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Modification of Adriamycin-induced Cytotoxicity by Recombinant Human Interferon-γ and/or Verapamil in Human Stomach Cancer Cells

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Recombinant human-interferon-γ (rH-IFN-γ) and verapamil (VRP), either alone or in combination, were evaluated in MTT assay for their modification effects on adriamycin-induced cytotoxicity against MKN-45, human stomach adenocarcinoma cells. VRP as a single agent did not inhibit the survival of MKN-45 at doses of up to 5.0 μg/ml. The survival of MKN-45 was inhibited by rH-IFN-γ dose-dependently and further inhibited by the addition of VRP. However, the maximum growth inhibition of MKN-45 in any combination treatment with rH-IFN-γ and VRP was less than 50% except in the highest concentration combinations (% survival: 47.9% at 10^4 U/ml of rH-IFN-γ and 3.0 μg/ml of VRP). Adriamycin caused a concentration-dependent cytotoxicity and its cytotoxicity was significantly enhanced by the addition of rH-IFN-γ and further enhanced by the combined use of rH-IFN-γ and VRP. The modification effects of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity were evaluated in terms of modification index (MI), demonstrating that rH-IFN-γ significantly increased in adriamycin-induced cytotoxicity and that the combined use of rH-IFN-γ and VRP enhanced the adriamycin-induced cytotoxicity to a greater extent than did rH-IFN-γ alone: MI values at 10^2 U/ml and 10^3 U/ml of rH-IFN-γ were 1.7 and 3.1, respectively; those at 1.5 μg/ml and 3.0 μg/ml of VRP in the presence of 10^3 U/ml of rH-IFN-γ were 4.4 and 6.0, respectively. These results suggest that the addition of rH-IFN-γ and VRP may increase in therapeutic efficacy of adriamycin in the treatment of cancer.

Key Words: *Recombinant human-interferon-γ, Verapamil, Adriamycin-induced cytotoxicity, MKN-45 (Human stomach adenocarcinoma)*

INTRODUCTION

Adriamycin is one of the most active chemotherapeutic agents, either alone, or more often, in combination with other agents for a variety of cancers. Adriamycin-based combination chemotherapy has markedly improved the response rate and survival in certain kinds of cancers, such as leukemia, lymphoma and ovarian cancer (Young et al., 1981). In patients with stomach cancer, the response rate has been improved by adriamycin-based combination chemotherapy, however, the prolongation of life has usually not been remarkable (Kim, 1984; Preusser et al., 1988). The poor therapeutic benefit of adriamycin-based combination chemotherapy in stomach cancer is believed to be mainly due to the existance of tumor cells innately resistant to adriamycin, the emergence of resistant cells after initial response, and no further administration of chemotherapeutic agents because of the severe side effects, such as myelosuppression and cardiac toxicity (Brstow et al., 1987; Kaye and Merry, 1985). It is of great importance, therefore, to develop methods not only of enhancing the cytotoxicity of chemotherapeutic drugs to improve the response rate further but also

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of overcoming the acquired resistance to achieve the prolongation of survival without adding to the side effects.

With a view toward improved therapeutic results, attempts have been made to modify the cytotoxicity of chemotherapeutic drugs by combined use with other drugs, resistance modifiers (Hong et al., 1988). We have previously reported that recombinant human tumor necrosis factor and recombinant human-interferon-γ (rH-IFN-γ) overcame acquired resistance to cisplatin in human cancer cells (Hong et al., 1987). More recently, we have shown that recombinant human tumor necrosis factor and rH-IFN-γ significantly enhanced the sensitivity to cisplatin in human stomach cancer cells (Kim et al., 1989). It has been reported by several investigators that verapamil (VRP), a calcium channel blocker, has the ability to enhance the adriamycin-induced cytotoxicity to a great extent in cells resistant to adriamycin (Hindenburg et al., 1987; Tsuruo et al., 1983; Twentyman et al., 1986).

If some of the mechanisms of resistance to chemotherapeutic agents are shared between cisplatin and adriamycin and between innate resistance and acquired resistance, rH-IFN-γ and VRP may overcome the innate resistance to adriamycin. On this supposition, we have attempted to investigate the modification effects of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity *in vitro* against MKN-45, human stomach cancer cells, and have demonstrated that rH-IFN-γ and VRP significantly enhanced adriamycin-induced cytotoxicity.

MATERIALS AND METHODS

Cell line

MKN-45, a human stomach adenocarcinoma cell line, was kindly donated by Dr. N. Saijo of the Nationl Cancer Center Hospital, Tokyo, Japan. MKN-45 was maintained as a monolayer culture in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (RPMI-FBS) in an atmosphere of humidified 5% CO_2 at 37°C. The cells were passed serially once or twice per week as necessitated by the growth rate.

Drugs

Adriamycin, VRP and rH-IFN-γ were obtained from Dong-A Pharm. Co., Ltd., Korea, Lucky Pharm. Corp., Korea and Sigma Chemical Co., USA, respectively. The specific activity of rH-IFN-γ was 2×10^6 IU/mg protein. THe stock solutions of these drugs were made with distilled water and stored at −70°C until use. Just before each experiment, these stock solutions were diluted in RPMI-FBS to the reguired concentrations.

MTT assay

The MTT assay used to measure cytotoxicity in this study was essentially the one devised by Dr. Mosmann (1983) and has been previously described in detail (Hong et al., 1988). In brief, tumor cells diluted with RPMI-FBS to the final concentrations of 1×10^4 cells/well were added to each well (135 μl/well) of a 96-well microtest plate (Beckton Dickinson, Oxnard, CA, USA) and preincubated in a humidified atmosphere of 5% CO_2 at 37°C for 4 hours. Cell numbers for seeding and incubation time were determined after the confirmation of the linear relationship between absorbance and number of cells in standard and growth curves of MKN-45. They were then treated continuously with 15 μl of various concentrations of adriamycin, rH-IFN-γ and VRP. After 4 days of incubation, 15 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml in phosphate buffered saline) (Sigma Chemical Co., St. Louis, MO, USA) solution were added to each well and the plates were further incubated for 4 hours. To solubilize the intracellular crystals formed, 150 μl of acid isopropanol (100 μl of 0.04N HCl in isopropanol) were added to each well and the contents of each well were subjected to repeated pipetting. The absorbance was measured at 570 nm using a spectrophotometer (Titertek Multiskan Plus MKII, Flow Laboratories, Finland). The percent survival was determined by the formula: [(mean absorbance in three test wells-absorbance in background well)/(mean absorbance in three control wells-absorbance in background well)]×100(%). Each experiment was performed in triplicate and repeated three times. IC_{70} was calculated from the dose-response curve as the drug concentration at which the survival of tumor cells tested is reduced to 30% of the control.

Statistical analysis

The significance of differences in results between experimental groups was determined by unpaired and paired Student's t-test.

RESULTS

Effects of rH-IFN-γ and VRP, alone or in combination

The cytotoxic effects on MKN-45 produced by rH-IFN-γ and VRP, either alone or in combination, were investigated by MTT assay (Fig. 1A). MKN-45 was resistant to doses of up to 5.0 μg/ml of VRP. Growth of MKN-45 was inhibited by rH-IFN-γ, dose-dependently,

and further inhibited by the addition of 3.0 μg/ml of VRP ($p<0.05$) (Fig. 1B). However, the maximum inhibition of the survival of MKN-45 in any combination treatment with rH-IFN-γ and VRP was less than 50% except in the highest concentration combinations (% survival: 47.9±2.9% at 10^4 U/ml of rH-IFN-γ and 3.0 μg/ml of VRP).

Table 1. Combination effects of rH-IFN-γ with verapamil on IC_{70} to adriamycin in human stomach adenocarcinoma, MKN-45

Drugs	IC_{70}
ADM alone	0.66±0.06[a]
ADM+VRP 1.5	0.50±0.08
ADM+VRP 3.0	0.44±0.12
ADM+IFN-γ 10^2	0.40+0.10*
ADM+IFN-γ 10^3	0.21±0.03**
ADM+IFN-γ 10^3+VRP 1.5	0.15±0.02**
ADM+IFN-γ 10^3+VRP 3.0	0.11±0.02**

ADM; adriamycin, IFN-γ 10^2; 10^2 U/ml of recombinant human interferon-γ, VRP 1.5; 1.5 μg/ml of verapamil
a) mean of IC_{70} (μg/ml)±SD of three experiments
*$p<0.05$, **$p<0.01$ as compared with ADM alone

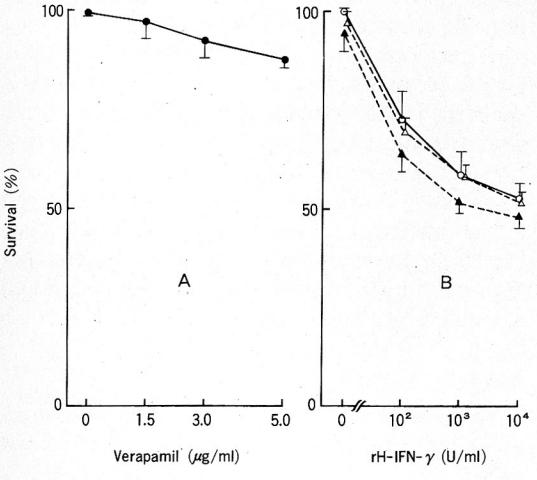

Fig. 1. Effects of verapamil (A) and rH-IFN-γ and/or verapamil (B) on the survival of MKN-45, human stomach adenocarcinoma. The solid and dotted lines in figure B represent the absence and presence of verapamil: ○, rH-IFN-γ alone; △, rH-IFN-γ +1.5 μg/ml of verapamil; ▲, rH-IFN-γ +3.0 μg/ml of verapamil. Each point and bar represent the mean and SD of three experiments.

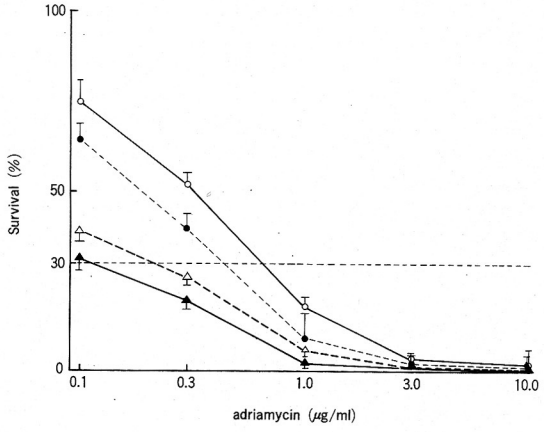

Fig. 2. Enhancement of adriamycin-induced cytotoxicity by rH-IFN-γ and verapamil in MKN-45, human stomach adenocarcinoma: ○, adriamycin alone; ●, adriamycin +3.0 μg/ml of verapamil; △, adriamycin +10^3 U/ml of rH-IFN-γ; ▲, 10^3 U/ml of rH-IFN-γ +3.0 μg/ml of verapamil. Each point and bar represent the mean and SD of three experiments.

Effects of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity

The enhancement of adriamycin-induced cytotoxicity by rH-IFN-γ and/or VRP in MKN-45 is presented in Fig. 2. Combined treatment of rH-IFN-γ and/or VRP with adriamycin augmented the adriamycin-induced cytotoxicity. The enhancing effects of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity were evaluated using IC_{70} to adriamycin (Table 1). rH-IFN-γ at doses of 10^2 and 10^3 U/ml of significantly decreased IC_{70} of MKN-45 ($p<0.05$). IC_{70} to adriamycin was not decreased by VRP alone. However, when used in a combination of rH-IFN-γ with VRP, IC_{70} decreased to a greater extent than did rH-IFN-γ alone. The modification effects of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity were evaluated in terms of modification index (MI: the ratio of IC_{70} to adriamycin to IC_{70} to adriamycin in the presence of rH-IFN-γ and/or VRP), demonstrating that rH-IFN-γ significantly increased in adriamycin-induced cytotoxicity and that the combined use of rH-IFN-γ and VRP enhanced the adriamycin-induced cytotoxicity to a greater extent than did rH-IFN-γ alone: MI values at 10^2 U/ml and 10^3 U/ml of rH-IFN-γ were 1.7 and 3.1, respectively; those at 1.5 μg/ml and 3.0 μg/ml of VRP in the presence of 10^3 U/ml of rH-IFN-γ were 4.4 and 6.0, respectively (Fig. 3). These results demonstrate that adriamycin-induced cytotoxicity can be enhanced by rH-IFN-γ and further en-

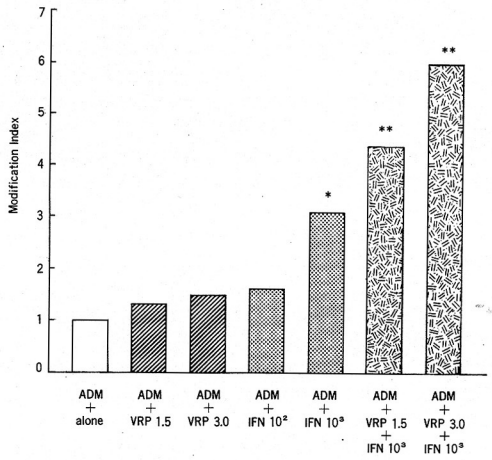

Fig. 3. Enhancement of adriamycin-induced cytotoxicity against MKN-45, human stomach adenocarcinoma, by rH-IFN-γ and/or verapamil. Modification Index was determined as the ratio of IC_{70} to adriamycin alone to IC_{70} to adriamycin in the presence of rH-IFN-γ and/or verapamil. ADM, adriamycin; VRP 1.5 and VRP 3.0, 1.5 and 3.0 μg/ml of verapamil, respectively; IFN 10^2 and IFN 10^3, 10^2 and 10^3 U/ml of rH-IFN-γ, respectively.
*$p<0.05$ **$p<0.01$ as compared with ADM alone

hanced by the addition of VRP.

DISCUSSION

Since adriamycin came into use it has played a major role in combination chemotherapy for many solid tumors and adriamycin-based chemotherapy has remarkably improved the response rate and survival in certain types of tumor (Young et al., 1981). In many solid tumors including stomach cancer, however, innate resistance to adriamycin is one of the major obstacles to curative chemotherapy (Kaye and Merry, 1985; Preusser et al., 1988).In recent years, a great deal of effort has been made to potentiate the cytotoxicity of adriamycin by overcoming this innate resistance. The present study was undertaken to examine the modification effect of rH-IFN-γ and VRP on adriamycin-induced cytotoxicity.

IFN-γ is a glycoprotein which has a variety of funtions including antiviral, immunomodulatory and antitumor activity. The antitumor effect of rH-IFN-γ is considered to be due to mainly indirect cytotoxicity by the activation of the immune system, while some direct cytotoxicity has been reported in certain cancer cell lines (Bonnem and Oldham, 1987; Fransen et al., 1986). IFN-γ has also been regarded as a potential mediator to modify the cytotoxicity of chemotherapeutic agents (Balkwill and Moodie, 1984; Kim et al., 1989). We have previously reported that rH-tumor necrosis factor and rH-IFN-γ overcame the acquired resistance to cisplatin in human lung cancer cells resistant to cisplatin and the innate resistance to cisplatin in MKN-45, the same cell line used in this study (Hong et al., 1987; Kim et al., 1989).

The mechanism by which adriamycin-induced cytotoxicity is enhanced by IFN-γ is not clearly understood at present. It has been suggested that the cytotoxicity may be enhanced by IFN-γ through the modification of the charcteristics of the cell membrane and submembrane, such as changes in cell membrane fluidity, microfilaments, membrane-bind proteins, and cell cycles (Chatterjee et al., 1982; Imai et al., 1981; Lundblad and Lundgren, 1981; Wang et al., 1981).

On the other hand, acquired resistance to adriamycin is often reported to show cross-resistance to other anticancer agents, known as multiple drug resistance. VRP, an inhibitor of calcium transport, was reported to have the ability to overcome the acquired resistance to adriamycin by an altered membrane transport, resulting in increased intracellular retention of adriamycin in resistant cells (Tsuruo et al., 1986; Twentyman et al., 1986). VRP may bind P-glycoprotein innately expressed in tumor cells and subsequently decrease in drug efflux. If the mechanism of acquired resistance is some part of the innate resistance and some of the mechanisms of resistance are shared between chemotherapeutic agents, innate resistance to adriamycin may be overcome by rH-IFN-γ and VRP.

In the present study, the role of rH-IFN-γ and VRP as a modifier of sensitivity of cancer cells to adriamycin has been investigated and the cytotoxicity of adriamycin was significantly enhanced by the use of rH-IFN-γ, being more enhanced by the combined use of rH-IFN-γ and VRP. To elucidate the mechanism of modification by rH-IFN-γ and VRP, additional study will be necessary. From the results presented here, we deduced that, by the simultaneous use of rH-IFN-γ and VRP with adriamcyin, better therapeutic results can be expected, although the clinical usefulness of these modifiers also depends upon whether concentrations large enough to overcome resistance can be obtained *in vivo* without any serious side effects. Therefore, we think that this preliminary study gives the rationale for the additional study of rH-IFN-γ and VRP as modifiers of sensitivity to adriamycin.

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REFERENCES

Balkwill FR, Moodie EM: *Positive interactions between human interferon and cyclosphamide or adriamycin in a human tumor model system. Cancer Res 44:904-908, 1984.*

Bonnem EM, Oldham RK: *Gamma-interferon: Physiology and speculation on its role in medicine. J Biol Response Mod 6:275-301, 1987.*

Brstow MR, Billingham ME, Mason JW, Daniels JR: *Clinical spectrum of anthracycline antibiotic cardiotoxicity. Cancer Treat Rep 62:873-879, 1987.*

Chatterjee S, Cheung HC, Hunter E: *Interferon inhibits Sendai virus-induced cell fusion: an effect on cell membrane fluidity. Proc Natl Acad Sci 79:835-839, 1982.*

Fransen L, Van der Heyden J, Ruysschaert R, Fiers W: *Recombinant tumor necrosis factor: its effect and its synergism with interferon-γ on a variety of normal and transformed human cell lines. Eur J Cancer Clin Oncol 22:419-426, 1986.*

Hindenburg AA, Baker MA, Gleyzer E, Stewart VJ, Case N, Taub RN: *Effect of verapamil and other agents on the distribution of anthracyclines and on reversal of drug resistance. Cancer Res 47:1421-1425, 1987.*

Hong W-S, Saijo N, Sasaki Y, Shinkai T, Eguchi K, Sakurai M, Takahashi H, Nakano H, Nakagawa K, Twentyman PR: *in vitro growth inhibition of cisplatin-resistant human lung cancer cell lines by recombinant human tumor necrosis factor and/or recombinant human interferon-γ by virtue of collateral sensitivity. Jpn J Cancer Res 78:1274-1280, 1987.*

Hong W-S, Saijo N, Sasaki Y, Minato K, Nakano H, Nakagawa K, Fujiwara Y, Nomura K, Twentyman PR: *Establishment and characterization of cisplatin-resistant sublines of human lung cancer cell lines. Int J Cancer 41:462-467, 1988.*

Imai K, Ah-Kau NG, Glassy MC, Ferrone S: *Differential effect of interferon on the expression of tumor-associated antigens and histocompatibility antigens on human melanoma cells. J Immunol 127:505-509, 1981.*

Kaye S, Merry S: *Tumour cell resistance to anthracyclines. a review. Cancer Chemother Pharmacol 14:96-103, 1985.*

Kim C-M, Hong W-S, Lee J-O, Kang T-W, Kim Y-H, Song J-K, Yun T-K, Kim C-Y: *Enhancement of cytotoxicity of cisplatin in vitro by recombinant human tumor necrosis factor and/or recombinant human interferon-α, -β and -γ. Jpn J Cancer Res 80:904-909, 1989.*

Kim NK: *Chemotherapy of advanced gastric carcinoma with 5-fluorouracil, adriamycin, mitomycin-C (FAM), and 5-fluorouracil, adriamycin, cisplatin (FAP) combinations, Experience in Korea. In: Ogawa M. et al., eds. Adriamycin, its expending role in cancer treatment. Excerpta Medica 629th ed, Amsterdam. 137-145, 1984.*

Lundblad D, Lundgren E: *Block of a glioma cell line in S by interferon. Int J Cancer 27:749-754, 1981.*

Mosmann T: *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J immunol Meth 65:55-63, 1983.*

Preusser P, Wilke H, Achterrath W, Fink U, Neuhaus P, Meyer J, Meyer H-J, van de Loo J: *Phase-II-study with EAP (etoposide, adriamycin, cisplatinum) in patients with primary inoperable gastric cancer and advanced disease. In: Schlag P, et al., eds. Recent results in cancer research Vol. 110. Springer-Verlag, Berlin-Heidelberg. 198-206, 1988.*

Tsuruo T, Iida H, Nojiri M, Tsukagoshi S, Sakurai Y: *Circumvention of vincristine and adriamycin resistance in vitro and in vivo by calcium influx blockers. Cancer Res 43:2905-2910, 1983.*

Tsuruo T, Iida-Saito H, Nojiri M, Kawabata H, Oh-Hara T, Hamada H, Utakoji T: *Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and in isolated clones. Jpn J cancer Res 77:682-692, 1986.*

Twentyman PR, Fox NE, Wright KA, Bleehen NM: *Deviation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells. Br J Cancer 53:529-537, 1986.*

Wang E, Pfeffer LM, Tamm I: *Interferon increases the abundance of submembranous microfilaments in Hela-S3 cells in suspension culture. Proc Natl Acad Sci 78:6281-6285, 1981.*

Young RC, Ozols RF, Myers CE: *The anthracycline antineoplastic drugs. N Engl J Med 305:139-153, 1981.*