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Methioninase Gene Therapy of Human Cancer Cells Is Synergistic with Recombinant Methioninase Treatment

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ABSTRACT

Results obtained over the past 40 years have demonstrated that tumor cells of all types tested have an elevated growth requirement for methioninase compared with normal cells. Recombinant methioninase (rMETase) cloned from *Pseudomonas putida* has been found previously to be an effective antitumor agent attributable to deprivation of the extracellular methionine source of the tumor. To degrade intracellular methioninase, we have now developed an adenoviral vector inserted with the *P. putida* methioninase (*MET*) gene (rAd-MET). The *in vitro* efficacy of rAd-MET was tested on the OVCAR-8 human ovarian cancer cell line, the HT1080 human fibrosarcoma cell line, and human normal fibroblasts. rAd-MET transduction of OVACAR-8 and HT1080 resulted in high levels of methioninase expression up to 10% or more of the total protein of the cells, depending on the multiplicity of infection. The IC_{50} of rAd-MET for OVCAR-8 cells in 96-well plates was approximately 2×10^6 plaque-forming units (pfu)/well. The IC_{50} of control adenovirus (control-rAd) was 4×10^7 pfu/well, 20 times higher than rAd-MET. In the presence of the IC_{50} of 2×10^6 pfu/well of rAd-MET, the addition of 0.025 units/ml of rMETase, which is 25% of the IC_{50}, resulted in a 90% inhibition of tumor cell number. This indicated that rAd-MET enhanced the efficacy of rMETase. In contrast, 2×10^6 pfu/well of control-rAd in combination with 0.025 units/ml of rMETase had an efficacy of only 10% inhibition of cell number. The synergistic effect of the combination of rMETase and rAd-MET was quantitated by calculating the combination index (CI). The CIs for all combinations of rAd-MET and rMETase tested on OVCAR-8 were <0.7 with a mean of 0.5, indicating synergy. Similar synergy of rAd-MET and rMETase was seen on HT1080 human fibrosarcoma cells with a mean of 0.74. In contrast, the CIs of all combinations of rMETase and control adenovirus concentrations tested on both cell lines had a mean CI of ~1, which indicated that this combination had only an additive effect. The normal fibroblasts, on the other hand, appeared relatively resistant to the *MET* gene because in the presence of rMETase, 2.5×10^7 pfu/well of rAd-MET or control rAd had almost an identical effect on cell survival. The selectively strong synergy of rAd-MET and rMETase on cancer cells allows reduced levels of each agent to be used, thus decreasing potential side effects.

INTRODUCTION

Methionine dependence, the elevated methionine requirement for tumor cell proliferation, is the property of the majority of tumor cell types tested (1–9). There have been several therapeutic strategies to target the methionine dependence of cancer cells. Methionine starvation therapy, such as with a methionine-free diet or methionine-depleted total parenteral nutrition, prolonged the survival time of tumor-bearing rodents (10). Methionine-free total parenteral nutrition in combination with chemotherapeutic drugs extended the survival of patients with high-stage gastric cancer (11).

METase[2] from *Pseudomonas putida*, which degrades extracellular methionine to α-ketobutyrate, ammonia, and methanethiol (12), has been demonstrated to have antitumor efficacy *in vitro* and *in vivo* (13–15). METase has synergistic efficacy in combination with 5-fluorouracil (15) and cisplatin (16).

Recently, we have introduced the *P. putida MET* gene (12) into human lung cancer cells using a retroviral vector (17). We demonstrated prolonged survival of mice surgically orthotopically implanted with the *MET* gene-transduced lung tumor in combination with rMETase treatment compared with animals similarly implanted with the lung tumor without the *MET* gene (17). However, the relatively low transduction efficacy of retroviruses makes it difficult to fully explore the potential of the *MET* gene as a therapeutic strategy.

To increase the transduction efficacy of the *MET* gene, we have constructed a recombinant adenovirus containing this gene (rAd-MET). In this report, we describe the synergistic efficacy of the combination of rAd-MET and exogenous administration of rMETase on human ovarian cancer cells and human fibrosarcoma cells compared with the efficacy of the combination on normal fibroblasts *in vitro*.

MATERIALS AND METHODS

Construction of METase-expressing rAd. The *MET* gene cloned from *P. putida* (12) was amplified by the PCR using the pAC-1 rMETase plasmid (13) as a template and two oligonucleotide primers, 5′-GGAAGATCTATGCACG-GCTCCAACAAGCTCCCA-3′ and 5′-CGCGGATCCTTAGGCACTCGC-CTTGAGTGCCTG-3′.

The 1.2-kb *MET* gene was ligated into the transfer vector pQBI-AdCMV5GFP (Quantum, Montreal, Quebec, Canada) downstream of the CMV-5 promoter at the *Bgl*II/*Pme*I site. In the resulting pQBI-AdCMV5 MET-CMVGFP shuttle vector, the *MET* gene and *GFP* gene were driven by the CMV-5 promoter and CMV promoter, respectively (Fig. 1). Shuttle vectors were cotransfected into 293A cells (Quantum) using the calcium phosphate method (18) along with the AdCMVLacZΔE1/ΔE3 viral DNA cut with *Cla*I (Quantum).

Tranfected cells were overlaid with 1.25% SeaPlaque agarose (FMC Bio-Products, Rockland, ME) and incubated at 37°C for 14–21 days. Primary plaques were isolated and used to infect 293A cells to generate a primary crude viral lysate. *MET* gene expression in the viral lysate was confirmed with a METase activity assay (12). After a second plaque purification, a single plaque was amplified in 293A cells. AdCMV5GFPΔE1/ΔE3, as a control vector (control-rAd), was purchased from Quantum. rAds, expanded in 293A cells, were purified by CsCl gradient centrifugation and subsequent fractionation on a Sephadex G25 column (Amersham Pharmacia Biotech, Piscataway, NJ).

Production of rMETase. The pAC-1 rMETase high expression clone, derived from *P. putida*, was used for the production of rMETase in host *Escherichia coli* cells (13). pAC-1 was constructed with the pT7-7 vector containing the T7 RNA polymerase promoter for high expression of the *rMETase* gene in *E. coli* (13). rMETase was purified with a DEAE Sepharose

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[2] The abbreviations used are: METase, methioninase; rMETase, recombinant METase; rAd, recombinant adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CI, combination index.

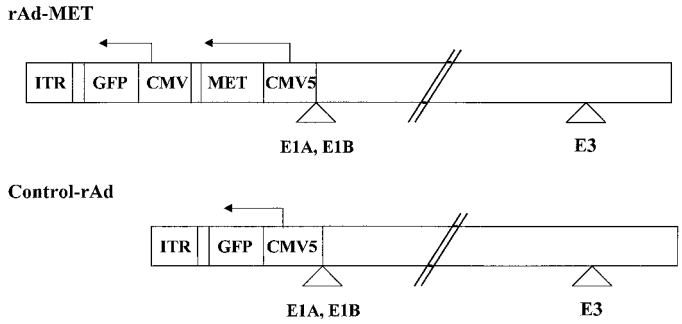

Fig. 1. Schematic structure of rAd-MET. The 1.2-kb *MET* gene, derived from the pAC-1 rMETase plasmid cloned from *P. putida* (13), was ligated into the transfer vector pQB1-AdCMU5GFP. The *MET* gene and the *GFP* gene are driven by the CMV5 and CMV promoters, respectively. *ITR*, inverted terminal repeat.

FF column. The rMETase was 98% pure in HPLC analysis and a single-band of M_r 43,000 on SDS-PAGE. The specific activity was ~20 units/mg protein.

Cells and Cell Culture. OVCAR-8 human ovarian cancer cells (19) and HT1080 human fibrosarcoma cells (20) were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C, and 5% CO_2. FS6 human normal foreskin fibroblasts were maintained in DMEM with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C, and 5% CO_2.

rAd-MET-transduced METase Activity. OVCAR-8 and HT1080 cells (2×10^6) were transduced with rAd-MET at MOIs of 10, 30, 100, and 300. Cells were harvested 48 h after infection. The cells were lysed by sonication for 1 min in 0.5 ml PBS. rMETase activity was measured after centrifugation at 13,000 rpm, in the supernatants, using the method of Tanaka *et al.* (12), which determines α-ketobutyrate production in the presence of 10 mM methionine using 3-methyl-2-benzo-thiazoline hydrazone. Specific rMETase activ-

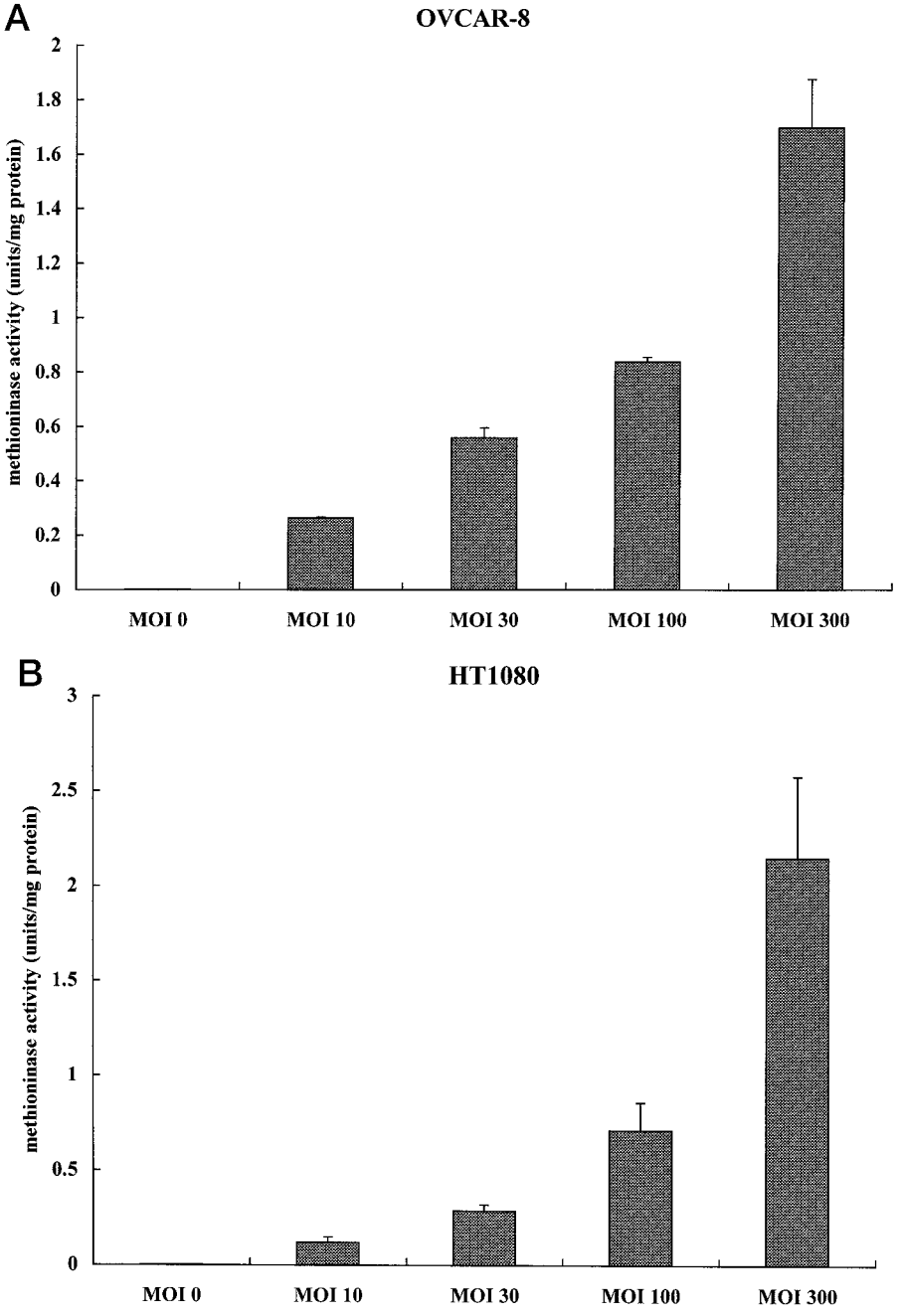

Fig. 2. Methioninase expression of OVCAR-8 human ovarian cancer cells infected with rAd-MET at various MOIs. 2×10^6 OVCAR-8 cells (*A*) and HT1080 cells (*B*) were infected with rAd-MET at various MOIs and incubated for 48 h. The cells were harvested and sonicated in PBS. The METase activity of cell lysates was determined by α-ketobutyrate production (12) in the presence of 10 mM methionine. The METase-specific activity was calculated as units/mg protein. *Bars*, SD. METase activity was positively dependent on the MOI.

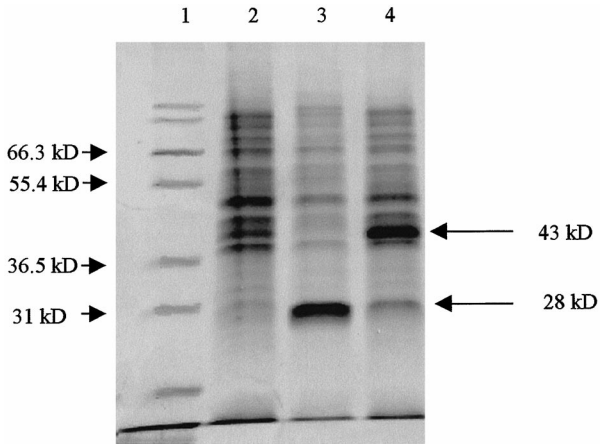

Fig. 3. SDS-PAGE of cell lysates. The cell lysates of nontransduced cells (*Lane 2*), control-rAd-transduced cells at MOI 100 (*Lane 3*), and rAd-MET-transduced cells at MOI 100 (*Lane 4*) were loaded on 12% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R. *Lane 1* contains the molecular weight markers. The METase protein was seen as a significant band at M_r 43,000 (rAd-MET-transduced cells in *Lane 4*). GFP protein was also detected at M_r 28,000 in *Lane 3* and *Lane 4*.

ity was calculated as units/mg protein, with one unit of enzyme defined as the amount that catalyzed the formation of 1 μmol of α-ketobutyrate/min. The protein concentrations of cell lysates were determined with the Lowry Reagent kit (Sigma Chemical Co., St. Louis, MO).

SDS Gel Electrophoreses. OVCAR-8 cells (2×10^6) were transduced with rAd-MET or control-rAd at a MOI of 100. Cells were harvested 48 h after infection. The cells were lysed by sonication for 1 min in 0.5 ml of PBS. After centrifugation at 13,000 rpm, the supernatants containing a total of 15 μg of total protein were analyzed with 12% SDS-PAGE (Novex, San Diego, CA) and stained with Coomassie Brilliant Blue R.

MTT Thiazolyl Blue Test. Cell killing efficacy was evaluated with the MTT assay (21). OVCAR-8, HT1080, and FS6 cells were seeded in 96-well plates at a density of 4000 cells/well. Twenty-four h later, 100 μl of medium containing various titers of rAd-MET, control-rAd, or various concentrations of rMETase were added to the cells. Cells were incubated for 72 h at 37°C as described above. The medium was replaced with 0.5 mg/ml MTT in fresh medium. After 2 h incubation at 37°C, the supernatants were removed, and 200 μl of isopropanol were added to each well, which were measured for absorbance at 540 nm in a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA).

IC_{50} Determination for rMETase, rAd-MET, and control-rAd. Cells were treated in quadruplicate either with rMETase at doses ranging from 0.025 to 5.4 units/ml, or with rAd-MET or control-rAd at doses ranging from 1×10^5 to 1×10^8 pfu/well. Efficacy was evaluated with the MTT test. The dose-effect curves were plotted for each agent in multiply-diluted concentrations by using the median-effect equation (22).

$$D = \mathrm{IC}_{50}[fa/(1 - fa)]^{1/m} \quad \text{(A)}$$

where D is the dose, IC_{50} is the dose required for 50% effect, fa is the fraction affected by dose D, and m is a coefficient of the sigmoidicity of the dose-effect curve.

From Eq. A, we obtain the following equation:

$$\log[fa/(1 - fa)] = m \log(D) - m \log(\mathrm{IC}_{50}) \quad \text{(B)}$$

whereby m and IC_{50} are determined by the median-effect plot; $x = \log(D)$, $y = \log [\mathrm{fa}/(1 - \mathrm{fa})]$. m and $\log(IC_{50})$ are the slope and the x-intercept of the regression line of this plot, respectively.

Combination Therapy Using rAd-MET and rMETase. Cells were seeded at 4000 cells/well in 96-well plates. Twenty-four h later, rAd-MET or control-Ad was added at different doses ranging from 5×10^5 to 8×10^6 pfu/well for tumor cell lines and from 10^6 to 10^8 pfu/well for normal fibroblasts. Three h later, rMETase was added at different doses in a range of concentrations from 0.025 to 0.1 units/ml for tumor cell lines and from 0.06 to 5.4 units/ml for normal fibroblasts. A total of 12 different combinations (three concentrations of rMETase and four titers of rAd-MET or control-rAd) were evaluated on OVCAR-8. Thirty-five different combinations were evaluated on HT1080 (seven concentrations of rMETase and five titers of rAds). Forty-five different combinations were evaluated on the normal fibroblasts (five concentrations of rMETase and nine titers of rAds). Three days after the transduction, surviving cells were quantified with the MTT assay.

CI for Evaluation of Synergism and Antagonism. To measure the synergistic efficacy of two drugs, we used the CI isobologram method developed by Chou and Talalay (22). The CI is defined as follows:

$$CI = D_1/Dx_1 + D_2/Dx_2 \quad \text{(C)}$$

$$Dx_1 = \mathrm{IC}_{50\text{-}1}[fa/(1 - fa)]^{1/ml}$$

$$Dx_2 = \mathrm{IC}_{50\text{-}2}[fa/(1 - fa)]^{1/m2}$$

where D_1 is the dose for drug-1, D_2 is the dose for drug-2, Dx_1 is the simulated dose of drug-1, which has the same efficacy when using drug-1 alone. Dx_2 is the simulated dose of drug-2; CI $<$ 1 indicates synergism; CI $>$ 1 indicates antagonism; and CI = 1 is additive.

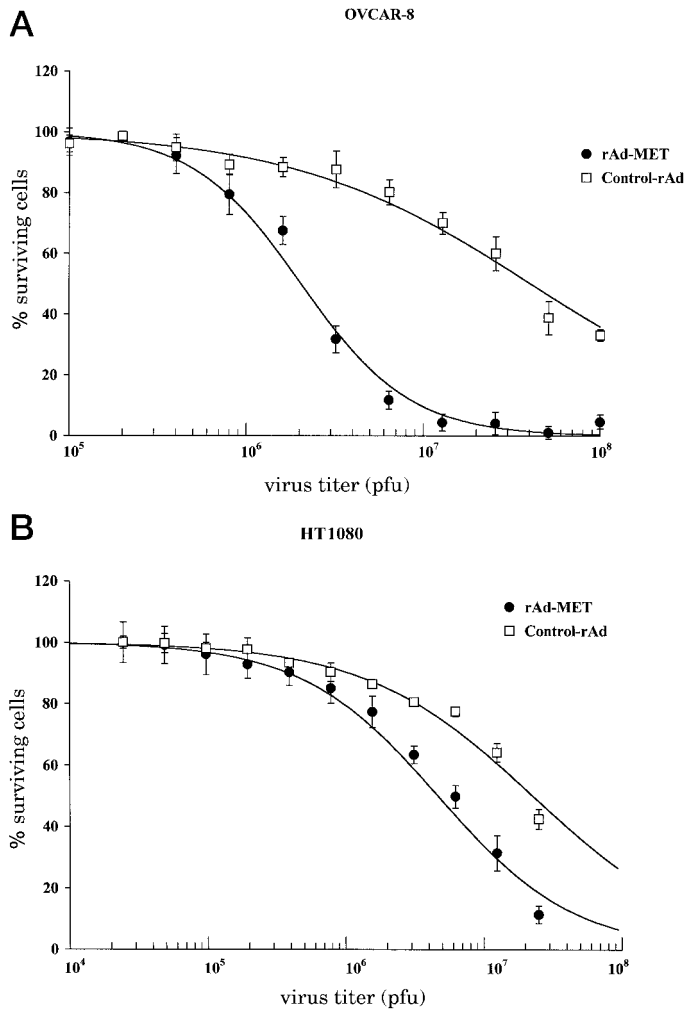

Fig. 4. Efficacy of rAd-MET and control-rAd on OVCAR-8 and HT1080 cells. Cells (4000) were infected with rAd-MET (*A*, *closed circles*) or control-rAd (*A*, *open squares*) at various titers. Three days after infection, the percentage of surviving cells was determined with the MTT assay. Dose-response curves for rAd-MET and control-rAd were plotted. *A*, OVACAR-8; *B*, HT1080. The IC_{50}s of rAd-MET were 2×10^6 and 4.6×10^6 pfu for OVCAR-8 and HT1080, respectively. The IC_{50}s of control-rAd were 4×10^7 and 2.3×10^7 pfu for OVCAR-8 and HT1080, respectively. *Bars*, SD.

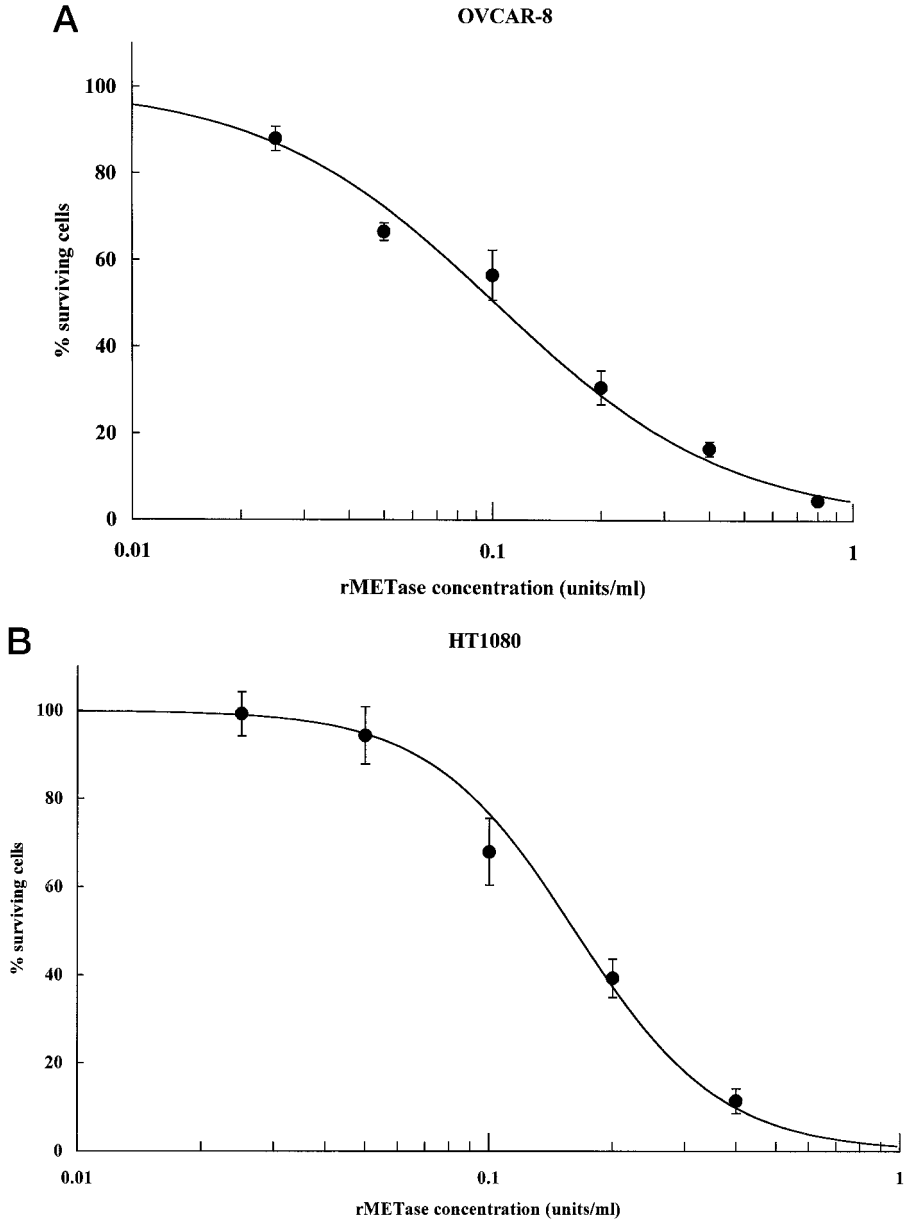

Fig. 5. Efficacy of rMETase on OVCAR-8 and HT1080 cells. Cells (4000) were treated with various concentrations of rMETase. Dose-response curves for rMETase were plotted as in Fig. 3. *A*, OVCAR-8; *B*, HT1080. The IC_{50}s of rMETase for OVCAR-8 and HT1080 were 0.1 and 0.15 unit/ml, respectively. *Bars*, SD.

RESULTS AND DISCUSSION

METase Expression in rAd-MET-infected Cells. The activity of the rAd-*MET* gene was measured in cell lysates of OVCAR-8 and HT1080 cells infected with this vector. The METase activities were positively dependent on the MOI of rAd-MET (Fig. 2). At the lowest MOI of 10, the METase activity in OVCAR-8 was 0.26 unit/mg protein, which was nine times higher than the activity we reported using retroviral vectors (17). At an MOI of 300, the METase activity for both OVCAR-8 and HT 1080 was ~2 units/mg protein in the crude lysate indicating METase was expressed at ~10% of the total protein of the cells. High-level rAd-MET expression in infected OVCAR-8 cells can also be seen by the predominant M_r 43,000 METase monomer band in SDS-PAGE (Fig. 3).

Efficacy of rAd-MET. The percentage of surviving cells was determined after transduction with various MOIs of rAd-MET or control-rAd (Fig. 4). The IC_{50}s for rAd-MET on OVACAR-8 and HT1080 were 2.0×10^6 and 4.6×10^6 pfu/well, respectively. In contrast, the IC_{50}s for control-rAd in OVACAR-8 and HT1080 were 4.0×10^7 and 2.3×10^7 pfu/well, respectively, which was 20 and 5 times higher than that of rAd-MET, respectively. The IC_{50}s for rAd-MET and control-rAd on normal fibroblasts were 4.7×10^7 and over 1×10^8 pfu/well, respectively.

Efficacy of rMETase. A dose-response relationship was observed for rMETase on both OVCAR-8 and HT1080 cells with an IC_{50} of 0.1 unit/ml and 0.15 unit/ml, respectively (Fig. 5). In contrast, the IC_{50} on the normal fibroblasts was 1.68 units/ml, >10 times higher than tumor cells.

Combination Therapy of Ad-MET and rMETase. In the presence of the IC_{50} of 2×10^6 pfu of rAd-MET, the addition of 0.025 unit/ml of rMETase, which was one-fourth of the IC_{50} dose, inhibited OVCAR-8 cell growth by 90% (Fig. 6*A*). This level of rMETase on untransduced cells or control-Ad-transduced cells had an efficacy of only 10% (Fig. 6*A*). The normal fibroblasts, on the other hand, appeared relatively resistant to the *MET* gene because in the presence of rMETase, 2.5×10^7 pfu/well of rAd-MET or control rAd had almost an identical effect on cell survival (Fig. 6*B*).

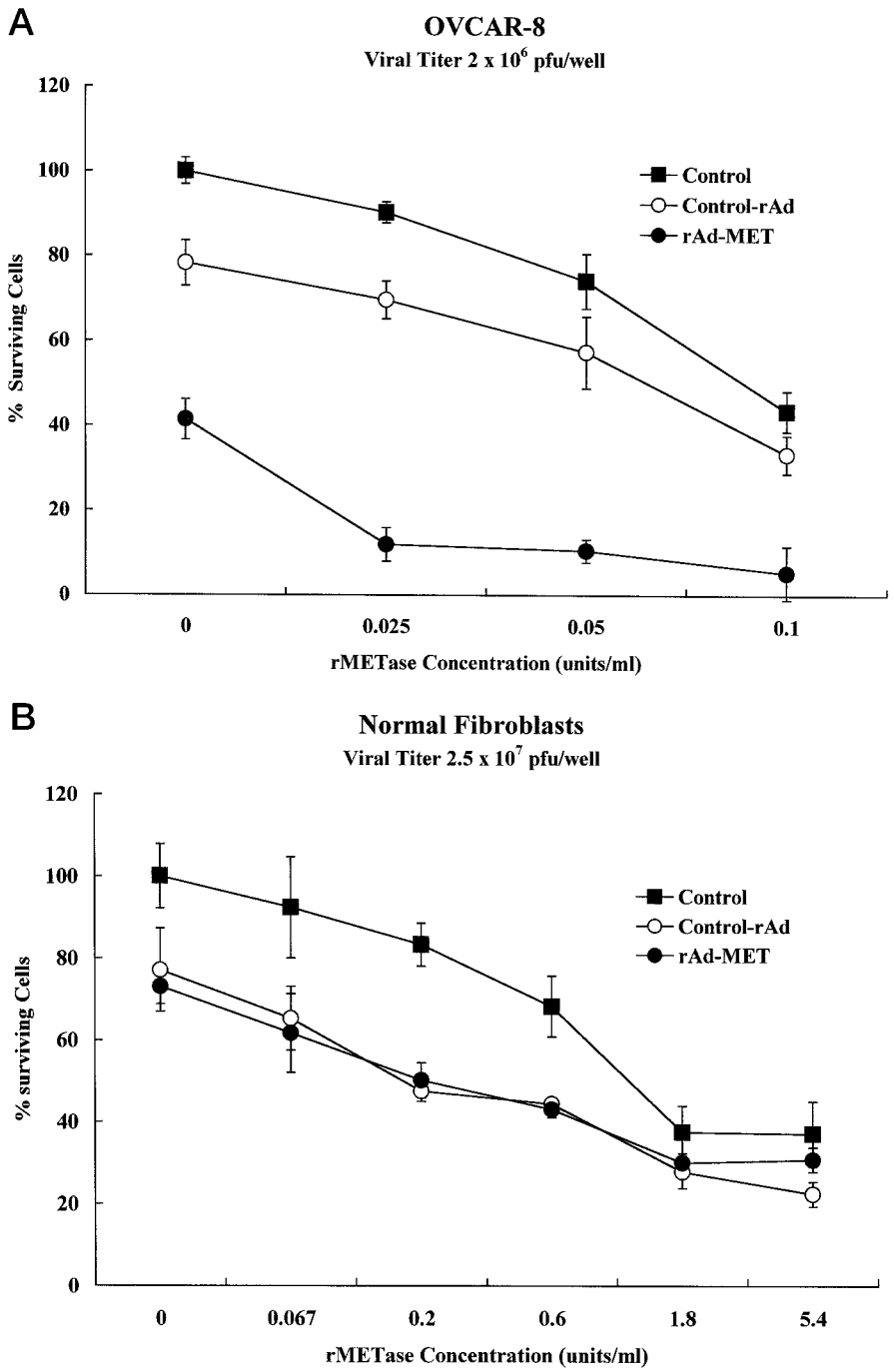

Fig. 6. Combination therapy of rAd-MET and rMETase. *A*, rAd-MET-transduced OVACAR-8 cells (2×10^6 pfu/well), control-rAd-transduced cells (2×10^6 pfu/well), or untransduced cells were treated with various concentrations of rMETase up to 0.1 units/ml. *B*, rAd-transduced normal fibroblasts (2.5×10^7 pfu/well) were treated with rMETase up to 5.4 units/ml. *Bars*, SD.

Synergistic efficacy of rAd-MET and rMETase was determined by calculating the CI. All of the CIs of the combinations of rMETase and rAd-MET tested on OVCAR-8 were <0.7, with a mean of 0.5 ± 0.13 (Fig. 7*A*). Therefore, this combination has strong synergy. In contrast, the CIs of rMETase and control-rAd for OVCAR-8 had a mean of $0.97 + 0.13$. This combination had only an additive effect. The mean value of CIs for the combinations of rMETase and rAd-MET on HT1080 was 0.74 ± 0.13, indicating synergy (Fig. 7*B*). In contrast, the combinations of rMETase and control-rAd on HT1080 had a mean of 1.11 ± 0.20. Therefore, rMETase had rAd-MET had synergistic efficacy on OVCAR-8 and HT1080, whereas rMETase and control-rAd did not. There were significant differences in the mean CI values between the test and control combinations on both cell lines using the Student *t* test ($P < 0.0001$). The CIs of the combination of rMETase and rAd-MET and combination of rMETase and control-rAd on normal fibroblasts were 0.89 ± 0.43 and 1.25 ± 1.30, respectively. There were no significant differences in the mean CIs between the two combinations on normal fibroblasts, again suggesting relative resistance of the normal fibroblasts to the *MET* gene.

rMETase protein therapy degrades extracellular methionine but cannot block the synthesis of methionine inside the cells, whereas *MET* gene delivery via rAd-MET degrades intracellular methionine but cannot block the uptake of methionine. Perhaps methionine depletion in both the extracellular space and within the cell accounts for the synergy in the cancer cells. Tisdale (23) has speculated that exogenous methionine and intracellularly biosynthesized methionine may be differentially used in cancer cells. Future experiments will attempt to elucidate the synergistic mechanism by determining the

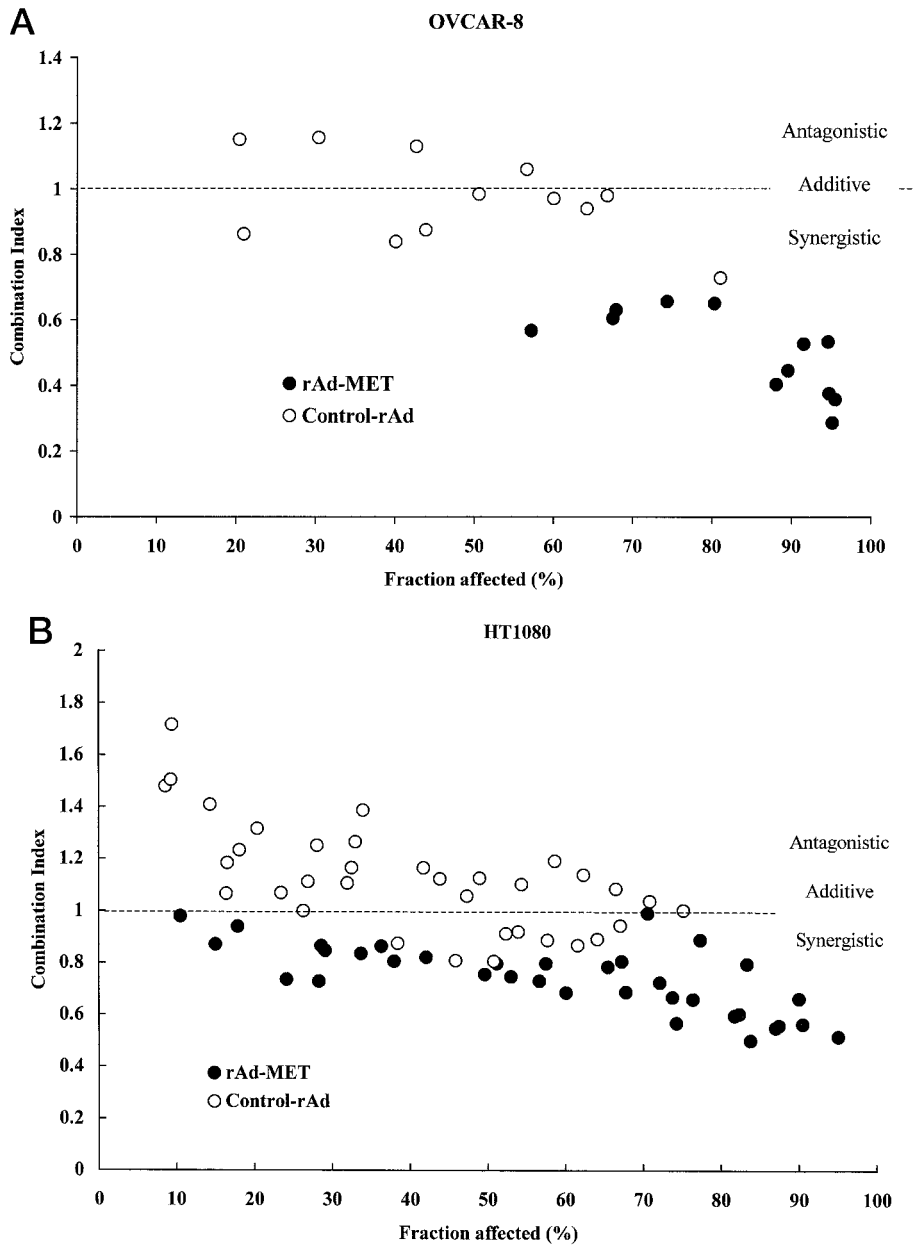

Fig. 7. The CI of the combination of rAd-MET and rMETase. The CIs of combinations of rMETase and rAd-MET or control-rAd were plotted using the CI isobologram method (22) for OVCAR-8 (*A*) and HT1080 (*B*). All of the CIs of the rMETase and rAd-MET combination were <1.0 for both cancer cell lines (0.5 ± 0.13 for OVCAR-8; 0.74 ± 0.13 for HT1080). In contrast, all of the CIs of rMETase and control-rAd had a mean CI of ~ 1, indicating additivity and not synergy.

differential effects of rMETase and rAd-MET on cellular methionine and methylation metabolism in tumors.

The rAd-*MET* cancer gene therapy combined with rMETase treatment has many powerful features including: (*a*) the high efficiency of transduction and the high expression of the rAd-*MET* gene in the tumor cells compared with the retroviral gene delivery; (*b*) the ability to deplete both intracellular and extracellular methionine, which strongly targets the increased methionine requirement of tumor cells; (*c*) the potential to target growing and resting tumor cells, which can be rendered more selective in the future using cancer-specific promoters to drive the *MET* gene; (*d*) the strong synergy of rAd-MET and rMETase, allowing reduced levels of each agent to be used, thus decreasing potential side effects; and (*e*) the relative resistance of the normal fibroblasts to the *MET* gene.

REFERENCES

1. Hoffman, R. M., and Erbe, R. W. High *in vivo* rate of methionine biosynthesis in transformed human and malignant rat cells auxotropic for methionine. Proc. Natl. Acad. Sci. USA, *73:* 1523–1527, 1976.
2. Hoffman, R. M., Jacobsen, S. J., and Erbe, R. W. Reversion to methionine independence in SV40-transformed human and malignant rat fibroblasts is associated with altered ploidy and altered properties of transformation. Proc. Natl. Acad. Sci. USA, *76:* 1313–1317, 1979.
3. Mecham, J. O., Rowitch, D., Wallace, C. D., Stern, P. H., and Hoffman, R. M. The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem. Biophys. Res. Commun., *117:* 429–434, 1983.
4. Hoffman, R. M. Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis: a review and synthesis. Biochim. Biophys. Acta. *738:* 49–87, 1984.
5. Stern, P. H., Wallace, C. D., and Hoffman, R. M. Altered methionine metabolism occurs in all members of a set of diverse human tumor cells. J. Cell Physiol., *119:* 29–34, 1984.
6. Stern, P. H., and Hoffman, R. M. Elevated overall rates of transmethylation in cell lines from diverse human tumors. In Vitro, *20:* 663–670, 1984.
7. Stern, P. H., Mecham, J. O., Wallace, C. D., and Hoffman, R. M. Reduced free methionine-dependent SV40 transformed human fibroblasts synthesizing apparently normal amount of methionine. J. Cell Physiol., *117:* 9–14, 1984.
8. Hoffman, R. M., and Jacobsen, S. J. Reversible growth arrest in SV40-transformed human fibroblasts. Proc. Natl. Acad. Sci. USA, *77:* 7306–7310, 1980.
9. Breillout, F., Antoine, E., and Poupon, M. F. Methionine dependency of malignant tumors: a possible approach for therapy. J. Natl. Cancer Inst., *82:* 1628–1632, 1990.
10. Goseki, N., Yamazaki, S., Endo, M., Onodera, T., Kosaki, G., Hibino, Y., and Kuwahata, T. Antitumor effect of methionine-depleting total parenteral nutrition with

doxorubicin administration on Yoshida sarcoma-bearing rats. Cancer (Phila.), *69:* 1865–1872, 1992.
11. Goseki, N., Yamazaki, S., Shimojo, K., Kando, F., Maruyama, M., Endo, M., Koike, M., and Takahashi, H. Synergistic effect of methionine-depleting total parenteral nutrition with 5-fluorouracil on human gastric cancer: a randomized prospective clinical trial. Jpn. J. Cancer Res., *86:* 484–489, 1995.
12. Tanaka, H., Esaki, N., and Soda, K. Properties of L-methionine-γ-lyase from *Pseudomonas ovalis.* Biochemistry, *16:* 100–106, 1977.
13. Tan, Y., Xu, M., Tan, X. Z., Tan, X-Y., Wang, X., Saikawa, S., Nagahama, T., Sun, X., Lenz, M., and Hoffman, R. M. Overexpression and large-scale production of recombinant L-methionine-α-deamino-γ-mercaptomethane-lyase for novel anticancer therapy. Protein Expression Purif., *9:* 233–245, 1997.
14. Tan, Y., Xu, M., Guo, H., Sun, X., Kubota, T., and Hoffman, R. M. Anticancer efficacy of methioninase *in vivo*. Anticancer Res., *16:* 3931–3936, 1996.
15. Yoshioka, T., Wada, T., Uchida, N., Maki, H., Yoshida, H., Ide, N., Kasai, H., Hojo, K., Shono, K., Maekawa, R., Yagi, S., Hoffman, R. M., and Sugita, K. Anticancer efficacy *in vivo* and *in vitro*, synergy with 5-fluorouracil, and safety of recombinant methioninase. Cancer Res., *58:* 2583–2587, 1998.
16. Tan, Y., Sun, X., Xu, M., Tan, X-Z., Sasson, A., Rashidi, B., Han, Q., Tan, X-Y., Wang, X., An, Z., Sun, F-X., and Hoffman, R. M. Efficacy of recombinant methioninase in combination with cisplatinum on human colon tumors in nude mice. Clin. Cancer Res., *5:* 2157–2163, 1999.
17. Miki, K., Xu, M., An, Z., Wang, X., Yang, M., Al-Refaie, W., Sun, X., Baranov, E., Tan, Y., Chishima, T., Shimada, H., Moossa, A. R., and Hoffman, R. M. Survival efficacy of the combination of the methioninase gene and the methioninase gene product in a lung cancer orthotopic model. Cancer Gene Therapy, *7:* 332–338, 2000.
18. Keown, W. A., Campbell, C. R., and Kucherlapati, R. S. Methods for introducing DNA in mammalian cells. *In:* D. V. Goeddel (ed.), Methods in Enzymology, Gene Expression Technology, Vol. 185, pp. 527–537. San Diego: Academic Press, Inc., 1991.
19. Schilder, R. J., Hall, L., Monks, A., Handel, L. M., Fornace, A. J., Jr., Ozols, R. F., Fojo, A. T., and Hamilton, T. C. Metallothionine in gene expression and resistance to cisplatin in human ovarian cancer. Int. J. Cancer, *45:* 416–422, 1990.
20. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P., and Gardner, M. B. Characterization of newly derived human sarcoma cell line (HT-1080). Cancer (Phila.), *33:* 1027–1033, 1974.
21. Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., and Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res., *48:* 589–601, 1988.
22. Chou, T. C., and Talalay, P. Applications of the median effect principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism, antagonism of chemotherapeutic agents. *In:* New Avenues I Development Cancer Chemotherapy, Bristol-Myers Symposium Series, pp. 37–64. New York: Academic Press, 1987.
23. Tisdale, M. J. Utilization of preformed and endogenously-synthesized methionine by cells in tissue culture. Br. J. Cancer, *49:* 315–320, 1984.