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Combination Therapy with Antibody and Interleukin-2 Gene Transfer against Multidrug-resistant Cancer Cells

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In the present study, we examined the effect of interleukin-2 (IL-2) gene transfer into multidrug resistance (MDR) cancer cells on the therapeutic efficacy of MRK16. Human MDR ovarian cancer cells, AD10, were transduced with a bicistronic IL-2 retrovirus, Ha-IL2-IRES-Neo. The G418-resistant population, IL2-AD10, secreted IL-2 into the culture supernatant and did not form a tumor mass in nude mice. The IL2-AD10 cells were more susceptible to the cytotoxicity of murine spleen cells than AD10 cells *in vitro*. For examination of the effect of IL-2 gene transfer on the antitumor activity of MRK16 against P-glycoprotein-positive tumors, IL2-AD10 cells were co-transplanted s.c. with AD10 cells into nude mice in a ratio of 1 : 3, and the mice were treated with MRK16 on days 2 and 7. MRK16 markedly inhibited the growth of AD10 cells mixed with IL2-AD10 cells under conditions (0.3–1 μg/body) where it showed only marginal effects on the growth of AD10 tumors. These findings suggest that IL-2 gene transfer potentiates the antitumor activity of MRK16 against MDR tumors.

Key words: Multidrug resistance — Antibody-dependent cell-mediated cytotoxicity — Interleukin-2 — Natural killer — Internal ribosome entry site

Multidrug resistance (MDR) of tumors is a major problem in cancer chemotherapy. Cell surface P-glycoprotein encoded by the *MDR1* gene has been shown to function as an ATP-driven efflux pump for various cytotoxic drugs.[1–3] The expression of P-glycoprotein has been found to be elevated in intrinsically drug-resistant cancers as well as in some tumors that acquired drug resistance during chemotherapy with P-glycoprotein-related anticancer agents.[4,5] On the other hand, P-glycoprotein non-related drugs such as cisplatin, mitomycin and 5-fluorouracil may be effective against some solid tumors, but there is a consensus among clinicians that it is difficult to control solid tumors with chemotherapy alone. Therefore, it seems necessary to assist conventional chemotherapy with other approaches, such as immunotherapy and gene therapy in the treatment of MDR tumors.

Since MDR tumor cells express P-glycoprotein on their cell surface, P-glycoprotein could be a suitable molecular target. A murine monoclonal antibody MRK16 (IgG2a) recognizes a cell surface epitope of P-glycoprotein.[6] In a previous study, we showed that an anti-human P-glycoprotein monoclonal antibody, MRK16, inhibited the *in vivo* growth of MDR human tumors in nude mice.[7] The MRK16 antibody induced antibody-dependent cellular cytotoxicity (ADCC) reaction of murine and human lymphocytes and monocytes against human MDR cancer cells *in vitro*.[7–9] In these studies, a high dose of MRK16 was required to achieve complete remission of MDR tumors since the ability of anti-P-glycoprotein monoclonal antibody (mAb) to penetrate a solid tumor could be limited by both the affinity and molecular size of the mAb. MRK16 did not show any toxicity to normal mouse tissues because it recognizes a human-specific epitope of P-glycoprotein. It was also reported that analysis of the physiology, anatomy, and histology of mice homozygous for a disruption of the *mdr1a* gene encoding a drug-transporting P-glycoprotein did not reveal clear abnormalities at various ages. However, the mice displayed elevated drug levels in the brain upon systemic administration of P-glycoprotein-related anticancer agents, owing to impairment of the blood-brain barrier.[10] These data suggest that MRK16 would not be highly toxic when given to humans singly, but the systemic administration of high-dose MRK16 may have some toxic effects on P-glycoprotein-positive normal cells, such as endothelial or adrenal cells. Therefore, efforts should be made to reduce the dose of MRK16 required to treat MDR tumors.

Interleukin-2 (IL-2) is known to induce or augment various types of lymphocyte-mediated cytotoxicity, including activation of natural killer (NK) cells, which can mediate ADCC, against cancer cells.[11,12] Since the half-life of injected IL-2 is short and systemic administration

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of a high dose of IL-2 causes severe side effects,[13] genetic approaches have been applied to achieve high local concentrations of IL-2 at tumor sites. IL-2 gene transfer into tumor-infiltrating lymphocytes[14] or tumor cells[15] and re-infusion of IL-2-modified cells have been shown to potentiate cell-mediated antitumor immunity in murine models.

In the present study we designed a combination therapy with antibody and IL-2 gene transfer against MDR tumors, since there have been few reports on animal models concerning gene transfer for modulation of the lymphocyte ADCC reaction. We examined the effect of local IL-2 secreted from an IL-2-gene-transduced variant of AD10 MDR tumor cells on the therapeutic efficacy of MRK16 *in vivo*, and showed that MRK16 exerted a higher therapeutic effect against MDR tumors in the presence of IL-2-secreting tumor cells.

MATERIALS AND METHODS

Cell lines and cell culture Human ovarian cancer A2780 cells and their adriamycin-resistant variant, AD10,[16] and the amphotropic retrovirus packaging cell line PA317[17] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of vectors The basic structure of the pSXLC/pHa retrovirus system that utilizes a putative internal ribosome entry site (IRES) was described previously.[18] To co-express the human IL-2 gene with a neomycin-resistance gene, we constructed a bicistronic vector plasmid, pHa-IL2-IRES-Neo. The cloning strategy is summarized in Fig. 1. We first inserted human IL-2 cDNA into the SalI site of pSXLC-Neo, which has an IRES-dependent neomycin-resistance gene. The resulting plasmid was termed pSXLC-IL2-Neo. The IL-2-IRES-Neo insert of pSXLC-IL2-Neo was isolated after SacII, XhoI digestion and transferred into the pHa retroviral vector. In the resulting expression construct, pHa-IL2-IRES-Neo, a single mRNA is transcribed under the control of an upstream promoter, and the two gene products are translated independently from a bicistronic mRNA: the IL-2 gene is translated in a cap-dependent manner, and the neomycin-resistance gene is translated under the control of the IRES. As a control vector, pHa-MCS-IRES-Neo, which carries the IRES-Neo insert of pSXLC-Neo, was also constructed.

Generation of retrovirus-producing cells DNA transfection was carried out by the high-efficiency calcium phosphate coprecipitation method.[19] PA317 cells were transfected with pHa-IL2-IRES-Neo or pHa-MCS-IRES-Neo and then selected with 600 μg/ml G418. The resulting G418-resistant cells were pooled and used as retrovirus-producing cells.

IL-2 gene transduction AD10 cells were plated at 5×10^4 cells per 100 mm dish in medium containing 6 μg/ml Polybrene (Aldrich, Milwaukee, WI), and transduced

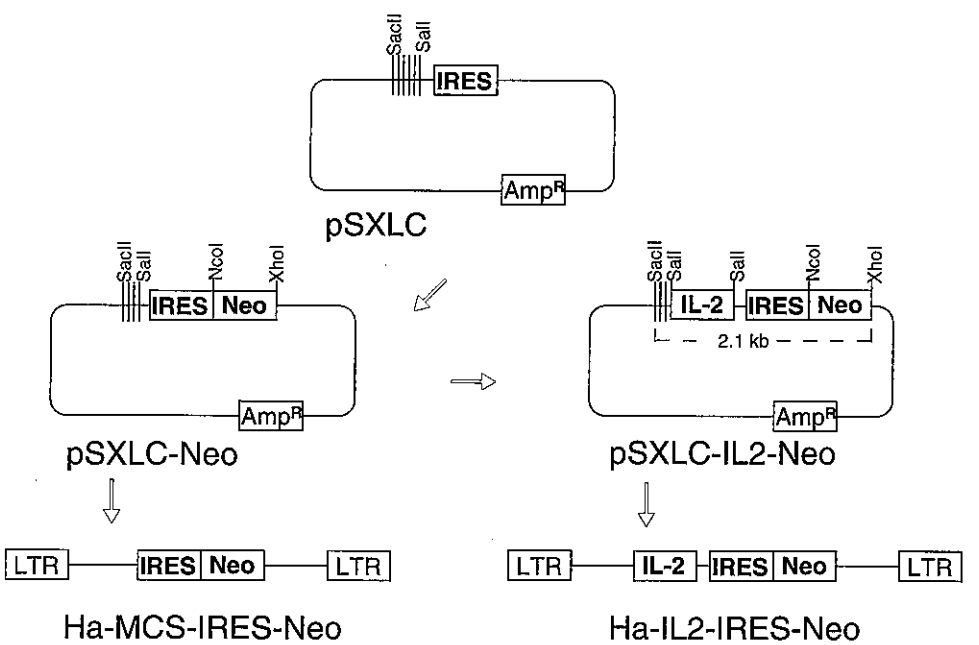

Fig. 1. Structures of Ha-MCS-IRES-Neo and Ha-IL2-IRES-Neo retroviruses. Drawings are not to scale. LTR, long terminal repeat of Harvey murine sarcoma virus; IL-2, human interleukin-2 cDNA; IRES, internal ribosome entry site; Neo, neomycin resistance gene; MCS, multicloning site.

with Ha-IL2-IRES-Neo or Ha-MCS-IRES-Neo retrovirus supernatant. The transduced cells were then selected with 600 μg/ml G418. The resulting G418-resistant cells were pooled and used as transduced cells. Ha-IL2-IRES-Neo-transduced cells and Ha-MCS-IRES-Neo-transduced cells were termed IL2-AD10 and Neo-AD10, respectively. Amounts of IL-2 in the supernatant of the transduced cells were determined using an IL-2 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

FACS analysis Cells (10^6) harvested after trypsinization were treated with MRK16 (100 μg/ml), washed and incubated with fluorescein-conjugated goat anti-mouse IgG (diluted 1 : 10, Cappel, Durham, NC). The cells were washed twice and fluorescence staining was analyzed using a FACSort (Beckton-Dickinson FACS System, San Jose, CA).

***In vitro* cell proliferation assay** Cell proliferation was measured by the MTT dye reduction method.[20] Briefly, cells were plated in 96-well plates at 10^4 cells per well and treated with various concentrations of drugs. After 72 h incubation at 37°C, 50 μl of MTT (Sigma, St. Louis, MO) solution (5 mg/ml) was added to each well, and incubation at 37°C was continued for 2 h. Then the culture media were removed and 100 μl of dimethylsulfoxide was added to dissolve the dark blue crystals. Absorbances were measured with an MTP-32 Microplate Reader (Corona Electric, Ibaragi) at test and reference wavelengths of 550 and 630 nm, respectively.

Cell-mediated cytotoxicity The target cells were labeled with ^{51}Cr as described previously.[8, 9] Murine spleen cells were isolated from BALB/c-*nu* mice and used as effector cells. The ^{51}Cr-labeled target cells (10^4 cells) were mixed with various numbers of effector cells in 96-well plates, centrifuged for 3 min at 100*g*, and incubated in the presence or absence of IL-2 and/or MRK16 at 37°C for 8 or 24 h. The radioactivities released into the supernatants were counted in a gamma counter. Determinations were carried out in triplicate. The percentage of specific cytolysis was calculated from the release of ^{51}Cr from test samples and control samples as follows:

$$\% \text{ Specific release} = (E-S)/(M-S) \times 100$$

where E is the release in the test sample (cpm), S is the spontaneous release (cpm in the supernatant from target cells incubated in medium alone), and M is the maximum release (cpm released from target cells lysed with 1 N HCl). The spontaneous release observed with different target cells ranged from 11% to 25%.

Animal studies Eight-week-old female athymic BALB/c-*nu/nu* mice (Clea Japan, Inc., Tokyo) were housed in laminar-air-flow units. The mice were inoculated s.c. with AD10 tumor sublines (2×10^7 cells/mouse), and treated i.v. with MRK16 on days 2 and 7. Tumor volumes (V) were calculated by means of the equation $V = ab^2/2$, where a and b are the values in mm of the longest and shortest diameter, respectively.

Statistical analysis The statistical significance of differences in tumor uptake was analyzed by applying Fisher's exact probability test.

RESULTS

Establishment of an IL-2-secreting, multidrug-resistant human ovarian cancer cell line, IL2-AD10 AD10 cells were transduced with Ha-IL2-IRES-Neo or Ha-MCS-IRES-Neo retrovirus and selected with G418. After drug selection for 2 weeks, G418-resistant cells consisting of >1000 G418-resistant colonies were pooled and used as transduced cells. IL2-AD10 cells secreted 72 ng/10^6 cells/48 h IL-2 into the culture media, whereas no IL-2 was detected in the supernatants of the Neo-AD10 or parental AD10 cells. IL2-AD10 cells secreted IL-2 stably for at least 12 months (data not shown). IL2-AD10 and Neo-AD10 cells showed similar levels of vincristine resistance and similar rates of *in vitro* growth to those of AD10 cells (Table I). The expression of P-glycoprotein in the transduced cells was examined by indirect staining with MRK16. As shown in Fig. 2, IL2-AD10 and Neo-AD10 expressed similar amounts of P-glycoprotein to that of the parental AD10 cells.

The presence of proviral DNA sequences in the transduced cells was confirmed by Southern hybridization. The expected 2.1-kilobase IL-2-IRES-Neo fragment was detected without rearrangement or deletion in IL2-AD10 cells but was not detected in Neo-AD10 or parental AD10 cells (data not shown).

Tumorigenicity of IL-2-gene-transduced AD10 cells For examination of the effect of IL-2 secretion by IL2-AD10 cells on their tumorigenicity in nude mice, parental AD10, Neo-AD10 and IL2-AD10 cells were inoculated s.c. into the right flank of mice at 2×10^7/mouse. As

Table I. *In vitro* Characteristics of AD10 Variant Cell Lines

Cell line	Production of IL-2[a] (ng/10^6 cells/48 h)	IC_{50} to vincristine[b] (μg/ml)	Doubling time *in vitro*[b] (h)
A2780	ND	0.0025	ND
AD10	<0.1	1.3	23
Neo-AD10	<0.1	1.0	24
IL2-AD10	72	1.4	22

a) Cells were plated at 10^6 cells in 10 ml per 100 mm dish. After 48 h, supernatants were collected and assessed by ELISA.
b) Cell proliferation was measured by the MTT dye-reduction method.
ND, not done.

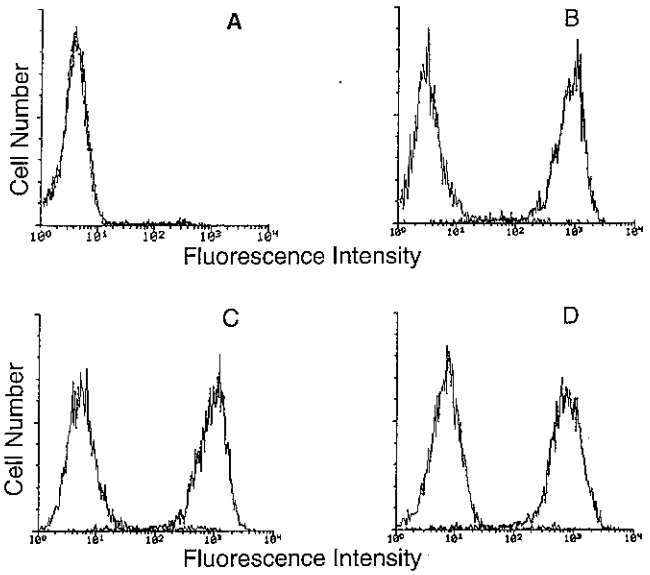

Fig. 2. Representative FACS analyses of P-glycoprotein expression. Cells were harvested after trypsinization, stained with the anti-P-glycoprotein monoclonal antibody MRK16, washed, and stained with fluorescein-conjugated anti-mouse IgG. A, parental A2780; B, AD10; C, Neo-AD10; D, IL2-AD10.

Table II. Tumorigenicity of AD10 Variant Cell Lines

(A) Inoculation of AD10 variant cell lines

Cell lines	Take rate on day 42 (%)
AD10 (2.0×10^7)	6/6 (100)
Neo-AD10 (2.0×10^7)	6/6 (100)
IL2-AD10 (2.0×10^7)	0/6 (0)

(B) Co-inoculation of AD10 and IL2-AD10 cells

AD10	IL2-AD10	Take rate on day 42 (%)
2.0×10^7	0	4/4 (100)
2.0×10^7	6.7×10^6	4/4 (100)
2.0×10^7	2.0×10^7	3/4 (75)

Cells were injected s.c. into 8-week-old BALB/c-*nu* mice. Tumor take rate was determined on day 42.

shown in Table II, formation of palpable tumors was observed in all the nude mice implanted s.c. with 2×10^7 AD10 or Neo-AD10 cells. On the other hand, IL2-AD10 cells did not form a tumor mass. Next, nude mice were implanted s.c. with a mixture of AD10 and IL2-AD10 cells. Co-inoculation of IL2-AD10 cells (6.7×10^6) did not inhibit tumor formation by AD10 cells (2×10^7), though a higher dose of IL2-AD10 cells (2×10^7) inhibited tumor formation by AD10 cells (2×10^7) in the mice (Table II).

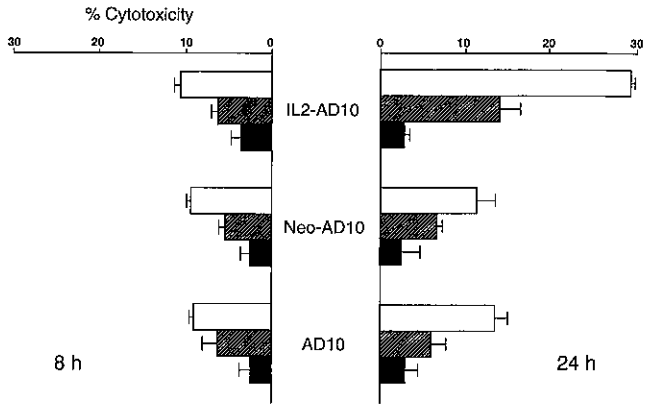

Fig. 3. Effect of IL-2-gene transduction on susceptibility to cell-mediated cytotoxicity. Spleen cells of athymic nude mice (effector cells) were incubated with ^{51}Cr-labeled target cells for 8 h or 24 h at the indicated E : T (effector : target) ratios. The radioactivity released was counted in a gamma counter. Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments. E : T ratios; □ 100 : 1, ▨ 50 : 1, ■ 25 : 1.

Recultured tumor cells from mice injected with a mixture of IL2-AD10 and AD10 cells at a ratio of 1 : 3 expressed a similar level of P-glycoprotein to tumor cells from mice injected with AD10 cells alone (data not shown).

Susceptibilities of the parental and transduced cells to the cytotoxicity of murine spleen cells The effect of IL-2-gene transfer on the susceptibility to the cytotoxicity of murine spleen cells was examined (Fig. 3). On incubation for 8 h, the spleen cells showed similar cytotoxicity towards IL2-AD10, Neo-AD10 and the parental AD10 cells, indicating that IL-2 gene transfer did not affect the sensitivity of the target cells. On the other hand, after 24 h incubation, IL2-AD10 cells showed higher susceptibility to the cytotoxicity of murine spleen cells than Neo-AD10 or parental AD10 cells (Fig. 3). These results suggest that IL-2 secreted by IL2-AD10 cells activated murine effector cells *in vitro*. In this assay, it took a long time before the level of IL-2 from IL2-AD10 cells became sufficient to activate murine spleen cells.

Therapy by MRK16 of human MDR cancer consisting of a mixture of parental AD10 cells and IL2-AD10 cells The effect of IL2-AD10 cells and the secreted IL-2 on the therapeutic efficacy of MRK16 against AD10 tumor cells was examined after the co-inoculation of IL2-AD10 cells (6.7×10^6) and AD10 cells (2×10^7) s.c. into nude mice. The mice were then treated with MRK16 (0–10 μg/mouse) on days 2 and 7. As shown in Table III, treat-

Table III. Effect of Co-inoculation of IL-2-producing IL2-AD10 Cells with the Parental AD10 Cells on Therapeutic Efficacy of MRK16 for Tumor Formation in Nude Mice

AD10	IL2-AD10	MRK16 treatment (μg/body)	Mice with tumor/total		Total take rate (%)
			Exp. 1	Exp. 2	
2.0×10^7	0	0	4/4	4/4	8/8 (100)
		0.1	ND	4/4	4/4 (100)
		0.3	4/4	4/4	8/8 (100)
		1	4/4	4/4	8/8 (100)
		3	1/4	4/4	5/8 (62.5)
		10	0/4	ND	0/4 (0)
2.0×10^7	6.7×10^6	0	4/4	4/4	8/8 (100)
		0.1	ND	4/4	4/4 (100)
		0.3	2/4	3/4	5/8 (62.5)
		1	1/4	3/4	4/8 (50)[a]
		3	0/4	0/4	0/8 (0)[b]
		10	0/4	ND	0/4 (0)

a) $P=0.039$ vs. value for parent AD10 alone.
b) $P=0.013$ vs. value for parent AD10 alone.
Cells were injected s.c. in the right flank of 8-week-old female nude mice on day 0. The mice were treated i.v. with MRK16 on days 2 and 7 at doses of 0, 0.3, 1, 3, 10 μg/body. Tumor take rate was determined on day 45.
ND, not done.

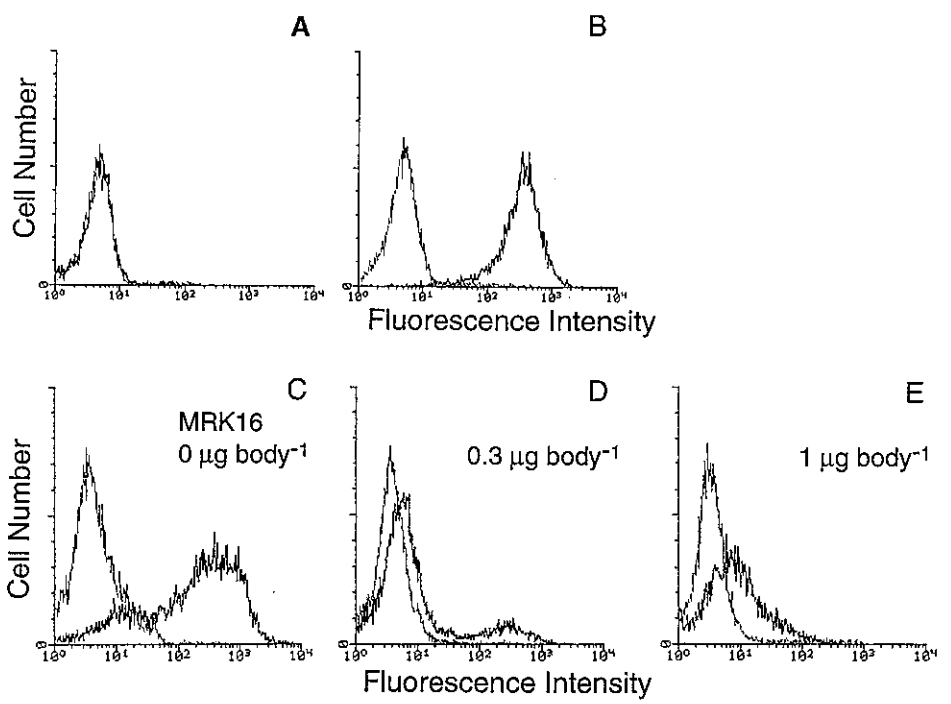

Fig. 4. Representative FACS analyses of P-glycoprotein expression. Cells were harvested after trypsinization, stained with the anti-P-glycoprotein monoclonal antibody MRK16, washed, and stained with fluorescein-conjugated anti-mouse IgG. AD10 cells (2×10^7) and IL2-AD10 cells (6.7×10^6) were co-inoculated s.c. into 8-week-old female nude mice on day 0. The mice were treated i.v. with MRK16 on days 2 and 7 at doses of 0, 0.3, 1, 3 and 10 μg/body. Tumors were excised on day 45 for *in vitro* recultivation. A, A2780 cells cultured *in vitro*; B, AD10 cells cultured *in vitro*; C, cells from mixed tumor (0 μg/body MRK16); D, cells from mixed tumor (0.3 μg/body MRK16); E, cells from mixed tumor (1 μg/body MRK16).

ment with MRK16 was more effective against mixed tumors of AD10 and IL2-AD10 than against AD10 tumors. For instance, systemic treatment with 1.0 μg of MRK16 did not inhibit tumor formation by AD10 cells, but significantly inhibited tumor formation by AD10 cells mixed with IL2-AD10 cells ($P<0.05$). Treatment with 0.3 μg of MRK16 resulted in 62.5% tumor formation by AD10 cells mixed with IL2-AD10 cells and markedly inhibited the growth of palpable tumors (data not shown). Co-injection of Neo-AD10 cells with parental AD10 cells did not affect the therapeutic efficacy of MRK16 (data not shown).

The tumors in nude mice injected with a mixture of IL2-AD10 and AD10 cells were excised on day 45 and cultured *in vitro*, and their expressions of P-glycoprotein were examined by FACS with MRK16 (Fig. 4). Tumor cells from mice that did not receive MRK16 treatment expressed appreciable cell surface P-glycoprotein except for some P-glycoprotein-non-expressing cells (revertants), whereas most of the surviving tumor cells from mice treated with MRK16 only expressed a low level of P-glycoprotein.

IL-2 secreted from IL2-AD10 cells stimulates cell-mediated cytotoxicity The biological activity of IL-2 secreted from IL2-AD10 cells was examined *in vitro* by cell-mediated cytotoxicity assay against the parental AD10 cells. The murine spleen cells were mixed with ^{51}Cr-labeled AD10 cells at a ratio of 100 : 1 and incubated for 8 h in the presence or absence of MRK16 (0.25 μg/ml) and/or 20-fold-diluted culture supernatant of IL2-AD10 cells (final concentration of IL-2, 0.35 ng/ml determined by ELISA). Recombinant IL-2 (1.14×10^7 U/mg, a gift from Takeda Pharmaceuticals, Osaka) was used as a positive control. As shown in Fig. 5, the culture supernatant of IL2-AD10 cells enhanced the MRK16-independent cytotoxic activity of murine spleen cells against AD10 cells and the addition of MRK16 further potentiated the cytotoxicity. The effect of 20-fold diluted IL2-AD10 supernatant on murine spleen cells was similar to that of 10 U/ml recombinant IL-2 (Fig. 5).

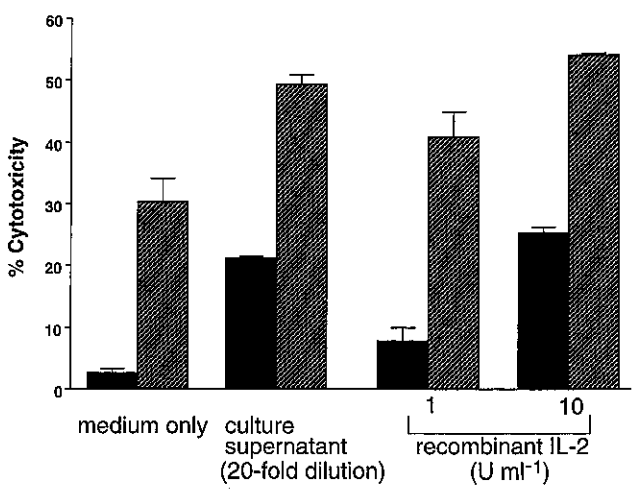

Fig. 5. Augmentation of cell-mediated cytotoxicity by culture supernatant of IL2-AD10 cells. The supernatant of IL2-AD10 cells (10 μl) was added to cultures (190 μl) of spleen cells of nude mice and ^{51}Cr-labeled AD10 cells at an E : T ratio of 100 : 1, and the cells were incubated for 8 h in the presence of MRK16 (0.25 μg/ml). The radioactivity released was counted in a gamma counter. Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments. ■ Ab (−), ▨ Ab (+).

DISCUSSION

In this study we demonstrated that IL-2 gene transfer into MDR cells enhanced the therapeutic efficacy of anti-P-glycoprotein monoclonal antibody MRK16 against human MDR tumors in nude mice. MRK16 markedly inhibited the tumor growth of AD10 cells mixed with IL-2-secreting AD10 cells even at low doses.

In humans, tumor antigens that can induce tumor-specific cytotoxic T lymphocytes (CTLs) are not easily identifiable since cancer cells possess a variety of defects in the machinery of antigen presentation. Therefore immunotherapeutic approaches that depend upon a strategy based on the availability of antigenically unique tumor cells, such as cancer vaccines, are not generally applicable to MDR cancers. Recently immunodominant epitopes that are recognized by CTLs have been identified on human cancer cells. Immunization of cancer patients with peptides of a specific antigen, such as MUC1 peptides, is under clinical trial, but this approach also has a potential difficulty. The immune response to peptides of a specific antigen is clonal, but in tumors, antigen expression is heterogeneous. However, the use of mAbs against tumor cells is an attractive strategy for cancer treatment.[21] We previously showed that a murine monoclonal antibody, MRK16, inhibited the *in vivo* growth of MDR human tumors in nude mice.[7]

Recently, local injection of the wild-type *p53* gene using a retrovirus vector for treatment of human lung cancer was reported.[22] It was difficult to achieve complete remission with this system because only some of the tumor cells could be transduced with the vector system. However, this result still indicated that it is possible to transduce some tumor cells because they are dividing. Therefore, it should be possible to deliver an IL-2-expressing system at a local tumor site using a retrovirus or adenovirus vector system. A phase I trial of an IL-2 plasmid DNA/lipid complex as an immunotherapeutic agent involving direct gene transfer is under way in Arizona. Alternatively, tumor-infiltrating lymphocytes

could be better vehicles to deliver an IL-2 expression system to a tumor site.

Based on these reports, we tried to use IL-2 gene therapy to potentiate the antitumor activity of MRK16 against MDR tumors, in order to lessen the required dose of MRK16, which might be toxic to P-glycoprotein-positive normal tissues. The first step toward this IL-2-antibody combined therapy was to examine whether local IL-2 secretion potentiates the therapeutic efficacy of MRK16 antibody. For this purpose, we used a co-transplantation method with IL-2-modified tumor cells and P-glycoprotein-positive tumor cells, because of the difficulty in estimating the transduction rate *in vivo*. We made use of a nude mice model to remove the influence of tumor-specific CTLs.

To introduce IL-2 cDNA into AD10 cells, we constructed a bicistronic vector Ha-IL2-IRES-Neo (Fig. 1). It is possible to enrich IL-2-secreting cells by simple G418 selection, since co-expression of the two gene products is guaranteed in this system. It was not necessary to isolate clones of transduced cells to achieve a high level of secretion of cytokines. Use of a transduced cell population seems better than the use of isolated individual clones, since the latter may not be representative of the whole tumor cell population. When a mixture of many G418-resistant clones is used as a transduced population, the expression of IL-2 will not be affected by the integration site in each cell, and therefore long-term stable expression can be expected. Indeed, the IL2-AD10 cells examined in the present study secreted IL-2 *in vitro* stably for at least a year.

We found that only IL2-AD10 cells (2×10^7) did not form a tumor mass in nude mice (Table II), and that after a long incubation time (24 h), IL2-AD10 cells showed higher susceptibility to the cytotoxicity of murine spleen cells than Neo-AD10 or parental AD10 cells *in vitro* (Fig. 3). These results suggest that IL-2 secreted by IL2-AD10 cells activated murine effector cells *in vivo*. Athymic nude mice lack functional T cells, but have higher NK cell activity than those of other mouse strains. Since NK cells constitutively express intermediate-affinity IL-2 receptors,[23] this population were probably composed mainly of effector cells which were activated from IL2-AD10 cells by IL-2.

As shown in Table III, systemic treatment with MRK16 inhibited the tumor formation by AD10 cells mixed with IL2-AD10 cells under conditions (0.3–1 μg/body) where MRK16 did not show an effect on the tumor formation by AD10 cells. These findings indicate that local production of IL-2 at the tumor site and systemic treatment with MRK16 have additive effects *in vivo* and that it is possible to reduce the required dosage of MRK16. Consistent with previous studies, MRK16 induced ADCC against AD10 cells through murine spleen cells and we confirmed that direct cytotoxicity and ADCC are additive when measured against the same cell line (Fig. 5). These findings do not conflict with a report that the effector functions involved in direct natural cytotoxicity vs. ADCC within the same population of cells are not equal.[24]

FACS analysis of surviving tumor cells after MRK16 therapy indicated that only cells with low-level expression of P-glycoprotein could survive and form a tumor mass (Fig. 4). This result shows that P-glycoprotein-MRK16 therapy excluded tumor cells expressing a high level of P-glycoprotein. Expression of P-glycoprotein in AD10 cells is not stable, and a decrease in P-glycoprotein expression is often seen under drug-free conditions. P-Glycoprotein-non-expressing cells (revertants) may have some growth advantage in nude mice (Fig. 4C). However, this down-regulation of P-glycoprotein does not seem to affect the results of this study. Conventional chemotherapy may be effective against P-glycoprotein-negative tumors. Therefore, it may be possible to use anticancer agents together with IL-2 gene transfer and MRK16 to treat tumors showing heterogeneous expression of P-glycoprotein, which is always a problem in cancer chemotherapy. We previously reported that systemic administration of MRK16 in combination with P-glycoprotein-related cytotoxic agents to nude mice restores the chemosensitivity of human multidrug-resistant tumor xenografts.[25] Considering the MDR-reversing potential of MRK16, the use of anticancer agents together with IL-2 gene transfer and MRK16 seems to be an attractive strategy for the future.

In summary, our results indicate that IL-2 gene transfer into MDR cells can enhance the therapeutic efficacy of MRK16. This finding suggests that a combination of MRK16 and IL-2-gene transduction may be a promising therapeutic strategy against human MDR cancers and may reduce side effects.

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