Tohoku J. Exp. Med., 1998, **186**, 313-322

Modulating Activity of Indomethacin to Vincristine Cytotoxicity in Various Human Carcinoma Cells

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KOBAYASHI, S., OKADA, S., HASUMI, T., SATO, N. and FUJIMURA, S. *Modulating Activity of Indomethacin to Vincristine Cytotoxicity in Various Human Carcinoma Cells.* Tohoku J. Exp. Med., 1998, **186** (4), 313-322 —— The modulating activity of indomethacin to vincristine (VCR) was investigated in thirty human pulmonary carcinoma cell lines (adenocarcinoma 9, large cell carcinoma 9, squamous cell carcinoma 6, small cell carcinoma 6) and five other cell lines (colon carcinoma 2, melanoma 1, teratocarcinoma 1, thymoma 1). The survival of these cell lines treated with VCR with or without indomethacin (2 μg/ml) for 72 hours were examined using MTT assay, and IC_{50} values were calculated. When the cutoff level of potent combined effect in clinical use was set at 2-fold increase of sensitivity, the positive rate was 100% for adenocarcinomas and large cell carcinomas, 25% for squamous cell carcinomas, 33% for small cell carcinomas. Mean modulating index was 2.91 in adenocarcinomas, 1.92 in squamous cell carcinomas, 3.06 in large cell carcinomas and 1.67 in small cell carcinomas. Of the cell lines of other tumors, three cell lines (colon carcinoma 1, melanoma 1, teratocarcinoma 1) showed the potent combined effect of VCR and indomethacin, while indomethacin was not effective in modulating activity to VCR in a thymoma cell line and fibroblast cells. In conclusion, it is considered that modulating activity of indomethacin for VCR is a general effect for various human cancer cells, and combined use with VCR and indomethacin may be a useful modulation therapy for the advanced lung cancer. ———— indomethacin; modulator; vincristine; cell line; pulmonary carcinoma

Use of a biochemical modulator combined with anticancer drugs has been considered useful for ordinary chemotherapy in patients with advanced cancer, as a means of enhancing the synergistic therapeutic effect against cancers. During the preliminary studies aimed at finding useful modulators for pulmonary carcinoma cells, we had examined the combined effect of each of 10 anti-

Received September 21, 1998; revision accepted for publication December 24, 1998.

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inflammatory agents for each of 10 anticancer drugs against two human pulmonary adenocarcinoma cell lines in vitro and found that indomethacin is an effective modulator of sensitivity to vincristine (VCR), resulting in more than a 3-fold increase in cytotoxicity of VCR in combination with 2 μg/ml indomethacin as compared with that produced by VCR alone (Kobayashi et al. 1997).

Since the modulating effect of indomethacin to VCR was not yet reported, the present study was undertaken in an attempt to examine the potential of indomethacin to enhance the cytotoxic activity of VCR against pulmonary carcinoma cell lines originated from tumors with different histological types (and various other carcinoma cell lines), and to identify that the combined use of VCR and indomethacin is useful for ordinary combined modality therapy in patients with advanced lung cancer.

Materials and Methods

Thirty human pulmonary carcinoma cell lines (adenocarcinoma 9, large cell carcinoma 9, squamous cell carcinoma 6, and small cell carcinoma 6) were derived from the resected primary tumors of 19 patients, the metastatic lymph nodes of 10 patients and subcutaneous metastatic tumor of one patient. Of five other cell lines tested as control, 2 colon carcinoma cells, 1 melanoma cell line and 1 teratocarcinoma cell line were derived from resected pulmonary metastatic tumors, and a thymoma cell line was derived from a resected primary tumor (Table 1). All these cell lines were cultured in our laboratory and kept in continuous culture for more than 3 years in Ham F12 medium with 10% fetal bovine serum, at 37°C in a humidified atmosphere containing 5% CO_2. The doubling time (D.T.) in vitro varied greatly among individual cell lines, ranging from 0.7 day to 14 days (Table 1). The mean D.T. for the 30 pulmonary carcinoma cell lines in vitro was 4.2 days. A subcultured fibroblast cell strain derived from interstitial tissue of a resected lung tumor was also examined as control cells.

We examined the drug sensitivity of these cancer cell lines after exposure to VCR in combination with indomethacin for 72 hours, by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with 96-well flat-bottomed microplates, and with Terasaki microplates. The MTT assay with 96-well microplates was done using the method described Carmichael et al. (1987). Each cell line was suspended in culture medium (5×10^5/ml) with 0.25% trypsin solution, cell suspensions (180 μl) were placed in the individual wells of a 96-well microplate, 20 μl of drug solution was added, and culturing was done in an incubator at 37°C in a humidified atmosphere containing 5% CO_2. After a 72 hours of incubation, 20 μl of MTT reagent (MTT 4 mg/ml + 0.1 M sodium succinic acid) was added, and incubation was performed for 4 hours at 37°C. Then the formazan had formed was extracted with 150 μl of dimethyl sulfoxide. Absorbent values were measured at a wavelength of 595 nm with a microplate spectrophotometer (model 550, Bio-Rad Laboratories, Hercules, CA, USA), and surviving

TABLE 1. *Modulating activity of indomethacin to VCR cytotoxicity in 30 human pulmonary carcinoma cell lines and 5 other cell lines*

Cell line	Age, Sex	Primary	Pathology	vitro D.T. (days)	IC_{50} VCR (ng/ml) (Ind. [−]	Ind. 2 μg/ml)	MI
77-1	59 M	MLN	Adeno	1.4	4.61±0.21	1.69±0.12	2.73
85-3	68 M	Tumor	Adeno	2.9	>25	>25	
87-1	52 M	Tumor	Adeno	12.1	3.74±0.18	1.34±0.11	2.79
87-3	80 M	MLN	Adeno	14.0	3.46±0.25	1.12±0.17	3.09
87-6	67 M	Tumor	Adeno	6.7	18.66±0.34	8.57±0.24	2.18
87-13	67 F	Tumor	Adeno	11.7	2.96±0.13	0.71±0.06	4.17
88-3	63 F	Tumor	Adeno	1.7	6.67±0.27	3.08±0.17	2.17
88-4	60 F	Tumor	Adeno	1.7	5.89±0.16	1.60±0.08	3.68
92-2	74 F	Tumor	Adeno	1.1	4.19±0.31	1.71±0.19	2.45
85-1	70 M	Tumor	Squamous	0.9	7.65±0.39	2.11±0.26	3.63
86-8	67 M	Tumor	Squamous	5.8	>25	>25	
86-11	62 M	Tumor	Squamous	1.0	>25	>25	
88-5	62 M	Tumor	Squamous	2.0	5.13±0.18	3.59±0.16	1.43
88-14	63 M	Tumor	Squamous	4.1	2.63±0.11	1.72±0.11	1.53
91-4	61 M	MLN	Squamous	3.4	10.26±0.43	9.18±0.38	1.12
85-5	61 M	Tumor	Large	2.1	22.32±0.55	9.81±0.36	2.28
87-12	57 M	MLN	Large	2.0	2.59±0.15	0.62±0.09	4.18
87-17	57 M	SCMT	Large	3.7	14.99±0.36	3.24±0.17	4.63
88-10	51 M	Tumor	Large	7.0	1.77±0.19	0.41±0.10	4.32
88-12	64 M	MLN	Large	13.5	5.70±0.20	2.33±0.18	2.45
88-13	38 M	Tumor	Large	2.0	3.62±0.31	1.80±0.20	2.01
88-29	69 M	Tumor	Large	9.0	>25	>25	
90-5	68 M	MLN	Large	3.7	3.82±0.14	1.51±0.14	2.53
92-3	79 M	Tumor	Large	1.9	3.97±0.12	1.94±0.11	2.05
78-2	44 M	MLN	Small	1.8	2.58±0.16	2.12±0.15	1.22
84-2	61 M	Tumor	Small	1.3	4.65±0.18	2.02±0.18	2.30
84-5	54 M	MLN	Small	0.9	1.58±0.17	0.52±0.07	3.04
87-5	64 M	MLN	Small	1.8	6.09±0.24	5.51±0.23	1.11
87-16	62 M	Tumor	Small	2.1	5.10±0.18	4.63±0.14	1.10
88-9	63 M	MLN	Small	2.4	4.31±0.16	3.40±0.16	1.27
78-1	49 F	PM	Melanoma	5.0	6.02±0.22	2.69±0.20	2.24
79-1	40 M	PM	Terato-Ca.	0.7	4.22±0.14	1.50±0.11	2.81
84-4	55 F	PM	Colon-Ca.	7.8	>25	>25	
86-4	61 M	PM	Colon-Ca.	7.0	12.56±0.33	6.03±0.32	2.08
92-1	58 F	Tumor	Thymoma	1.4	15.64±0.20	13.25±0.17	1.18

D.T., Doubling Time; Ind., indomethacin; MLN, metastatic lymph node; PM, pulmonary metastasis; SCMT, subcutaneous metastatic tumor; MI, modulating index.

fraction and modulating index (MI) were calculated as follows: Surviving fraction = OD 595 of experiment/OD 595 of control. Modulating Index (MI) = IC_{50} value treated without indomethacin/IC_{50} value treated with 2 μg/ml of indomethacin. All determinations were carried out in triplicate. The significance of differences between the groups treated with VCR alone and the combined use of VCR with 2 μg/ml of indomethacin was evaluated by Student's t-test.

Results

Fig. 1 shows plates of an actual screening assay using Terasaki microplates (Kobayashi et al. 1993). The effect of a combination of VCR and indomethacin are investigated in 3 adenocarcinoma cell lines, 3 large cell carcinoma cell lines and 3 other cells as control (Fig. 1). The upper row of each cell line tested indicate the cytotoxicity of VCR alone, and the lower row indicate the cytotoxicity of VCR in combination with 2 μg/ml indomethacin solution. Two hundreds ng/ml VCR solution were added to each well of the far right line and serially diluted 2-fold to left. We can macroscopically reveal that indomethacin had a marked combined effect to VCR in many pulmonary carcinoma cells with different histological types and a teratocarcinoma cells, resulting in more than a 2-fold increase in cytotoxicity of VCR as compared with that produced by VCR alone, whereas indomethacin had no obvious combined effect in a thymoma cell line and a fibroblast cell strain.

The survival of these cell lines treated with VCR (0.06–25 ng/ml) with or without indomethacin (2 μg/ml) were examined using MTT assay with 96 well plates, and calculated IC_{50} values. As shown in Fig. 2, indomethacin significantly potentiated the cytotoxicity of VCR in human pulmonary carcinoma cells ($p < 0.05$). In Adeno 2 cell line, the IC_{50} for single agent VCR is 5.63 ± 0.21 (S.E.) ng/ml. When 2 μg/ml of indomethacin is added to the assay, the IC_{50} for VCR decreased to 1.49 ± 0.12 (S.E.) ng/ml which represents a 3.75-fold decrease. In Large 1 cell line, the IC_{50} for VCR alone and VCR in combination with 2 μg/ml indomethacin were 1.42 ± 0.2 and 0.58 ± 0.11 (S.E.) ng/ml respectively, which represents a 2.45-fold decrease. All p-values were < 0.05 using t-test for paired data in various concentrations of VCR in these two pulmonary carcinoma cell lines. On the other hand, in Fibroblast cell strain, indomethacin is not effective in significantly modulating the activity of VCR: the IC_{50} for VCR alone and VCR in combination with 2 μg/ml indomethacin were 16.8 ± 0.4 and 13.7 ± 0.3 (S.E.) ng/ml respectively, which represents a 1.23-fold decrease.

Table 1 shows the IC_{50} for VCR alone and VCR in combination with 2 μg/ml indomethacin in 35 cell lines examined by MTT method. The sensitivity of human pulmonary carcinoma cells for VCR varied. The IC_{50} for single agent VCR exceeded 25 ng/ml in 4 human pulmonary carcinoma cell lines and a colon carcinoma cell line. Therefore, the combined effect of VCR and indomethacin could not be estimated in these 5 cell lines (Table 1). When the combined effect

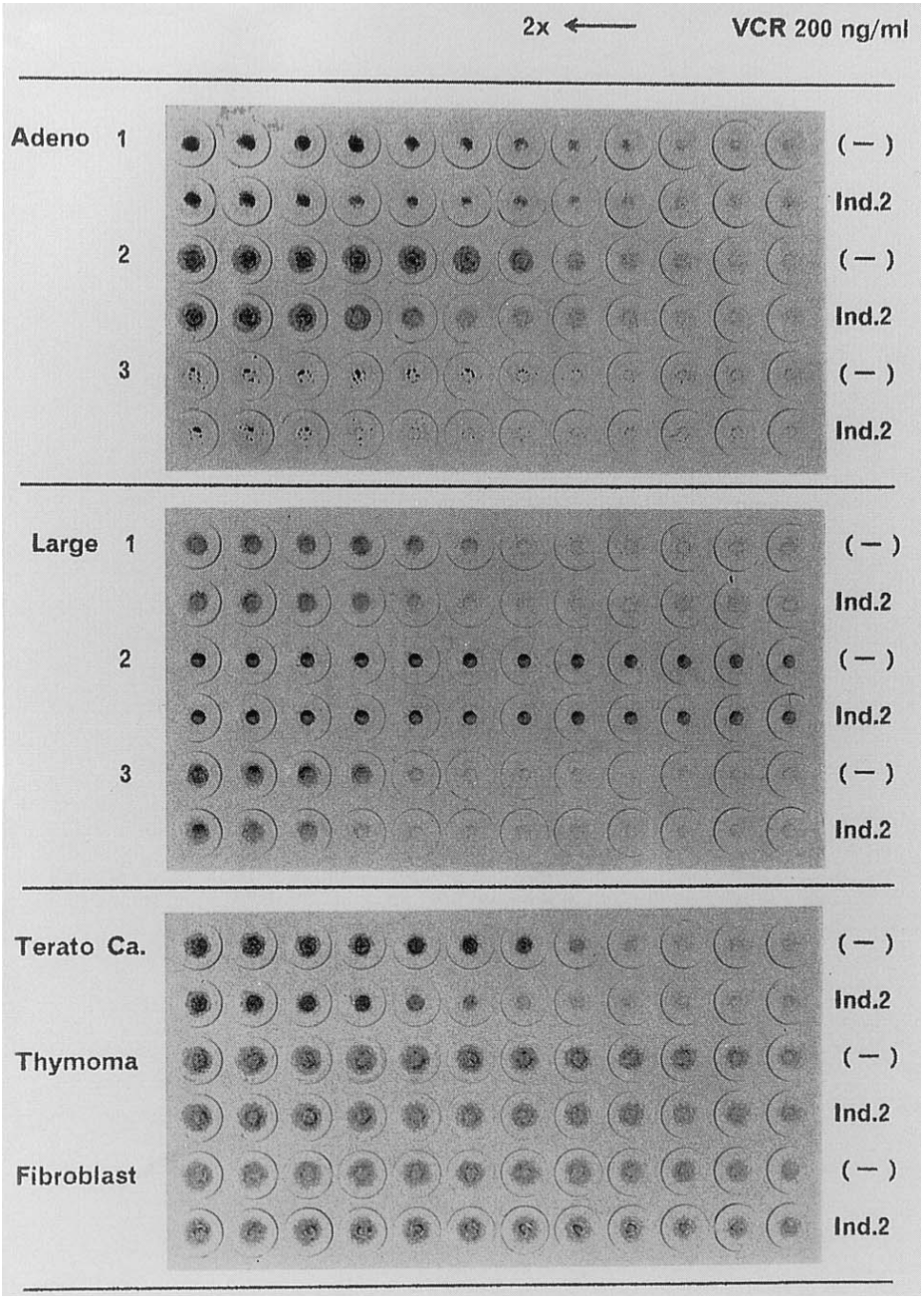

Fig. 1. The efficacy of the drug combination using screening assay with Terasaki microplates. The effect of a combination of 2 μg/ml of indomethacin and VCR are investigated in 3 adenocarcinoma cell lines, 3 large cell carcinoma cell lines and 3 other cells. The upper row of each cell line tested indicates the cytotoxicity of VCR alone, and the lower row indicates the cytotoxicity of VCR in combination with 2 μg/ml indomethacin solution. Two hundreds ng/ml VCR solution were added to each well of the far right line and serially diluted 2-fold to left.

of VCR and 2 μg/ml indomethacin was analyzed in relation to the histological type of 26 pulmonary carcinoma cell lines and 4 other cell lines, marked modulating activity of indomethacin to VCR (over 3-fold increase of sensitivity) was noted in three cell lines each of adenocarcinoma and large cell carcinoma, while marked modulating activity was observed in only one cell line each of squamous

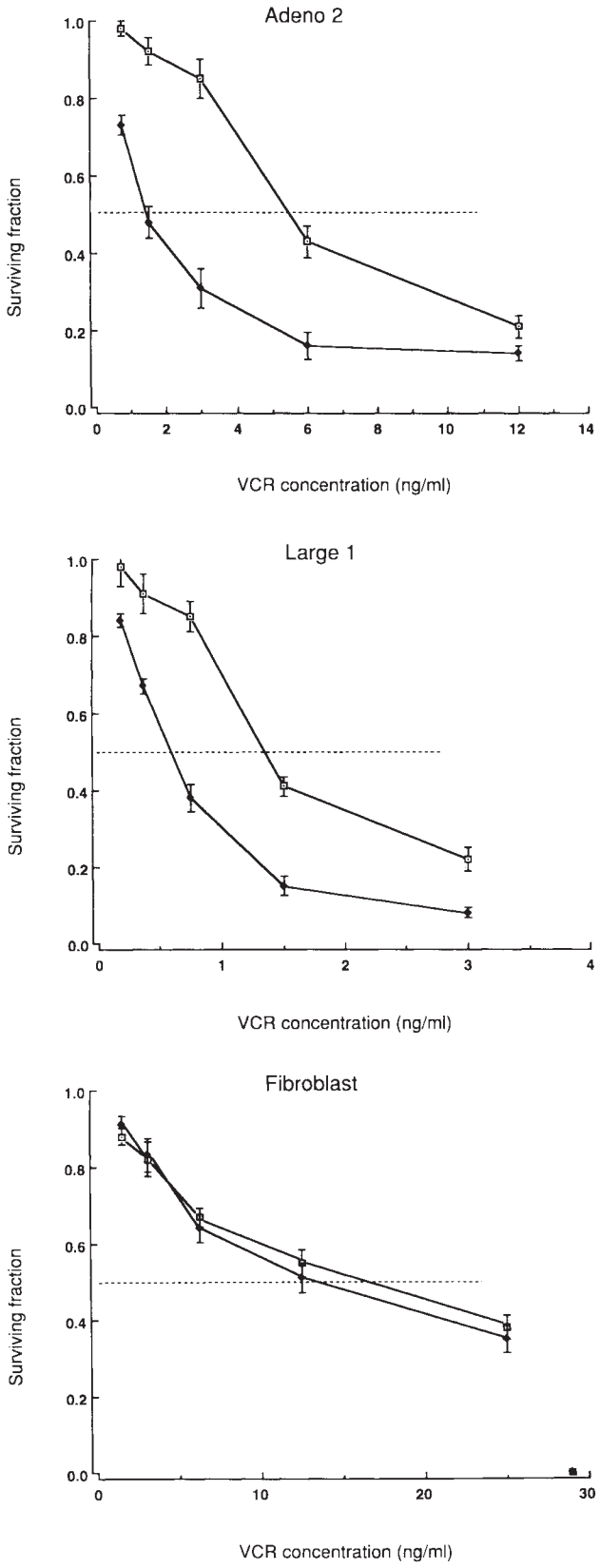

Fig. 2. The effect of indomethacin on the sensitivity of two pulmonary carcinoma cell lines and a fibroblast cell strain to VCR. The effect of VCR on each cell line in the absence (□) or presence of 2 μg/ml (◆) of indomethacin examined by MTT assay. Each point represents the mean of three experiments; bars, S.E. All p-value were <0.05 in various concentrations of VCR in two pulmonary carcinoma cell lines.

cell carcinoma and small cell carcinoma. When the cutoff level of potent combined effect in clinical use was set at 2-fold increase of sensitivity, the positive rate was 100% (8/8) for adenocarcinomas and large cell carcinomas, 25% (1/4) for squamous cell carcinomas, 33% (2/6) for small cell carcinomas (Fig. 3). Mean MI was 2.91 in adenocarcinomas ($p<0.01$), 1.92 in squamous cell carcinomas, 3.06 in large cell carcinomas ($p<0.01$) and 1.67 in small cell carcinomas ($p<0.05$). Of the cell lines of other tumors, which served as controls in the present study, three cell lines (colon carcinoma, melanoma and teratocarcinoma) showed the potent combined effect (over 2-fold increase of sensitivity) of VCR and indomethacin, while indomethacin were not effective in modulating activity to VCR in a thymoma cell line (Table 1 and Fig. 3). The normal human fibroblast cells, passaged over generations, also not revealed potent combined effect (Fig. 2).

Discussion

Although small cell lung cancer (SCLC) is sensitive to chemotherapy, the tumors almost invariably relapse and become clinically resistant to chemotherapy (Sehested et al. 1986; Brambilla et al. 1991). Non-SCLC are also usually clinically resistant to chemotherapy. The acquired or intrinsic resistance of carcinoma

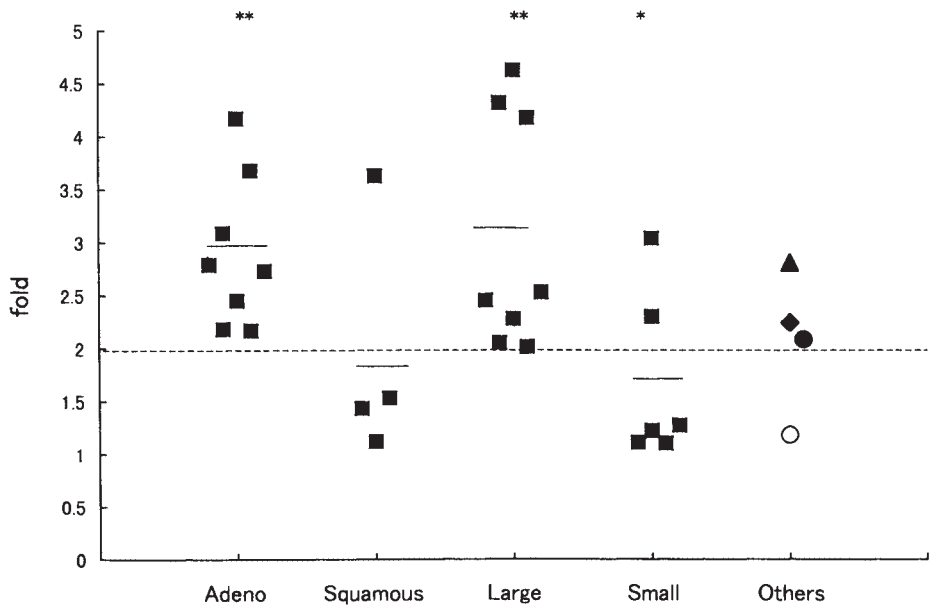

Fig. 3. The modulating activity of 2 μg/ml indomethacin to VCR analyzed in relation to the histological type of 26 pulmonary carcinoma cell lines and 4 other cell lines. When the cutoff level of potent combined effect in clinical use was set at 2-fold increase of sensitivity, the positive rate was 100% (8/8) for adenocarcinomas and large cell carcinomas, 25% (1/4) for squamous cell carcinomas, and 33% (2/6) for small cell carcinomas. Mean modulating index (MI) was 2.91 in adenocarcinomas, 1.92 in squamous cell carcinomas, 3.06 in large cell carcinomas and 1.67 in small cell carcinomas. ■, Lung Cancer; ◆, Melanoma; ▲, Teratocarcinoma; ●, Colon Cancer; ○, Thymoma. (**$p<0.01$; *$p<0.05$)

cells to multiple anti-cancer drugs is a major obstacle in the current chemotherapy for human cancers. Moreover, there is a limit to chemotherapy in terms of the patient's quality of life, due to severe adverse reactions (Stephens et al. 1994). In this sense, the efforts to minimize adverse reactions while using anti-cancer drugs in combination with effective modulating agents, thereby maintaining overall performance status and improving the quality of life, are considered important for the chemotherapy of advanced carcinoma in future.

A variety of agents have been investigated for biochemical modulators of anti-cancer drugs in resistant cells in vitro (Bellamy et al. 1988; Kramer et al. 1988). However, many of such modulators are not appropriate for a clinical use. The reasons for the failure include the toxicity of the modulators related to their own pharmacological effects and the inability to achieve optimal serum concentrations (Miller et al. 1991). There remains a need to identify safer and more potent modulators in clinical trials.

Indomethacin is known to work as an inhibitor of prostaglandin synthesis, and have been shown to inhibit the growth of animal experimental tumors (Sato et al. 1983; Lala et al. 1986). Furthermore, indomethacin is reported as a modulator to enhance the sensitivity of methotrexate (MTX) in mouse tumor cells and human breast cancer cell lines (Bennett et al. 1987). In addition, indomethacin have been also found to increase the sensitivity of MTX and etoposide (VP-16) in mouse tumor cells and human leukemia cell lines in vitro (Maca 1991). In a previous preliminary report, we have demonstrated that several anti-inflammatory agents, indomethacin, sulindac and mephenamic acid, significantly potentiate the cytotoxic activity of VCR, adriamycin (ADR), MTX and VP-16 in human pulmonary adenocarcinoma cell lines (Kobayashi et al. 1997). Especially, VCR in combination with indomethacin is the most potentiated combined effect for human pulmonary adenocarcinoma cells. To the best of our knowledge, the enhancement of VCR sensitivity by indomethacin was not yet reported in both animal experimental tumors and human tumors.

In this report, we clearly demonstrate that 2 μg/ml of indomethacin significantly potentiates the cytotoxic activity of VCR in a variety of human pulmonary carcinoma cells with different histological types. As shown in Table 1 and Fig. 3, when the combined effect of VCR and indomethacin was analyzed in relation to the histological type of pulmonary carcinoma, the mean enhancement rate was highest for large cell carcinoma (3.06-fold), followed by adenocarcinoma (2.91-fold), squamous cell carcinoma (1.92-fold) and then small cell carcinoma (1.67-fold). Indomethacin has been known as an most popular anti-inflammatory agent with safe clinical use. The concentration of indomethacin (2 μg/ml) required for the potentiation of combined anti-cancer effect with VCR can be safely achieved in patients. When indomethacin has been used at an ordinary dose (30 mg/m^2/8 hours), the plasma level of indomethacin achieved was 2 μg/ml (Adams et al. 1982). The concentration of indomethacin revealed more than a

2-fold cytotoxic efficacy on many human pulmonary carcinoma cells by combined use with VCR compared with only use of VCR in vitro.

The anticancer mechanism of indomethacin is still not well understood, and some hypotheses have been proposed to explain its action; inhibition of the endogenous synthesis of prostaglandin (Lala et al. 1986); acting as an immune-potentiator (Tilden and Balch 1982). The exact mechanism by which indomethacin enhances the cytotoxicity of anti-cancer drugs is not elucidated, though one possible mechanism that indomethacin modulates the sensitivity of anticancer drugs by decreasing the cellular efflux of the drugs by P-glycoprotein system have been proposed (Maca 1991). Although the mechanism by which indomethacin enhances the cytotoxicity of the anticancer drugs remains uncertain, the present data shows that indomethacin in combination with VCR is considered for use in cancer chemotherapy for the patients with advanced lung cancer. Especially, combined chemotherapy of VCR with indomethacin is considered of useful in the treatment of patients with large cell carcinoma, low differentiated adenocarcinoma and recurrent small cell carcinoma, since the chemotherapy in combination with VCR is recently used in non-SCLC (Dautzenberg et al. 1995; Takita et al. 1995), and currently of highly large therapeutic value in SCLC (Ettinger et al. 1990).

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