tumor was high (Fig. 3A): 850.8% at 12 h, 1200% at 1 day, 1074% at 3 days and 642.1% at 7 days. Thus, more than about 50% of the maximum ^{125}I-Nd2 measured in the tumor remained in the tumor even 7 days after administration. The %ID/g in blood was much lower than that for the i.v. group (Fig. 2, 1.1% at 12 h, 7.4% at 1 day, 8.8% at 3 days and 4.0% at 7 days). With the non-specific IgG1 the %ID/g in the tumor was 591.2% at 12 h, 265.0% at 1 day, 76.1% at 3 days and 67.8% at 7 days after administration (Fig. 3B and 4). The %ID/g of the non-specific antibody in the tumor, even at 1 day, was much less than that for the ^{125}I-Nd2 i.t. group. In the
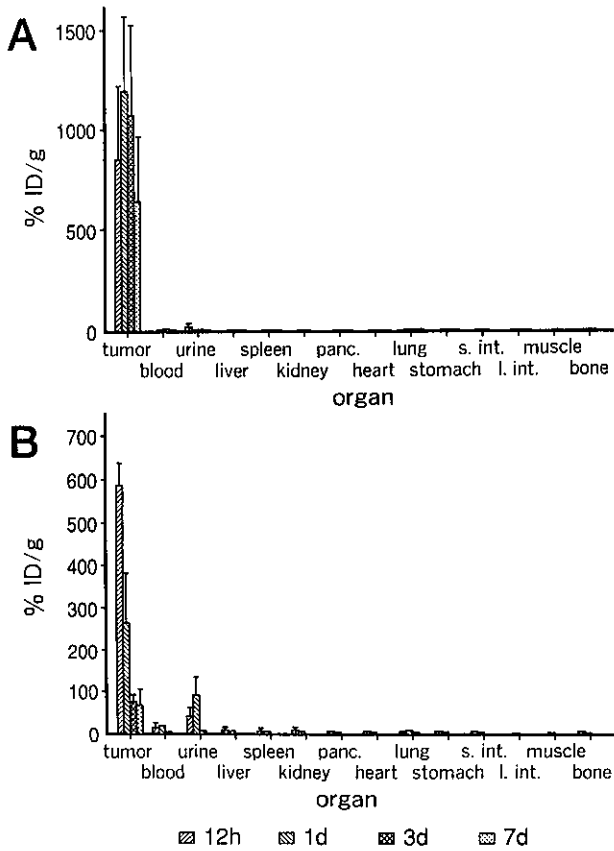
Fig. 3. Biodistribution of ^{125}I-Nd2 after intratumoral administration. A. %ID/g after i.t. administration of ^{125}I-Nd2. B. %ID/g after intratumoral administration of ^{125}I-labeled nonspecific IgG1. The bars are the means±SD (n=4).

Fig. 4. Change in %ID/g in tumor, blood and urine with time following intratumoral administration of ^{125}I-labeled Nd2 or non-specific IgG1. The values are the means±SD (n=4).

non-specific IgG1 group ^{125}I-Ig disappeared rapidly from the tumor (Fig. 4).

A comparison of the levels of ^{125}I-Nd2 remaining in the tumor after the different routes of administration (Fig. 2) showed that the intratumoral route produced the highest tumor retention among the three methods after seven days. Between day one and day seven, the %ID/g after i.t. administration was between 17 and 71 times that after i.v. administration.

Immunoreactivity of ADM-Nd2 and free Nd2 with antigen All the ADM (490 nm) eluted with Nd2 (280 nm), indicating successful ADM conjugation to Nd2 (Fig. 5A). The absorbances at 490 and 280 nm of the ADM-Nd2 conjugate were used to determine the molecular ratio of ADM to Nd2 (10–20:1). The ADM-Nd2 used in this study had an ADM-to-Nd2 molecular ratio of 16:1. ELISA of the ADM-Nd2 conjugate against purified mucin of SW1990 showed good reactivity of the immunoconjugate with antigen. Reactivity of the conju-
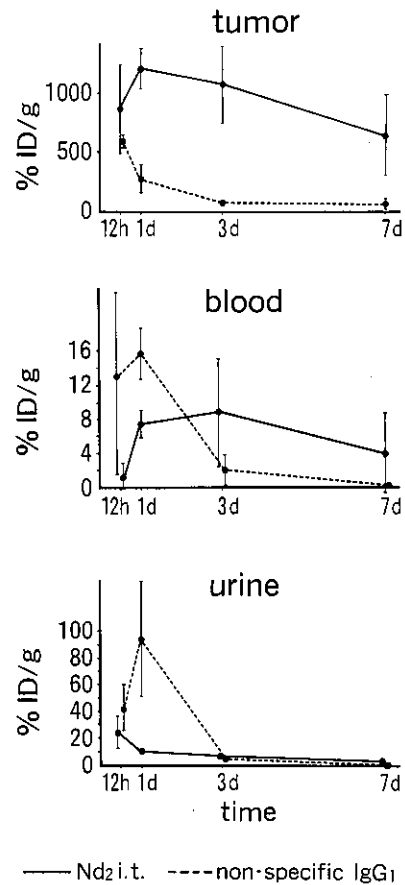
gate was slightly lower than that of the free Nd2, as might be expected if some ADM had been conjugated to the Nd2-binding site (Fig. 5B).

Cytotoxicity of ADM-Nd2 in vitro The number of surviving SW1990 cells treated in vitro with ADM-Nd2 decreased with increasing ADM concentration (Fig. 6). The effect of ADM-Nd2 was almost the same as that of ADM alone. Treatment of cells with Nd2 alone did not affect cell growth.

Antitumor effect of ADM-Nd2 in vivo In view of the similarities of %ID/g in the tumor and T/B ratios between i.v. and i.p. administration, only i.v. administration of ADM-Nd2 was compared to i.t. administration. Intravenous administration of ADM alone had the same effect as the administration of saline (Fig. 7A). However, even a single intravenous administration of ADM-Nd2 to SW1990-bearing nude mice arrested tumor growth until the 13th day. Thereafter, the tumor resumed growth.

A

450k   45k

280nm

490nm

0   18
retention time (min)

B

Nd₂

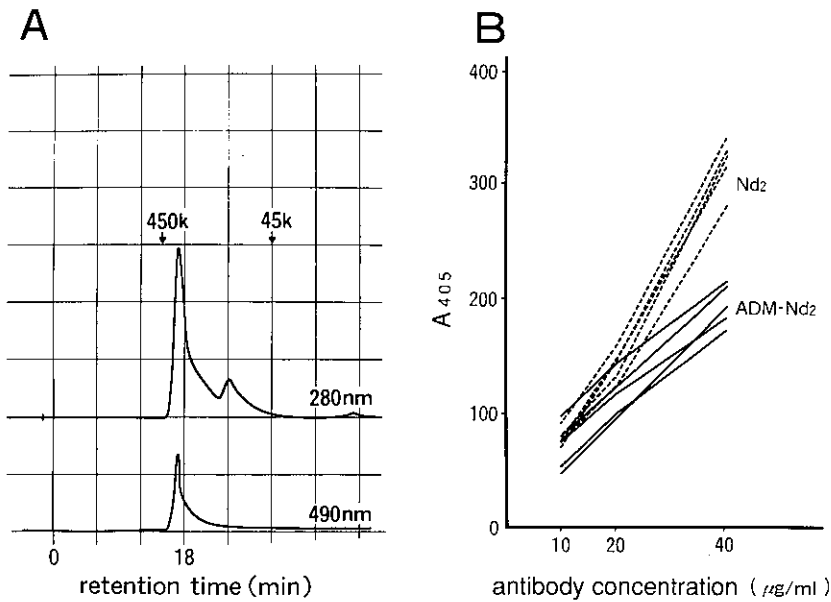ADM-Nd₂

10   20   40
antibody concentration (µg/ml)

Fig. 5. Conjugation of Nd2 and immuno-reactivity of ADM-Nd2 conjugate. A. HPLC analysis of ADM-Nd2 conjugate. After conjugation of Nd2 with ADM as described in "Materials and Methods," free ADM was separated from ADM conjugated to Nd2 by column chromatography on Sephadex G-50. ADM-Nd2 was then passed through a TSK G-3000SW column. B. Reactivity of ADM-Nd2 conjugate with its antigen. Reactivities of free Nd2 and ADM-Nd2 were compared by ELISA against purified mucin of SW1990 xenografts. The X-axis shows antibody concentration and the Y-axis absorbance. The lines correspond to adsorbed purified mucin concentrations of 3.1–0.019 ng/50 µl/well.

Nd₂ concentration (µg/ml)

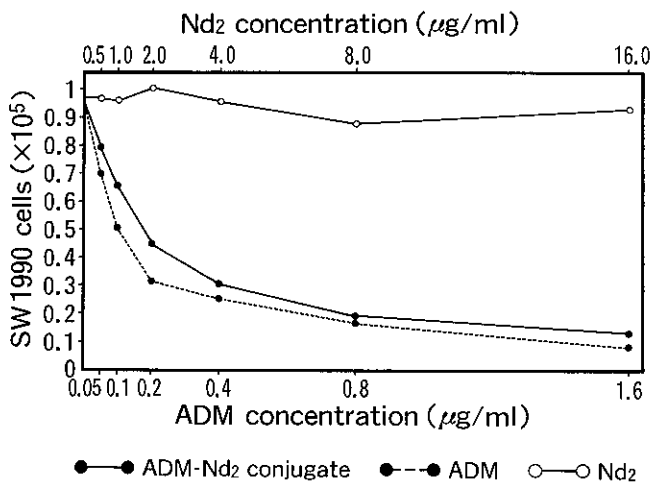●——● ADM-Nd₂ conjugate   ●--● ADM   ○——○ Nd₂

Fig. 6. Cytotoxic effect of free ADM and ADM-Nd2 against SW1990 cells *in vitro*. Cytotoxicity of ADM-Nd2 conjugate was compared to that of ADM alone. The points are the means of duplicate determinations.

After administration of either ADM alone or normal saline directly into the tumor (Fig. 7B), there was no inhibition of tumor growth. In all the mice given ADM-Nd2, however, there was little change in tumor size even after 45 days. At this time, cells of ADM-Nd2-treated tumors showed many types of degenerative changes, including the formation of large vacuoles, autolysis of cytoplasm, pyknosis and karyorrhexis.

A

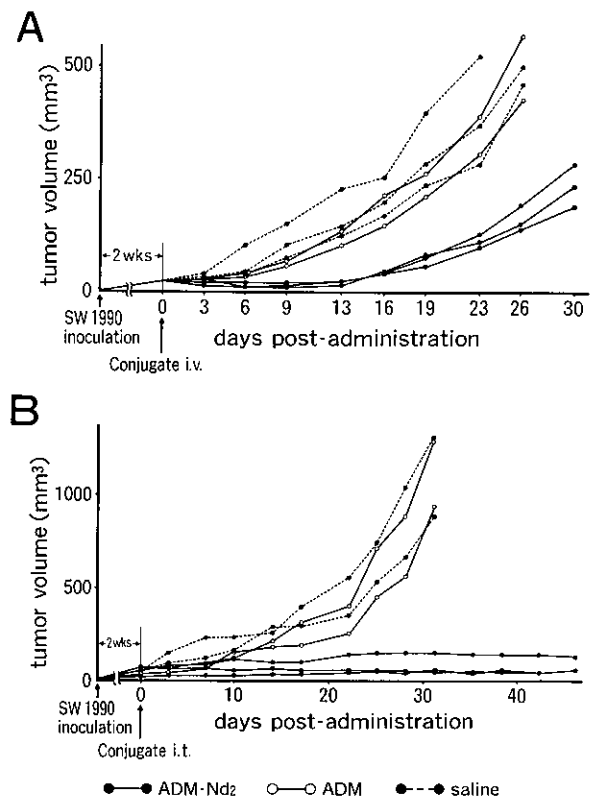## B

●——● ADM-Nd₂   ○——○ ADM   ●--● saline

Fig. 7. Effect of ADM-Nd2 on the growth rate of SW1990 xenografts. A. Intravenous administration. Tumor volume was calculated as described in "Materials and Methods." B. Intratumoral administration.

Thus, a single intratumoral administration of ADM immunoconjugate was highly effective in bringing about tumor arrest and necrosis. A single intravenous injection also inhibited tumor growth to some degree.

DISCUSSION

Early diagnosis of pancreatic cancer is difficult because of the occult position of the organ and because symptoms are ill-defined. Fewer than 20% of patients have disease confined to the pancreas at the time of diagnosis.[6] Thus, pancreatic cancer is presently a disease with few therapeutic possibilities. Monoclonal antibodies against tumor-specific antigens offer the capability of targeting both the primary tumor and any metastatic lesions. Recently, a number of different antibodies have shown promise in the localization of pancreatic tumors.[13, 23–25] However, delivery of sufficient killing radiation[9, 10] or cytotoxic drug to the tumor via antibodies after systemic administration, without systemic side effects, is hampered by a number of physiological factors.[8] One is the degree of tumor vascularization. Unfortunately, pancreatic tumors are usually poorly vascularized. Several authors[26, 27] have suggested that a more localized administration (i.p.) might be beneficial with tumors located in the peritoneal cavity. However, the tumor uptake of immunoconjugate appeared to be similar after i.p. and i.v. administrations, even when the tumor was i.p.[28] In this study we have shown that administration of Nd2 i.p. did not improve tumor accumulation over i.v. administration. Another factor reducing delivery to the tumor is the interstitial transport rate of the molecules.[8, 29] To increase the transport rate, as well as to reduce the antigenicity of murine antibodies, investigators have reduced the size of antibodies by the production of fragments.

While firm cell binding and internalization may not be as important with radioimmunoconjugates, they are important with many toxin or drug immunoconjugates. The effectiveness of intratumorally administered ADM-intact Nd2 suggests that it might provide a means to circumvent the problem of sparse tissue vascularization.

ADM (doxorubicin) is one of the most generally applicable anticancer agents available.[30] However, ADM, alone or in combination with a number of other drugs, is largely ineffective in improving the survival of patients with pancreatic cancer. We have shown in this and another study[12] that pancreatic cancer cells are susceptible to free ADM *in vitro*. The *in vitro* chemosensitivity study with prolonged exposure showed that ADM-Nd2 retained cytotoxicity to SW1990 cells. In view of the antigen-binding activity of Nd2, even a short exposure

should show a superior cytotoxic effect of the conjugate to that of ADM alone. Therefore, intratumoral injection of ADM-Nd2 might be effective *in vivo*. Indeed, the present study showed that *in vivo* free ADM had little effect on the pancreatic tumor, while the same dose of ADM when conjugated to Nd2 was highly effective. Other authors have reported a similar difference in efficacy *in vivo* between free ADM (or a related drug, daunorubicin) and ADM-conjugated antibody.[31–33] The precise cell killing mechanism of ADM is not known.[30] Its action seems to require its presence both outside and inside the cell.[34] In our present study, we conjugated ADM to the antibody via an acid-labile bond.[16, 18] It has been shown that antibody is irreversibly bound to the surface of the cell, and is slowly internalized and degraded.[35] This may occur by traversal through the lysosomal compartment, where free ADM would be released due to the acid milieu or to proteolysis of the antibody. Thus, use of an ADM conjugate to an antibody would ensure the presence of ADM both inside and outside the cell.

The immunoconjugate ADM-Nd2 arrested tumor growth regardless of whether it was administered i.v. or i.t. After a single i.v. administration, tumor growth was suppressed for almost two weeks while after a single i.t. injection, tumor growth was suppressed for up to 45 days. The i.t. group retained about 17 times more radio-labeled Nd2 than the i.v. group after 7 days. An antibody with no tumor reactivity was lost much more rapidly from the tumor, indicating that specific binding to tumor antigens accounted for the high tumor retention. Thus, a single dose of ADM-Nd2 was more effective when administered i.t. because the tumor received more of the initial dose, and retained a larger portion of it for a longer time.

In summary, this study has shown that intratumoral administration of ADM conjugated to the monoclonal antibody Nd2 is effective in arresting growth and in producing necrosis of pancreatic tumors. Intratumoral administration would not be an unusual procedure in the treatment of pancreatic cancer, since similarly invasive procedures are already employed, such as intraoperative and interstitial radiation. Concurrent intravenous administration may be effective in targeting metastatic lesions.

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