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The Inhibitory Effect of the Combination of Antineoplaston A-10 Injection with a Small Dose of *cis*-Diamminedichloroplatinum on Cell and Tumor Growth of Human Hepatocellular Carcinoma

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The inhibitory effects of a combination of Antineoplaston A-10 Injection with a small dose of *cis*-diamminedichloroplatinum (CDDP) on cell and tumor growth was tested in *in vitro* and *in vivo* settings. A human hepatocellular carcinoma cell line (KIM-1) was used for the cell growth and transplanted tumor growth studies. In the cell growth study, one-hour exposure of KIM-1 cells to CDDP in the medium at concentrations of 0.5, 1.0, and 2.0 μg/ml inhibited cell growth dose-dependently. Continuous exposure of cultured cells to Antineoplaston A-10 Injection at concentrations of 4, 6, and 8 mg/ml also inhibited tumor growth dose-dependently. The combination of 0.5 μg/ml CDDP and 6 mg/ml A-10 Injection inhibited cell growth more than did each agent individually. Electron microscopic study showed well-maintained organelle structures in Antineoplaston A-10 Injection-treated cells compared to CDDP-treated cells. α-Fetoprotein (AFP) production by 10^4 cells in 48 h increased in the A-10 Injection-treated and A-10 Injection + CDDP-treated groups as the concentration of these agents increased. In the tumor growth study, daily administration of Antineoplaston A-10 Injection 75 mg with once a week administration of 20 μg of CDDP for 5 weeks inhibited transplanted tumor growth in athymic mice after 33 days of treatment, while administration of 75 mg of A-10 Injection or 20 or 60 μg of CDDP alone showed no significant inhibition of tumor growth.

Key words: Antineoplaston A-10 Injection — CDDP — Cell growth — Tumor growth — Hepatocellular carcinoma

Antineoplastons are naturally occurring peptides and amino acid derivatives which show an inhibitory effect on cancer cell growth. These peptides were isolated from human blood and later from urine by Burzynski in 1973.

Burzynski[1)] proposed the existence of a biochemical defense mechanism separate from the immune system to correct program errors brought about by carcinogens in our environment. He was able to find substances involved in this putative mechanism, and named them antineoplastons. He fractionated them and identified Antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione), which he succeeded in synthesizing. Antineoplaston A-10 is quite insoluble in water and, when administered orally, is partly hydrolyzed in pancreatic juice to phenylacetylglutamine and phenylacetylisoglutamine at a ratio of 4:1. Since these two degradation products were also found to have an inhibitory effect on neoplastic cell growth, the sodium salt of these degradation products was formulated as A-10 Injection, because it is very soluble in water.

Although this injection has been confirmed to have an inhibitory effect on human breast cancer transplanted in athymic mice,[2)] it failed to show an inhibitory effect on rapidly growing human hepatocellular carcinoma (KIM-1).[3)]

A clinical observation of remarkable improvement in ovarian cancer that was brought about by using a combination of Antineoplaston A-10 Injection and a single intraperitoneal administration of CDDP suggested the possible potentiation of anti-cancer effects and the reduction of adverse effects when both agents were so used. Thus, we designed a study to test whether the use of a combination of Antineoplaston A-10 Injection and a small dose of CDDP was effective for this purpose.

MATERIALS AND METHODS

The human hepatocellular carcinoma cell line (KIM-1), established in the 1st Department of Pathology, Kurume University School of Medicine, was used for this study. The cells were maintained in T-25 flasks with serum-free GIT medium (Nihon Seiyaku Co., Tokyo) and passed by trypsinization every week or 2 weeks. The cells were divided into two groups for cell and tumor growth studies.

Cell growth study Cell suspension (0.1 ml), containing 5×10^5 cells/ml, was seeded in 1.5 ml of medium (Dulbecco's modified Eagle medium) in a Lab-Tek chamber and incubated for 24 h. These cells were then subjected to 3 kinds of treatment: CDDP, (Nihon Kayaku Co., Tokyo), Antineoplaston A-10 Injection (Burzynski Research Institute, Houston), and CDDP+Antineoplaston A-10 Injection. In the CDDP group, the cells were exposed to 0.5, 1.0, and 2.0 μg/ml of CDDP for one hour and incubation was continued after the CDDP had been rinsed off. The medium was renewed every two days up to the 9th day. In the A-10 Injection group, the cells were incubated in medium containing Antineoplaston A-10 Injection at concentrations of 4, 6, 8 mg/ml up to the 9th day. This medium was also renewed every two days. In the CDDP+A-10 Injection group, the cells received a one hour exposure to 0.5 μg/ml of CDDP and were then incubated in medium containing 4 or 6 mg/ml of A-10 Injection up to the 9th day. Cells were also incubated after one-hour exposure to 1.0 μg/ml of CDDP without A-10 Injection for comparison. Cells in all groups were counted on days 1, 3, 5, 7, and 9 with a Coulter cell counter.

Electron microscopic study was performed on the cells on the 5th and 9th days. All procedures were duplicated. AFP, measured in the medium on days 1, 3, 5, 7, and 9, was expressed as AFP ng/10^4 cells/48 h.

Tumor growth study The KIM-1 cell suspension was implanted subcutaneously in BALB/c athymic mice. About one month later, the tumors which had grown were excised and their parenchymal tissues were cut into small pieces 2×2 mm in size. These small pieces were implanted subcutaneously in 50 athymic mice, which were divided into the following 5 groups, each containing 10 mice: control, CDDP 20, CDDP 60, A-10 Inj. 75, and CDDP 20+A-10 Inj. 75. Only well-grown tumors whose estimated tumor volume (defined below) was over 100 mg were selected for the start of this study.

In the CDDP 20 and CDDP 60 groups, 20 and 60 μg of CDDP, respectively, was administered intraperitoneally every Tuesday for the first 5 consecutive weeks of this study. In the A-10 Inj. 75 group, Antineoplaston A-10 Injection 75 mg was administered once daily for the whole period of this study. In the CDDP 20+A-10 Inj. 75 group, 20 μg of CDDP was administered intraperitoneally every Tuesday for the first 5 consecutive weeks in addition to daily i.p. administration of 75 mg of A-10 Injection.

The diameters of the long and short axes of the tumors were measured with vernier calipers twice a week, and the weights of the mice were measured at the same intervals. The tumor volume, estimated by the formula: length$\times$width$\times$width/2, was expressed in mg.

The unpaired, two-tailed Student's t test was used for evaluating the statistical significance of differences between groups.

RESULTS

Cell growth inhibition by CDDP is shown in Figure 1A. Cell growth was inhibited most on the 2nd day, 35%, 75%, and 85% inhibition being seen at 0.5, 1.0, and 2.0 μg/ml of CDDP, respectively. Inhibition decreased each day thereafter and no inhibition was seen at 0.5 and 1.0 μg/ml of CDDP on the 9th day. Cell growth inhibition by Antineoplaston A-10 Injection is shown in Figure 1B. Cell growth was inhibited at a constant rate up to the 9th

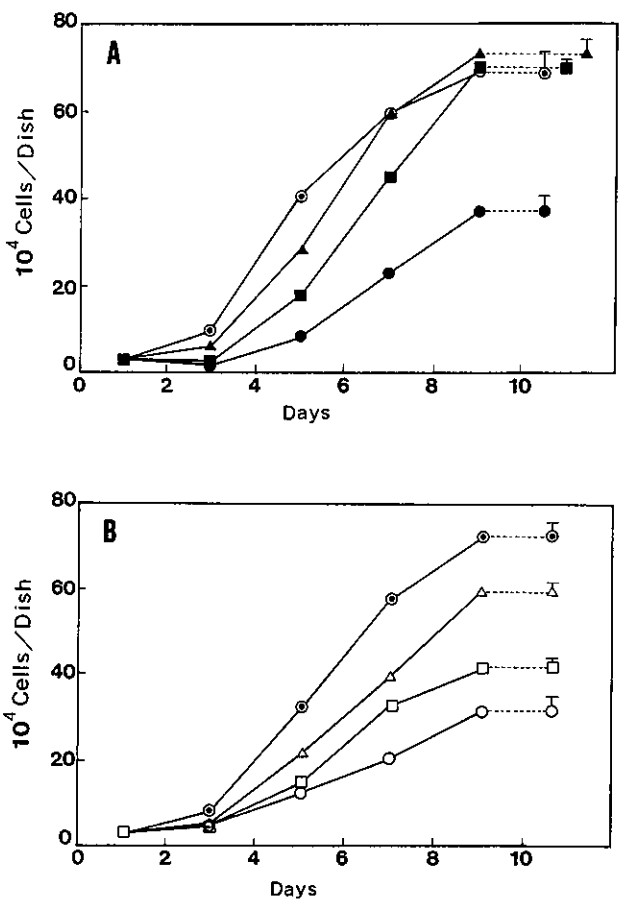

Fig. 1. Inhibition of cell growth by CDDP (A), and Antineoplaston A-10 Injection (B). One-hour exposure to 0.5 (▲) and 1.0 (■) μg/ml of CDDP inhibited cell growth most on the 2nd day, the inhibitory effect being reduced with time thereafter, while inhibition with 2.0 (●) μg/ml of CDDP was constant. Antineoplaston A-10 Injection 4.0 (△), 6.0 (□), 8 (○) mg/ml inhibited cell growth at a constant rate during the whole incubation period; ◉ represents the control. Bars indicate standard deviation.

day, the final cell growth inhibition being 37%, 47%, and 57% at 4, 6, and 8 mg/ml of A-10 Injection, respectively, on the 9th day. Cell growth inhibition by CDDP+ Antineoplaston A-10 Injection is shown in Figure 2.

There was no inhibition with A-10 Injection alone at 4 mg/ml or 6 mg/ml, or with CDDP alone at 0.5 μg/ml, while there was 50% inhibition with the combination of CDDP 0.5 μg/ml+A-10 Injection 6 mg/ml. The combination of CDDP 1.0 μg/ml+A-10 Injection 4 mg/ml inhibited cell growth by 36% compared to the control.

Electron microscopic study on the 5th and 9th days showed well-maintained organelle structures such as mitochondria and RER in surviving cells treated with A-10 Injection at concentrations of 4–6 mg/ml (Figure 3C). There were also some destructive changes, such as fat droplets and secondary lysosomes. In surviving CDDP-treated cells (Figure 3B), the extent of these destructive changes was markedly greater.

AFP production by 10^4 cells in 48 h increased as the concentration of A-10 Injection was increased (Figure 4).

There was no significant inhibition of tumor growth in the CDDP 20, the CDDP 60, and the A-10 Inj. 75 groups, but there was significant inhibition in tumor weight in the CDDP 20+A-10 Inj. 75 group on the 33rd

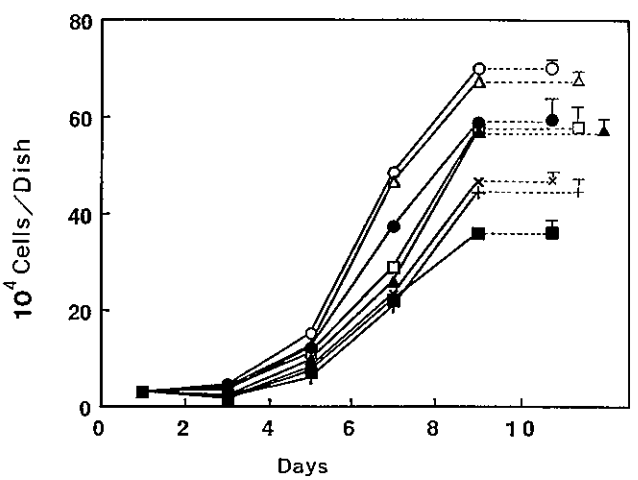

Fig. 2. Inhibition of cell growth by CDDP plus Antineoplaston A-10 Injection. On the 9th day, 0.5 μg/ml CDDP plus 6 mg/ml Antineoplaston A-10 Injection (■) inhibited cell growth by 50% compared to the control, while A-10 Injection 6 mg/ml alone, and CDDP 0.5 μg/ml alone showed no inhibition. ○, △, and □ represent control and 4 and 6 mg/ml of Antineoplaston A-10 Injection, respectively. ● and × represent 0.5 and 1.0 μg/ml of CDDP, respectively. ▲, ■, and + represent CDDP plus Antineoplaston A-10 Injection, 0.5 μg/ml +4 mg/ml, 0.5 μg/ml+6 mg/ml, and 1.0 μg/ml+4 mg/ml respectively. Bars indicate standard deviation.

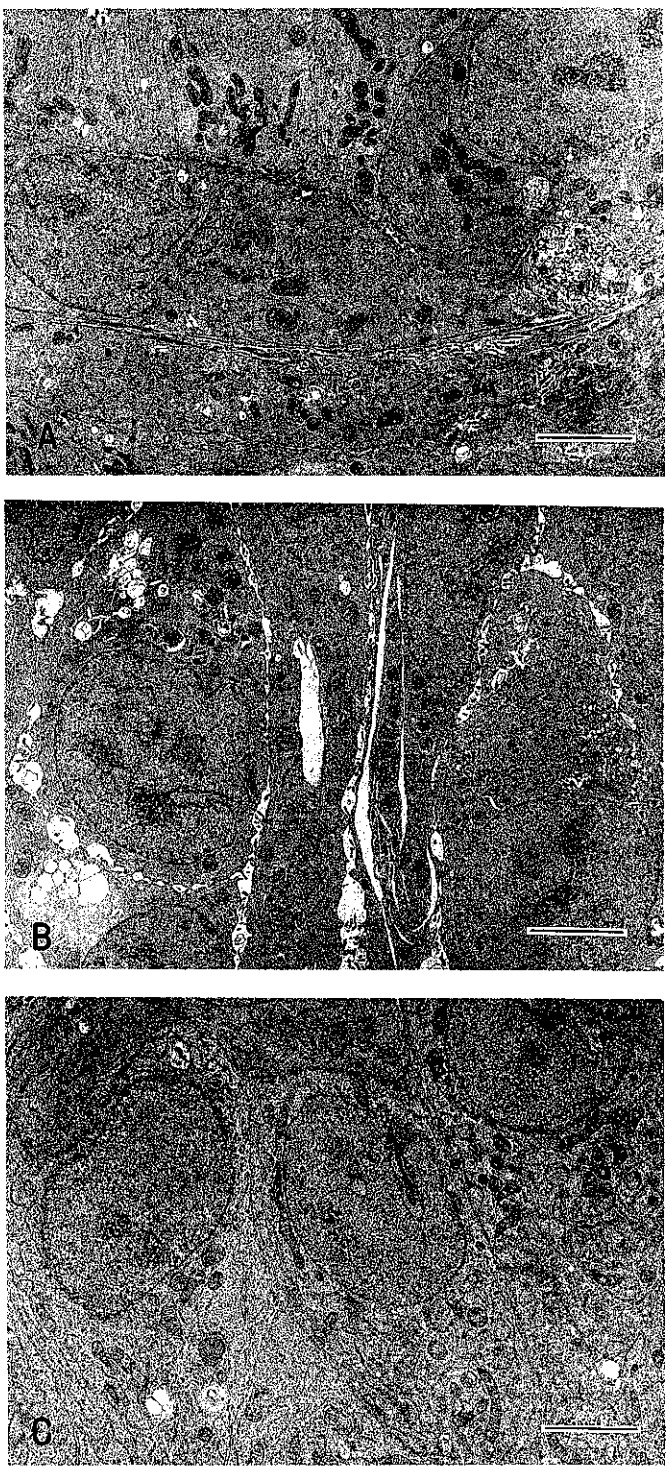

Fig. 3. Electronmicroscopic findings in control (A), CDDP (B), and Antineoplaston A-10 Injection (C) groups. Well equipped organelles such as mitochondria and rSER are seen more in cells treated with Antineoplaston A-10 Injection and less in cells treated with CDDP, compared to the control group. (×2,000; scale 5 μm)

day (1686±210 mg: m±SE) compared to the control (3057±567 mg) and thereafter until the end of the study ($P<0.05$) (Figure 5).

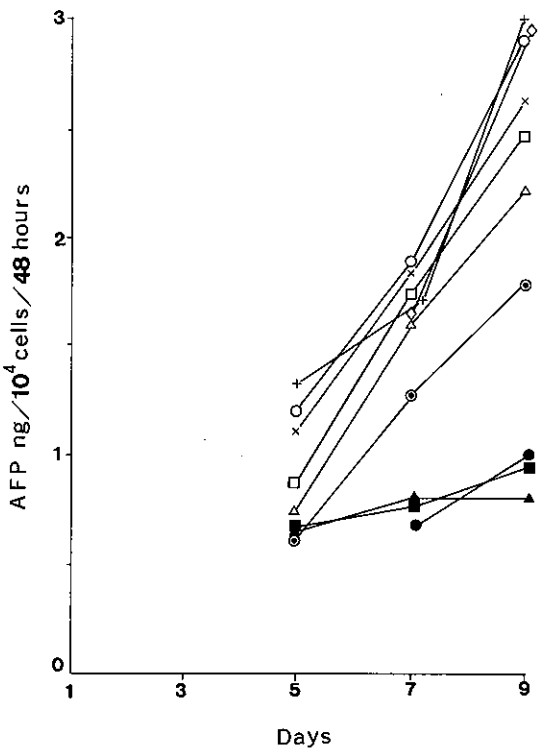

Fig. 4. AFP production per 10^4 cells in 48 h; cells treated with Antineoplaston A-10 Injection either alone or combined with CDDP produced more AFP as the concentration of the agents was increased, compared with cells treated with CDDP alone. ◉, ▲, ■, and ● represent control and 0.5, 1.0, and 2.0 μg/ml of CDDP, respectively. △, □, and ○ represent 4, 6, and 8 mg/ml of Antineoplaston A-10 Injection respectively. ×, +, and ◇ represent CDDP plus Antineoplaston A-10 Injection, 0.5 μg/ml +4 mg/ml, 0.5 μg/ml+6 mg/ml, and 1.0 μg/ml+4 mg/ml, respectively.

DISCUSSION

Burzynski[4,5] first described antineoplastons as naturally occurring peptides and amino acid derivatives which control neoplastic cell growth in humans. He succeeded in isolating and synthesizing some of these compounds. Antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione)[6] was the first antineoplaston to be synthesized. Antineoplaston A-10 is quite insoluble in water and, when administered orally, in partially hydrolyzed in pancreatic juice to phenylacetylglutamine and phenylacetylisoglutamine at a ratio of 4:1. Antineoplaston A-10 Injection[7] consists of the sodium salts of phenylacetylglutamine and phenylacetylisoglutamine in the ratio of 4:1, and was so formulated since these products were confirmed to have inhibitory effects on human cancer cell growth.

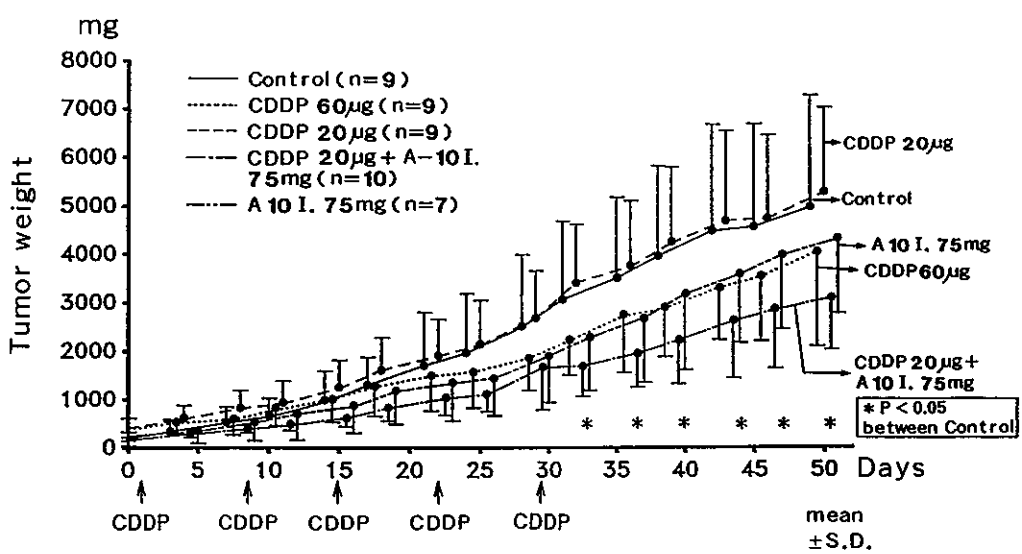

Fig. 5. Tumor growth curves for control, CDDP, Antineoplaston A-10 Injection, and CDDP plus Antineoplaston A-10 Injection groups. After 33 days of treatment, 20 μg CDDP plus 75 mg Antineoplaston A-10 Injection inhibited tumor growth significantly compared to control ($P<0.05$) and compared to 75 mg Antineoplaston A-10 Injection treatment after 43 days of treatment ($P<0.10$).

Although we found that Antineoplaston A-10 Injection had an inhibitory effect on KIM-1 cell growth in a previous tissue culture study,[8] this agent failed to show an inhibitory effect on rapidly growing KIM-1 tumor. The effective dose *in vitro* ranged from 4–8 mg/ml, which appears to be quite high compared to the effective doses of such conventionally available anticancer agents as CDDP.

If the mechanism of action of Antineoplaston A-10 Injection is similar to that of Antineoplaston A-10 itself, which was speculated by Hendry[9] to involve weak reversible intercalation of A-10 between base pairs of DNA, then Antineoplaston A-10 Injection must enter the cell to exert its effect. The high extracellular concentration required to show an inhibitory effect *in vitro* suggests the impermeability of the cell membrane to this compound. A consistently high concentration of A-10 Injection is not easily maintained in animal experiments since this agent is rapidly excreted by the kidney. CDDP, on the other hand, has an inhibitory effect even at such low concentrations as 1.0–2.0 μg/ml, suggesting that CDDP is able to cross the cell membrane very easily. It is not likely that CDDP has a specific receptor on the cell membrane, since this is not a naturally occurring substance in the human body. It is reasonable to believe that the concomitant use of A-10 Injection and a low dose of CDDP may augment anticancer effects and help reduce adverse effects, especially if a small dose of CDDP helps A-10 Injection to enter the cell. Better anticancer effects and fewer adverse effects would be expected with this combination, since A-10 Injection has virtually negligible adverse effects. The electron microscopic findings of well equipped organelles seen in A-10 Injection-treated cells, together with the constant rate of inhibition of cell growth and increase of secretory AFP, strongly indicate that Antineoplaston A-10 Injection matures the cell and slows cell growth. Further, it has been demonstrated by Samid *et al.*[10] that degradation products of Antineoplaston A-10 induced terminal differentiation in erythroleukemia. Antineoplaston A-10 Injection could inhibit tumor growth in transplanted athymic mice via the same mechanism.

It must be kept in mind that, while CDDP does destroy cells, it leaves any cells that survive treatment immature. This may give the cells the chance to regrow rapidly. It seems a promising approach to use both Antineoplaston A-10 Injection and CDDP in cancer chemotherapy, not only to augment their anticancer effect, but also to mature survivng tumor cells. With this combination it may become possible to treat severely ill cancer patients without the usual harmful side effects of chemotherapeutic agents.

In conclusion, our *in vitro* and *in vivo* experimental results indicate that the use of a combination of Antineoplaston A-10 Injection and a small dose of CDDP might be beneficial for patients on cancer chemotherapy.

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