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Enhancement of Both Intracellular Uptake and Antitumor Action of Cisplatinum on Human Neuroblastoma Cells by Encapsulation in Liposomes

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Phospholipid vesicles (phosphatidylcholine : phosphatidylserine : cholesterol = 6:2:3 in molar ratio) with a small unilamellar structure were used as drug carriers for introducing *cis*-diamminedichloroplatinum (CDDP) into human neuroblastoma cells, IMR-32, GOTO, Nagai, and TGW. DNA synthesis of IMR-32 cells among the human neuroblastoma cell lines was inhibited most strongly by CDDP-liposomes. CDDP-liposomes dose-dependently inhibited the DNA synthesis of IMR-32 in a similar fashion to that observed with free CDDP, but the drug concentration required to induce 50% inhibition of DNA synthesis for CDDP-liposomes (IC50: 0.7 μg CDDP/ml) was 1/3 of the IC50 for free CDDP (2.0 μg CDDP/ml). In support of the marked growth-inhibitory action of CDDP-liposomes, the intracellular incorporation rate of CDDP-liposomes was 3-fold higher when liposomes were used as carriers than when free CDDP was directly applied. CDDP-liposomes showed a stronger growth inhibition on IMR-32 cells at a high cell density than at a low density in culture. CDDP-liposomes were rapidly incorporated by IMR-32 cells within 5 min, resulting in the inhibition of DNA synthesis to 40% of the control. Swiss albino mouse 3T3 cells were less inhibited by CDDP-liposomes than by free CDDP, suggesting that encapsulation of CDDP in liposomes decreases cytotoxicity to normal cells.

Key words: Cisplatinum — Liposome — Neuroblastoma — Growth inhibition

Neuroblastoma is one of the most serious malignant tumors of infants, though it occasionally differentiates or matures to a benign form called ganglioneuroma.[1,2] For the last few years our laboratory has made considerable efforts to find a means for inducing differentiation of neuroblastoma cells *in vitro* using agents that increase intracellular cyclic AMP, such as papaverine, BL191, and prostaglandin, or neuroblastoma growth-inhibitory factor (NGIF) which is derived from glioblasts and specifically inhibits neuroblastoma cell growth.[3–5] It was found, however, that cyclic AMP-related agents were unsuitable for clinical applications due to severe side effects, and NGIF due to the presence of an intrinsic serum inhibitor.[4] Although the potent antitumor activity of CDDP has recently made it a widely used a chemotherapeutic agent against neuroblastoma,[6] its therapeutic potential is limited because of the nephrotoxicity and severe nausea and vomiting in patients.[7] Previous investigators have reported that the use of anionic or neutral liposomes as drug delivery vehicles can minimize the drug toxicity while maintaining or improving its antitumor effect.[8–10] Since our research has been directed toward obtaining a neuroblastoma-specific effect by the use of antitumor agents or differentiation-promoting substances in order to reduce the general toxicity, we employed liposomes as a drug carrier capable of specific drug-transport to neuroblastoma cells by coupling with either antibodies to neuronal cell components or NGIF. As a preliminary step for clinical application of antitumor agents, in this paper, we compare the growth-inhibitory effects of CDDP-liposomes and free CDDP on human neuroblastoma cells.

The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum; FBS, fetal bovine serum; NBS, newborn bovine serum; F-10 medium, Ham's F-10 medium; DMEM, Dulbecco's modified Eagle's medium; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidyl-L-serine; SDS, sodium dodecyl sulfate; ATCC, American Type Culture Collection.

MATERIALS AND METHODS

Materials CDDP and cholesterol were purchased from Wako Pure Chemical Industry, Ltd., Osaka. PC, PS, and 5(6)-carboxyfluorescein were from Sigma. [^{3}H]-Thymidine was from New England Nuclear, Boston, Mass.

Cell cultivations IMR-32 is a human neuroblastoma cell line which was purchased from the ATCC; its properties have been described fully elsewhere.[11] IMR-32 cells were cultivated in Ham's F-10 medium (Microbiological Associates, Walkersville, USA) containing 10% FBS (GIBCO, Chagrin Falls, USA), penicillin (40 units/ml) and streptomycin (200 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. Other human neuroblas-

toma cell lines, TGW, Nagai, and GOTO, from the Japanese Cancer Research Resources Bank (JCRB) were grown in RPMI medium 1640 (GIBCO) containing 10% FBS with the same supplements as in the case of IMR-32. Costar flasks (75 cm^2, No. 3275, Mass.) were used to maintain the cells. Costar 12-well plates (No. 3512) were used to examine cell growth and DNA synthesis, and petri dishes (diameter, 150 mm; Lux, No. 5212, Calif.) to measure the intracellular contents of CDDP. Cell viability was determined by counting the number of cells stained with 0.25% trypan blue. The cell number was counted with a hemocytometer. The cultures for a fluorohistochemistry (carboxyfluorescein) techniques were conducted in tissue culture glass chamber slides (No. 4804, Miles Scientific, Ill.). Swiss albino 3T3 cells were purchased from the ATCC, and cultured in F-10 medium supplemented with 10% FBS. Normal rat glioblasts in primary culture were obtained from 17-day Wistar rat embryos according to the method previously described.[12] As the secondary culture, the cells were maintained in monolayer culture for 6 days in F-10 medium with 10% NBS. The incorporation of [^{3}H]-thymidine as an index of DNA synthesis of the cultured cells was determined according to McLeester and Hall.[13]

Preparation of CDDP-containing liposomes The CDDP-containing liposomes were prepared from a lipid mixture composed of PC, PS, and cholesterol according to Forssen and Tokes.[10] Briefly, a complex of CDDP and PC was formed by adding a solution of CDDP (2 mg/ml) in 0.15 *M* NaCl to the dried PC (Type III-E, 22.9 mg) at a CDDP:PC molar ratio of 1:2. This mixture was sonicated for 5 min under a nitrogen stream with a microtip sonicator (Heat Systems, Model No. 225R, N.Y.) set at 80 W. This complex was then added to a dried mixture of PC, PS, and cholesterol at a molar ratio of 6:2:3. The second liposome suspension was either sonicated at 80 W for 5 min or mechanically mixed in a vortex mixer for 20 min. Encapsulated CDDP was separated from unencapsulated drug by gel filtration on Sephadex G-50 eluting with 0.02 *M* Tris-HCl-buffered saline, pH 7.4. 5(6)-Carboxyfluorescein was also encapsulated by the same procedure, except that 100 m*M* 5(6)-carboxyfluorescein solution in 0.02 *M* Tris-HCl-buffered saline, pH 7.4, was used in place of CDDP solution.

Quantification of CDDP The amounts of CDDP encapsulated in liposomes were determined on the basis of the absorbance of platinum at 266 nm by the use of a non-flame atomic absorption spectrometer (Shimadzu Seisakusho, Model No. AA-670G, Kyoto). Liposomes or cultured cells for the determination of CDDP were solubilized in 1% SDS solution. To measure the intracellular CDDP, near-confluent IMR-32 cells in a petri dish (diameter, 150 mm) were cultured in the presence of free CDDP or CDDP-liposomes for 24 h. After removal of the medium the culture was washed three times with 5 ml of ice-cold 0.02 *M* Tris-HCl-buffered saline, pH 7.4, and cells were scraped off with a rubber policeman and pelleted by centrifugation (2,000 rpm for 5 min). The cell pellets were solubilized with 0.1% SDS solution. The intracellular CDDP contents were expressed on the basis of protein concentration measured by Lowry's method.[14]

Negative staining for electron microscopy The prepared liposome suspension was placed on a copper grid coated with carbon film. After the removal of moisture with filter paper, 2.5% glutaraldehyde solution was dropped on the grid for fixation. The grid was washed with 0.05 *M* phosphate buffer, pH 7.0, containing 0.2 *M* sucrose, and negatively stained with 1.0% phosphotungstic acid subsequent to applying a droplet of 4% sucrose. The specimen was examined with an electron microscope (Hitachi, HU-12, Tokyo).

RESULTS

Properties of liposomes The morphological study of liposomes indicated that the liposomes obtained by sonication, which had vesicular sizes ranging from 50 to 100 nm (diameter) as found by transfer-electron microscopy under negative staining, were smaller than the liposomes obtained by mechanical shaking (diameter larger than 100 nm). The CDDP concentrations of liposomes after a passage through the Sephadex G-50 column were 16.4 μg/ml (n=5) for liposomes formed by mechanical shaking and 24.7 μg/ml (n=5) for liposomes formed by sonication. The growth-inhibitory effects of liposomes obtained by sonication were much stronger: i.e., 98.7% of DNA synthesis of IMR-32 cells was inhibited by the addition of 5.4 μg CDDP/ml encapsulated in liposomes prepared by sonication, while only 50.8% was inhibited by 7.4 μg CDDP/ml of liposomes prepared by mechanical shaking (Table I). We therefore employed liposomes prepared by sonication for the following experiments on cell-growth inhibition.

Inhibition of DNA synthesis and cell growth of neuroblastoma cells by CDDP-liposomes The concentration-dependent inhibition by CDDP of the proliferative capacity of various kinds of cultured cells was studied by the measurement of DNA synthesis (Fig. 1). Human neuroblastoma cell lines TGW, GOTO, and Nagai as well as IMR-32 were more sensitive to CDDP-liposomes than to free CDDP. IMR-32 cells were the most sensitive to CDDP-liposomes among the four human neuroblastoma cell lines. IMR-32 cells were incubated with various amounts of CDDP-liposomes (30 μg/ml) for 48 h from the second day to the fourth day after subculture (Fig. 2). Cell growth rates were markedly lowered and saturation densities were also suppressed in the presence of CDDP-liposomes. The minimum effective concentra-

Table I. Comparison on Neuroblastoma Growth-inhibitory Capacity between CDDP-liposomes Prepared by Sonication and by Mechanical Shaking

CDDP-liposomes	Concentration of CDDP (μg/ml)[a]	DNA synthesis[b] (cpm)	Inhibition rate[c] (%)
Liposomes by sonication	0.54	3656.5	26.6
	5.40	64.5	98.7
Liposomes by mechanical shaking	0.74	4836.5	2.9
	7.40	2450.5	50.8
Control	none	4982.8	0

a) The quantification of CDDP in liposomes was conducted with a non-flame atomic absorption spectrometer on the basis of platinum content as described in the text.
b) IMR-32 cells cultured in 12-well plates (cell density; 4×10^5/well) were incubated with the indicated concentrations of liposomes and [^{3}H]thymidine (0.1 μCi/ml).
c) Incorporation rates of [^{3}H]thymidine into DNA were determined by the method described in the text. All data are the mean values of duplicate experiments.

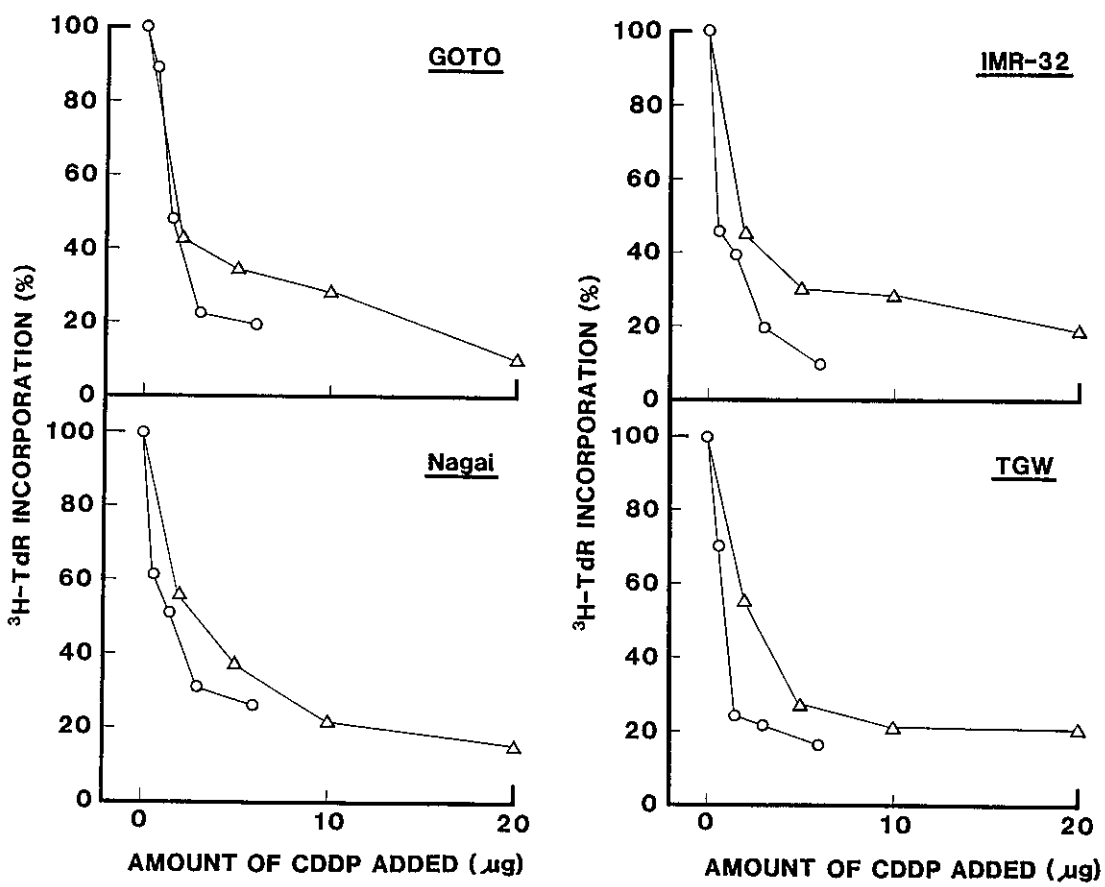

Fig. 1. Growth-inhibitory activity of CDDP-liposomes to human neuroblastoma cell lines. Four kinds of human neuroblastoma cell lines at near-confluence were exposed to various concentrations of free CDDP (△) or CDDP-liposomes (○) in the presence of [^{3}H]thymidine (0.1 μCi/ml) for 24 h. The IC50 values of CDDP-liposomes were 1.29 μg CDDP/ml for GOTO, 1.57 μg CDDP/ml for Nagai, and 0.63 μg CDDP/ml for TGW, respectively. The IC50 values of free CDDP were 1.71 μg/ml for GOTO, 2.86 μg/ml for Nagai, 2.0 μg/ml for IMR-32, and 2.57 μg/ml for TGW, respectively.

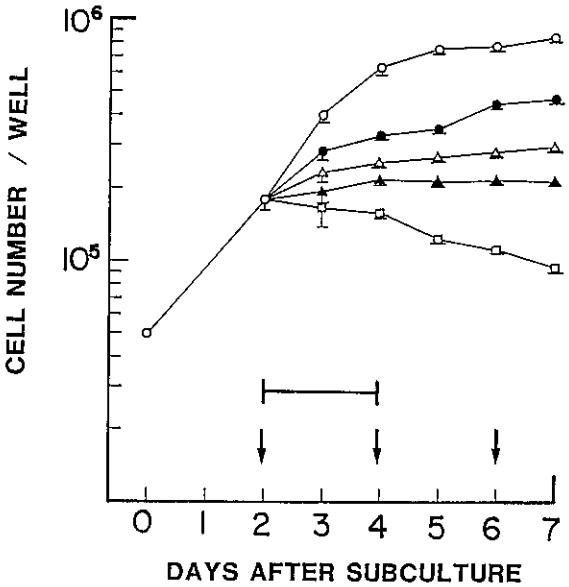

Fig. 2. Reduction of cell growth rates by CDDP-liposomes. IMR-32 cells were exposed to various amounts of CDDP-liposomes for 48 h from the second day to the fourth day after subculture. The growth medium was replaced every other day with a fresh F-10 medium containing 10% FBS. The amounts of CDDP-liposome solution (30 μg CDDP/ml) added in each experiment were 0 μl (○), 20 μl (●), 50 μl (△), 100 μl (▲), and 200 μl (□), which gave final CDDP concentrations of 0, 0.6, 1.5, 3.0, and 6.0 μg/ml, respectively. The arrows indicate the time-point of the replacement of medium and a solid bar indicates the period of drug exposure. All data are the mean values and standard deviations of triplicate cultures. Bars on the symbols indicate the standard deviation.

tion of CDDP-liposomes on cell growth was 20 μl/ml, corresponding to 0.5 μg CDDP/ml. It was of interest that the saturation density remained suppressed even after the replacement of CDDP-liposomes with a fresh medium, indicating the strong and persistent growth inhibitory action of CDDP. The effect of cell density in the culture system on the growth-inhibitory activity of CDDP-liposomes was examined as shown in Table II. Cells at high density (near confluence; 59.8×10^4/well) were much more sensitive to CDDP-liposomes than cells at low density (10.8×10^4/well).

Intracellular delivery of CDDP-liposomes Exposure time to CDDP-liposomes to induce the inhibition of DNA synthesis of IMR-32 cells was determined. After incubation with either CDDP-liposomes (30 μg CDDP) or free CDDP (100 μg) for various times, the culture media of IMR-32 cells were replaced with fresh F-10 media containing 10% FBS. IMR-32 cells were incubated continuously for 6 h in the presence of 0.1 μCi of [^{3}H]thymidine. As shown in Fig. 3, the minimum incubation time of CDDP-liposome with IMR-32 cells required to elicit full growth inhibition was 5 min. This result was confirmed by a fluorohistochemical method by the use of 5(6)-carboxyfluorescein-containing liposomes. Intense fluorescence was detected in the cytoplasm of IMR-32 exposed to carboxyfluorescein-containing liposomes for only 5 min (data not shown), suggesting that CDDP-liposomes were incorporated in a short time.

Action of CDDP-liposomes on normal cells CDDP-liposomes at the concentration of 10 μg CDDP/ml completely inhibited the DNA synthesis of IMR-32 cells. So, dose-dependent inhibition of normal cells such as Swiss 3T3 and rat glioblasts by CDDP-liposomes at concentrations lower than 10 μg CDDP/ml was examined. The

Table II. The Effect of Cell Density in the Culture System on the Growth-inhibitory Activity of CDDP-liposomes

Concentration of CDDP-liposomes (μg CDDP/ml)	Density of cultured cells			
	Low cell density		High cell density	
	Cell No. after 24 h ($\times10^4$/well)	Growth rate (%)	Cell No. after 24 h ($\times10^4$/well)	Growth rate (%)
0	20.2±0.9	187	69.5±0.8	116
0.6	19.0±0.7	176	52.8±1.5	88
1.5	17.8±0.6	165	42.2±1.2	71
3.0	11.7±0.2	108	31.8±1.9	53
6.0	8.0±0.7	74	18.2±0.6	30

IMR-32 cells at either a low density (10.8×10^4/well) or a high density (59.8×10^4/well) were exposed to various amounts of CDDP-liposome solution (30 μg CDDP/ml) from 0 to 6.0 μg CDDP/ml for 24 h. The data indicate the cell numbers and their increments after drug exposure for 24 h, and are the mean values and standard deviations of triplicate cultures.

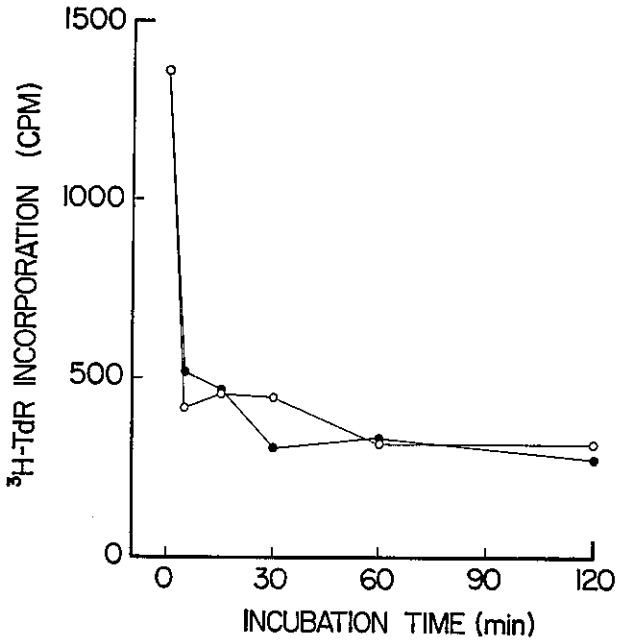

Fig. 3. Exposure time to CDDP-liposomes required to induce the inhibition of DNA synthesis of IMR-32 cells. After incubation with either CDDP-liposomes (30 μg CDDP/ml) (●) or free CDDP (○) (100 μg/ml) for the indicated time, the culture media of IMR-32 cells were replaced with fresh F-10 media containing 10% FBS. IMR-32 cells were incubated continuously for 6 h in the presence of 0.1 μCi [^{3}H]thymidine.

IC50 of CDDP-liposomes on IMR-32 was 0.7 μg CDDP/ml corresponding to one-third of the IC50 value of free CDDP (IC50: 2.0 μg CDDP/ml)(Fig. 4A). In contrast, toward mouse embryonic fibroblasts, Swiss 3T3 cells, CDDP-liposomes showed a lower inhibitory activity (IC50: higher than 10 μg CDDP/ml) than that of free CDDP (IC50: 3.0 μg CDDP/ml) (Fig. 4B). Normal rat glioblasts also exhibited lower sensitivity to CDDP-liposomes (IC50: 0.48 μg CDDP/ml) than to free CDDP (IC50: 0.23 μg CDDP/ml) like 3T3 cells (Fig. 4C), suggesting that encapsulation of CDDP in liposomes decreases the cytotoxicity of CDDP to normal cells.

Intracellular contents of CDDP The intracellular contents of CDDP incorporated into IMR-32 cells treated with 3.0 μg/ml of free CDDP or CDDP-liposomes for 24 h were estimated on the basis of the platinum content determined by a non-flame atomic absorption spectrometer. The rate of platinum incorporation by the cells incubated with CDDP-liposomes (1.2 μg CDDP/mg protein) was three times that with free CDDP (0.4 μg CDDP/mg protein) as indicated in Table III.

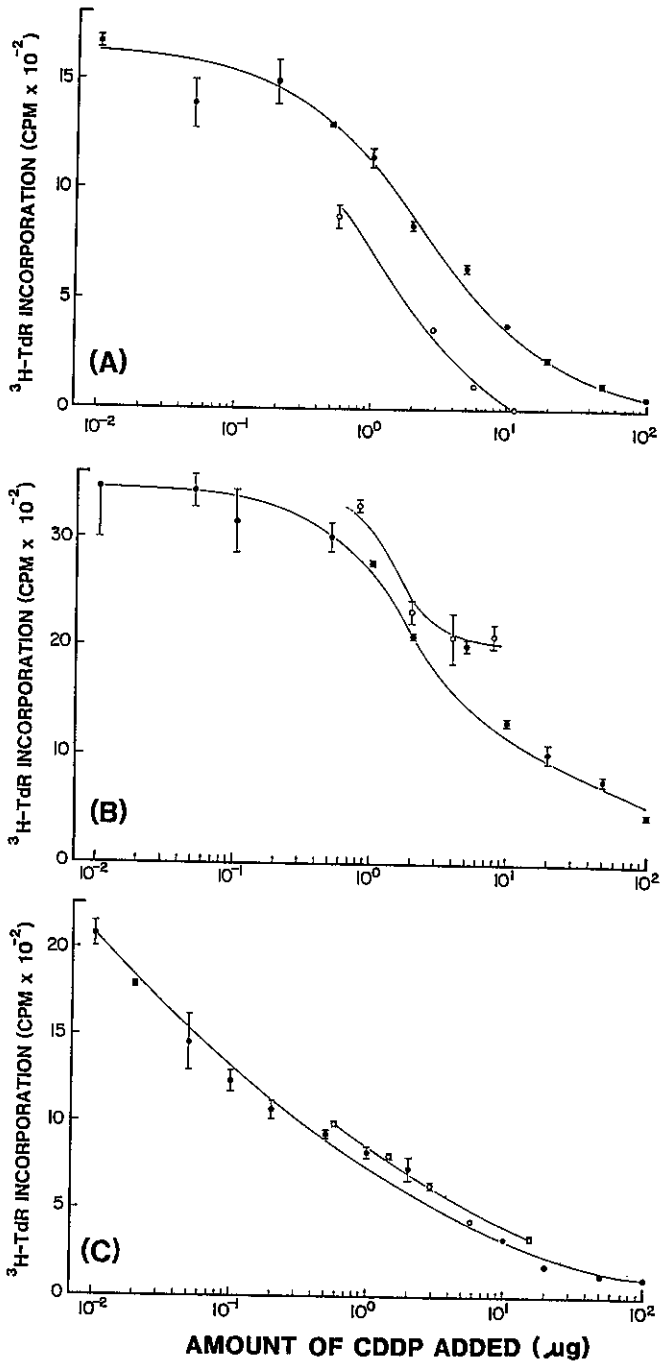

Fig. 4. Dose-dependence of the inhibition of DNA synthesis of IMR-32 cells (A), 3T3 cells (B), and normal rat glioblasts (C) by free CDDP and CDDP-liposomes. The cultured cells were prepared by the methods described in the text, and exposed to the indicated concentrations of free CDDP (●) or CDDP-liposomes (○) in the presence of [^{3}H]thymidine (0.1 μCi/ml) for 24 h. All data are the mean values and standard deviations of triplicate cultures.

Table III. Incorporation of CDDP-liposomes into Human Neuroblastoma Cells

	Free CDDP (μg/mg prot.)	CDDP-liposomes (μg/mg prot.)	CDDP-lipo./free CDDP
Exp. 1	0.46±0.04	1.37±0.15	2.98
Exp. 2	0.29±0.04	1.02±0.05	3.52

Near-confluent IMR-32 cells were cultured in the presence of free CDDP (3 μg/ml) or CDDP-liposomes (3 μg CDDP/ml) for 24 h. The quantification of platinum was conducted by the methods described in the text. The incorporation rates were expressed as the concentration of CDDP on the basis of mg cellular proteins. The values are the mean and standard deviations obtained from triplicate cultures for each experiment.

DISCUSSION

Liposome encapsulation of drugs is known to be generally useful for overcoming side effects and for maintaining a long-lasting effect *in vivo* and *in vitro*. We have demonstrated that the liposome is an effective carrier vehicle for introducing CDDP, a severely cytotoxic and nephrotoxic antitumor drug, into human neuroblastoma cells, IMR-32, *in vitro*.

Small unilamellar vesicles (SUV), which were used for the current experiments (prepared by sonication), differed from large unilamellar vesicles (LUV) prepared by mechanical shaking, and were superior to LUV in the following respects: (i) SUV could be easily and reproducibly prepared by the use of freshly prepared lipid solutions under a nitrogen gas stream at a constant temperature with the sonication periods indicated in the text; (ii) the incorporation efficiency of CDDP-liposomes into IMR-32 was greater, resulting in stronger growth inhibition.

CDDP-liposomes showed a marked growth-inhibitory action on other human neuroblastoma cell lines such as TGW, GOTO, and Nagai, besides IMR-32. There are numerous reports on enhancement of antitumor drug action by encapsulation: actinomycin D-containing liposomes on actinomycin-resistant Chinese hamster tumor cells,[15] CDDP-liposomes (containing a different lipid composition from ours) on Ehrlich ascites carcinoma cells,[16] and various antitumor drugs (actinomycin D, bleomycin, methotrexate, and cytosine arabinoside) encapsulated in liposomes on solid tumor growth *in vivo*.[17–20] In marked contrast, however, Mayhew *et al.*[21] and Mano *et al.*[22] found no difference in the growth-inhibitory activity of free and encapsulated drugs: cytosine arabinoside on mouse leukemia cell line, L1210,[21] and methotrexate on human choriocarcinoma cell line, BeWo.[22] Thus, the action of drugs encapsulated in liposomes on the cell growth of tumor cells seems to be rather variable. The action may mostly depend on the lipid composition of the target cell membrane, and the efflux and/or intracellular degradation of drugs subsequent to their incorporation into cells. Determination of the actual mechanism of growth inhibition must await precise pharmacodynamic experiments by the use of radiolabeled drugs.

It should be noted that the confluent IMR-32 cells were more sensitive to CDDP-liposomes than the growing cells, suggesting that CDDP, which usually acts at any phase of the cell cycle, once encapsulated into liposomes, may exhibit its growth-inhibitory action preferentially at a particular time-point of the G_1 and/or G_0 phase. The results may be indicative of alterations of the plasmalemmal lipid constituents during the cell cycle, resulting in a change of the affinity of liposomes for the cell membrane,[23] or of the structural transition of microtubules to a stable network at the G_1 or G_0 phase,[24] subsequently causing the promotion of endocytosis or intracellular transport of CDDP-liposomes.

The encapsulation of CDDP in liposomes enhanced CDDP actions to inhibit both DNA synthesis and cell growth of human neuroblastoma cells approximately three-fold, while it decreased the cytotoxicity of CDDP toward normal cells. It is clear, however, that the clinical application of vesicles to introduce drugs into neuroblastoma cells demands a means for strict tageting of the vesicles to these cells, such as coating of the vesicles with cell-specific immunoglobulins.[25] NGIF, which is a proteinaceous growth inhibitor (Mr 75,000) derived from normal glioblasts and shows a specificity to neuroblastoma cells,[3,4] could provide a tool for targeting of CDDP-liposomes by coupling NGIF to the liposomal surface, and this could be a promising approach for efficient drug delivery in neuroblastoma chemotherapy. An investigation along this line is in progress.

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