Lethal Effects of Radiation and Platinum Analogues on Multicellular Spheroids of HeLa Cells

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Multicellular tumor spheroids of HeLa cells have been grown in a static culture system. Samples of spheroids were exposed for 2 h to graded concentration of cis-platinum and its analogue, carboplatin, and then response assayed by survival of clonogenic cells. The purpose of present experiment is to clarify the effectiveness of these platinum compounds and to evaluate intrinsic radiosensitivity of cells using spheroids of HeLa cells as an experimental in vitro model. Variations of the drug sensitivity of monolayers as well as spheroids were also evaluated in cell-survival curves. In cis-platinum concentration-survival curve, there was a large shoulder extending as far as Cq=3.4 μM, after which there was exponential decrease in survival curve having a Co Value of 1.2 μM in spheroids. While the Co for the spheroids was essentially no significant change, but Cq value was larger than that of monolayers. This suggest that the effect of cis-platinum is greater in the monolayer with actively proliferaing cells than hypoxic one. In the carboplatin concentration-survival curves, the Co value of spheroids was 15.0 μM and the ratio with the Co from monolayer cell (32.5 μM) was 0.46, thus indicating that the spheroids had a greater sensitivity to carboplatin than monolayers. Therefore, the effect of carboplatin is mainly on the deeper layers of spheroids acting as hypoxic cell sensitizer. The enhanced effect was obtained for monolayer cells using combined X-ray and carboplatin treatment 2 hours before irradiation. The result shown in isobologram analysis for the level of surviving fraction at 0.01 indicated that the effect of two agents was truely supra-additive. From this experimental data, carboplatin has excited much recent interest as one of the most promising, since it is almost without nephrotoxicity and causes less gastrointestinal toxicity than cis-platinum. Interaction between carboplatin and radiation might play an important role for more effective local tumor control.

Key Words: Platinum analogues-radiation interaction, Spheroid, HeLa cells, Additive response

INTRODUCTION

Spheroid system displays fundamental characteristics of in vivo solid tumors and provides rapid, useful, and economical method for screening sensitizers and their therapeutic agents because it is intermediate in complexity between single-cell in vitro culture and tumors in experimental animals. Multicellular spheroid has several technical and theoretical advantages not found in monolayer cultures, and is, therefore, thought suitable as in vitro model tumor system.

Cells constituting a solid tumor in vivo have ben known to differ in various aspects from those growing exponentially as a monolayer in vitro. A number of in vitro models have been devised to simulate characteristics of solid tumors, including intercellular contact[1], heterogeneity of cell kinetics[2], heterogenous nature of intratumoral pH and nutrient supply, and existence of chronically hypoxic cells[3]. As a result, the effects of chemo-or radiotherapy, which are strongly influenced by these factors, cannot be accurately studied within the framework of experimental design using monolayer cells.

Multicellular spheroids developed by Sutherland et al[4] the spinner culture method and later by Yuhas et al[5] by culture on agar surface have been used widely as a model system for solid tumors. Multicellular spheroids of HeLa cells have structures very similar to the 3-dimensional cord structures in vivo, and they contain radioresistant hypoxic cells. Spheroid of HeLa cells therefore offer a suitable model system in vitro where the microenvironment and the cellular kinetics are similar to those of tumors in vivo. Three-dimensional growth of cells in spheroids facilitates direct and close range cell-cell interactions which may modify the cellular metabolism and thus responses to

therapeutic agents[6].

A number of factors are known to influence the lethal action of a particular drug in solid tumors. Among these there are inherent sensitivity of tumor cells constituting a solid tumor, age distribution in the cell cycle, growth fraction, drug uptake, and drug inactivation. Cis-platinum is an anticancer drug which is widely used for the treatment of malignant cancers, but has nephortoxicity and gastrointestinal toxicity. Carboplatin is a second generation platinum compound which in preclinical testing was found to be less nephrotoxic and emetogenic than cis-platinum, while retaining a broad spectrum of antitumor activity.

The purpose of the present experiments is to clarify the effectiveness of cis-platinum and its analogue, carboplatin, and to evaluate intrinsic radiosensitivity of cells using multicellular spheroids of HeLa cells as an experimental in vitro model. Further, variations of the sensitivity of monolayer as well as spheroids were evaluated in cell-survival curves.

MATERIALS AND METHODS

1. Cells

HeLa S3 cells were maintained as monolayer in Eagle's minimum essential medium (MEM) with Earle's solution containing all nonessential amino acids, and 1 mM sodium pyruvate and supplement with 10% fetal bovine serum (Flow laboratories), 100 units of penicillin and 100 μg of streptomycin per ml at 37℃ in 100% relative humidity of 5% CO_2 and 95% air.

2. Spheroid Culture

HeLa S3 cells grown for 2 days subculture were plated onto 0.5% Noble agar (Difco Co. Detroit, USA) in culture medium described above. After 10-min quiescence and formation of gel, it was washed once with the culture medium and then 10 ml culture solution, containing 5×10^5 cells, was placed on the agar surface of a plastic Petri dish (Nunc, Inc., Denmark, 90 mm diameter). Small spheroids (about 100 μm in diameter) formed 2 days after plating into dish. About 80% of the medium was replaced by fresh medium every 2 days for the first week and every day thereafter until the end of experiment. Spheroids (400 to 600 μm in diameter) with central necrosis after 11 to 13 days of culture were used for experimentation.

3. Irradiation

For irradiation of HeLa cells, 2 ml of single cell suspension obtained from the monolayer cells was placed into a 5 ml cryotube (A/S Nunc, Inc.) and irradiated in a horizontal postion. The radiation equipment was a X-ray apparatus (Pantak HF 100), which was operated at 100 kV and 30 mA. with 0.11 mm Cu and 0.5 mm Al filter and focal surface distance (FSD) of 50 cm. The dose rate was 0.63 Gy/min.

4. Treatment with Cis-Platinum and Carboplatin

A 200 mg potency of cis-platinum and a 100 mg potency of carboplatin were dissolved in 10 ml of physiological saline and 10 ml of 5% glucose, respectively diluted with culture medium containing 10% fetal calf serum immediately before use and added to the medium. The monoayer cells were treated in a culture medium containing these drugs and the spheroids in 5 ml of culture medium containing a known concentration of drug were incubated in 0.5% agar-coated Petri dishes (60 mm) which had been washed once with medium containing the desired concentration of the drug. After incubation, the spheroids were washed 3 times with culture medium.

5. Assay for Surviving Fraction

An appropriate number of spheroids were washed with 0.1% trypsin in Ca^{++}-Mg^{++} free Dulbecco's phosphate buffered saline and trypsinized in 0.1% trypsin containing 0.04% Versene in a shaking water bath for 30 min. After adding an equal volume of culture medium containing 10% serum, the cell clumps were dissociated by pipeting. Viable cells, which were not stained with erythrosine B[7], were counted using an improved Neubauer's hemocytometer. After appropriated dilution, the cells cultured in 60 mm Petri dishes. After 14 days of incubation, colonies consisting of more than 50

Table 1. Parameters of Dose-Survival Curves of HeLa Cells in Monolayer for X-Ray alone and Carboplatin (16.5μM) Administration 2 Hours before X-Ray Irradiation

	Do (Gy)	Dq (Gy)	n
X-ray alone	1.45	3.2	8.9
X-ray + carboplatin	1.40	2.3	4.2

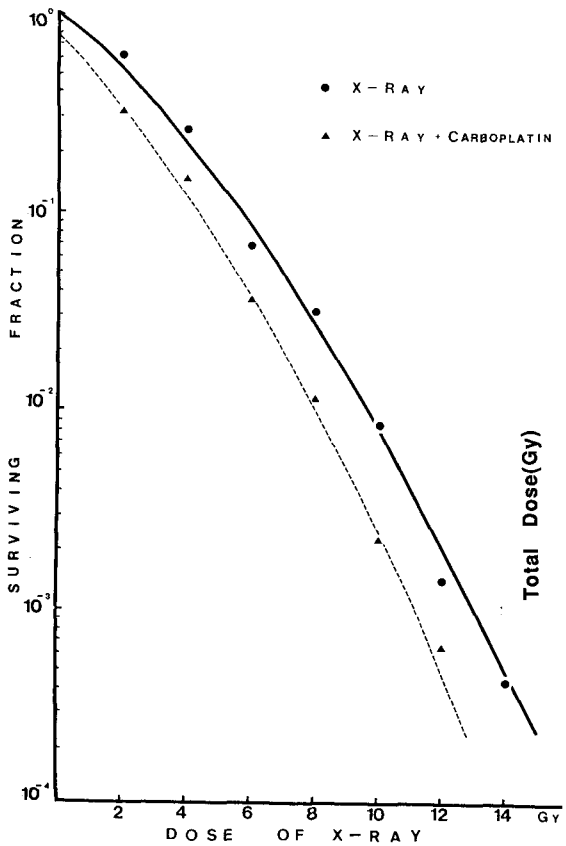

Fig. 1. Survival curves of HeLa cells in monolayer X-irradiated after treatment with a fixed concentration of Carboplatin (16.5 μM). Dq was decreaed by combined treatment, while Do was essentially unchanged.

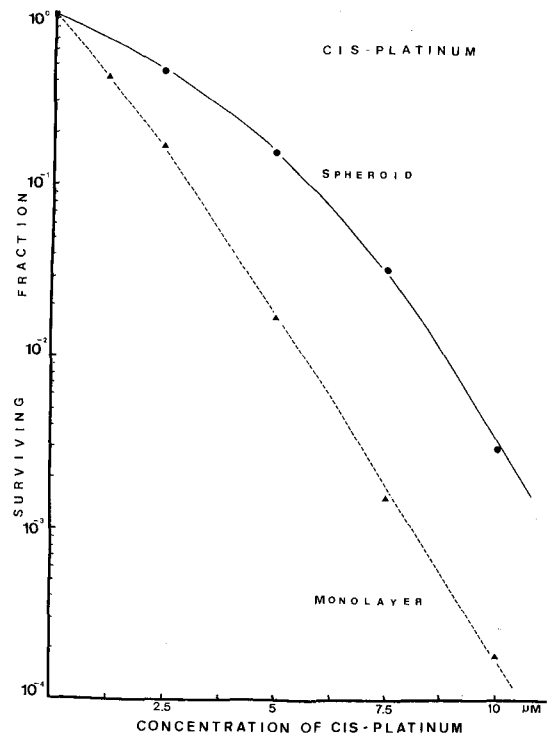

Fig. 2. Concentration-survival curves for HeLa cells in monolayer and spheroids treated by cis-platinum.

intact cells were counted to determine the surviving fraction. Under these conditions, the plating efficiency of HeLa cells was usually about 87% for monolayers and 73% for large spheroids. The resulting survival curve was fitted to linear-quadratic model by the least-squares method.

RESULTS

1. Effects of X-Irradiation

On the X-ray dose-survival curve, there was shoulder in the curve for X-rays alone extending as far as Dq=3.2 Gy, after which there was an exponential decrease in the survival with increasing X-ray doses and a Do value of 1.45 Gy (Fig. 1.). The Do value showed little change with the addition of carboplatin but the Dq value decreased by combined effect suggesting more than additive one of the individual therapies. This effect of X-ray treatment is thought to be due to an apparent decrease in the accumulation of capacity of sublethal damage to cells caused by the carboplatin.

2. Effects of Platinum Analogues

HeLa cells were treated for 2 hours at various final concentrations up to 10 μM and 200 μM of cis-platinum and carbiplatin, respectively.

a. Cis-platinum; In the monolayer cells, a small shoulder in the dose-survival curve was found, and with increasing concentration there was an exponential decrease in cell survival (Fig. 2). Here the Co value was 1.1 μM and the Cq value was 0.85 μM. While the Co for the spheroids was essentialy no significant change, but Cq value (3.4 μM) was larger than that of monolayers (Table 2). This suggests that the effect of cis-platin is greater in the monolayer with actively proliferating cells than hypoxic one.

b. Carboplatin; In the carboplatin concentration-survival curves, an exponential

Table 2. Survival Parameters of HeLa Cells for Graded Concentration of Cis–platinum and Carboplatin

	Monolayer			Spheroid		
	Co (μM)	Cq (μM)	n	Co (μM)	Cq (μM)	n
Cis–Platinum	1.1	0.85	2.1	1.2	3.4	18
Carboplatin	32.5	10.0	1.5	15.0	16.5	2.2

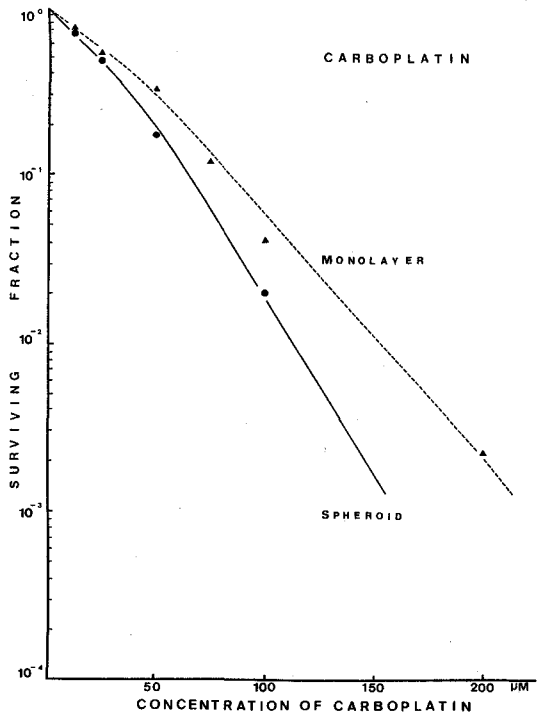

Fig. 3. Concentration-survival curves for HeLa cells in monolayer and spheroids treated by carboplatin.

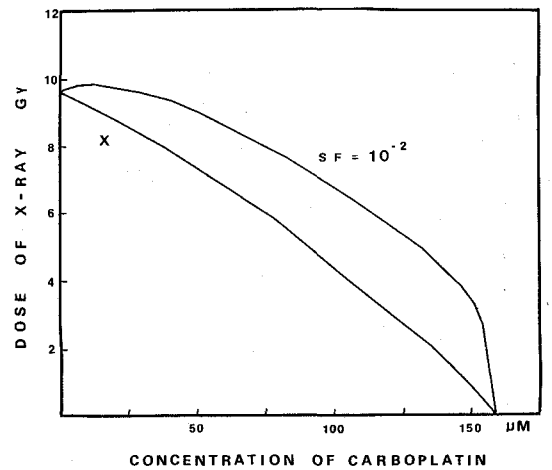

Fig. 4. Isobologram of HeLa cells in monolayer for X-ray irradiation following 2-h administration of Carboplatin was constructed from Fig. 1 and Fig. 3 for isoeffect level of surviving fraction et 0.01, where an additive envelope was surrounded by curves of interaction of two agents. Data of combined treatment represented by X is from Fig. 1.

decrease in the survival curve was seen in monolayers and spheroids (Fig. 3). The Co value of spheroids was 15.0 μM and the ratio with the Co from monolayer cells (32.5 μM) was 0.46, thus indicating that in comparision with monolayer cells the spheroids had a greater senstivity to carboplatin. Therefore, the effect of carboplatin is mainly on the hypoxic cells of spheroids acting as hypoxic cell sensitizer.

3. Isobologram Analysis

The enhanced effect was obtained for monolayers of HeLa cells using combined X-ray and carboplatin treatment, isobologram analysis[8] was performed when carboplatin was used 2 hour before X-irradiation. The isobologram constructed from Fig. 1 for isoeffect level of surviving fraction at 0.01 is shown in Fig. 4. The result shown in isobologram indicates that the effect of two agents was below the "envelope of additivity", where the X mark was drawn as an additive interaction of the two agents. From above finding it can be seen that combined carboplatin and irradiation produced effect was truely supra-additive.

DISCUSSION

1. Spheroids as an In Vitro Solid Tumor Model

A variety of in vitro and in vivo experimental models exist for research on properties of cancer cells during growth. In vitro models are attractive

compared with in vivo, due to their relative speed, ease and low expense. However, a cell population growing on a pertri dish is obviously very different from that in a tumor. The multicellular spheroid was developed as a useful model system of intermediate in complexity between conventional culture systems and tumors in vivo in which three-dimensional, spherical aggregates of cells simulates the conditions in intervascular microregions of tumors or micrometastatic foci. Spheroids have been grown readily from permanent cell lines[9] and human tumors[10]. In an investigation of establishment of human sarcoma experimental models success rates from patients of 50% for spheroids, 18% for xenografts, and 6% for monolayers were demonstrated[11]. This has been described in detail in spheroids of both rodent[2,4] and human origin[5,12,13]. Different methods of culturing spheroids, such as spinner-flask culture[2], gel culture[14], and liquid-overlay culture[5,12] have been developed.

Solid tumors in vivo often grow in cord structures, which consist of actively proliferating tumor cells close to the capillaries and of necrosis beyond a certain distance from the capillaries. These cord structures have been reported in human bronchogenic carcinoma[15] and in mouse mammary carcinoma[16]. Although the spheroids may have a histological resemblance, they may not simulate the functional aspect of the tumor cord structure. In fact, it has been reported by Durand[17] that a spheroid of loosely packed cells dose not necessarily contain radiobiologically hypoxic cells. However, spheroids of HeLa cells in this experiment consisted of a tightly packed cell population and contained hypoxic cells in large spheroids which was similar to other report[18]. Thus, the spheroids offer a suitable model system for testing the effectiveness of a particular drug on chronically hypoxic cells in poorly vascularized areas of solid tumors.

The role and mechanisms of variety of factors that may determine therapeutic responsiveness can be studied using spheroids. These include: a) intercellular contact effects, b) the fraction of truly self-renewing stem cells, c) presence of non-proliferating cells, d) redistribution of cell-cycle growth kinetics, e) development of heterogeneity due to genetic instability, f) environmental factors, and g) poor drug penetration[19].

Spheroids are used as models of the cells in poorly vascularized regions of fast-growing, malignant tumors. Increased knowledge of cells in such regions is of importance, because they may show increased radioresistance when derived of oxygen and they may also be difficult to reach with cytotoxic substances. Spheroids are at present being used to investigate both these phenomena[2,17,20,21].

2. Effects of Platinum Analogues

Although many antineoplastic drugs effectively kill cells close to capillaries, they may not be as effective in killing cells which are remote from the vascular supply.

Cis-diamminedichloroplatinum (II), cis-platinum, is a cytotoxic agent with a wide spectrum of activity against human cancers. Like most chemotherapeutic agents, however, its use is inevitably associated with a range of toxic side effects, notably nausea and vomiting, nephrotoxicity, myelosuppression, ototoxicity and neurotoxicity[22]. The antineoplastic activity of cis-platinum appears to be related to the chemical nature of the species which are bound to the central platinum atom and the relative position of these ligands to one another. Active platinum compounds must have two sites at which they can interact with intracellular targets, and these two sites must be in the cis configuration[23]. Cis-platinum can bind to DNA base in several possible ways. It can bind to single nucleophilic site (monofunctional binding). Bifunctionally, it can bind to two sites within a single DNA base or between two bases. Two bases bound a cross-linked by a single platinum containing molecule DNA strands (interstrand cross-link)[23]. Cis-platinum, in addition to being a potent cytotoxic agent, has also been shown to act as a radiosensitizer in bacteria[24] mammalian cells in vitro[25], and in animal tumors[26]. Its interaction with radiation also includes its ability to inhibit the repair of radiation-induced sublethal and potentially lethal damage.

The preclinical studies suggest that four drug-radiation mechanisms may be exploited separately or together such as a) hypoxic cell radiosensitization due to electron affinity of platinum drugs, b) DNA targeting due to their alkylating property, c) inhibition of recovery from radiation damage, d) thiol interaction[27]. In this experiments as compared with monolayers of HeLa cells the Co for the spheroids was no significant change, but Cq values (3.4 μM) was larger than that of monolayers (0.85 μM). This results suggested that the effectiveness of cis-platinum decreased for cells in spheroids, which can be attributed to a decrease in drug killing of cells in the deeper layers of the spheroids. Although the reduced effectiveness is probably due partly to lower concentration of the active form of

cis-platin, it may be specutated that other factors such as a hypoxic microenvironment must account for the observed resistance. This finding indicates that there are two possible reasons for this hypoxic resistance. First, hypoxic cells tend to located near necrotic regions in spheroids, and therefore these cells may be difficult to reach with cis-platinum. Secodly, clonogenic cells temporally rendered hypoxic may become non-cycling, or have their progression through the cell slowed down[28]. Because of the relatively severe side effects of cis-platinum in the clinic, and because of carboplatin appears to be considerably less toxic, one have tested the ability of these two analogues to radiosensitize HeLa cells.

Carboplatin (CBDCA) is a second-generation platinum coordination compound which was found to be less nephrotoxic and ementogenic than cisplitinum, while retaining broad spectrum of antitumor activity[29,30]. Dose-limiting toxicity is myelosuppression, primary thrombocytopenia, occuring between days 14 and 28 of the cycle[31].

Since carboplatin is less toxic than cis-platin and not limited by nephrotoxicity or gastrointestinal toxicities, higher levels of platinum may be administered to patients and mice with the attainment of higher peak plasma levels, and the potential for higher platinum levels in solid tumors relative to cisplatin[32]. It is hypothesized that the free solution compartment may be apply to mammalian cell radiosensitization. This hypothesis would predict that the use of less toxic analogs such as carboplatin at higher concentrations might provide more free solution platinum for interaction with radiation[32].

A rationale for coordinating the administration of carboplatin with radiation to achieve enhancement of cancer therapy is developed. Two major effects induce radiosensitization of hypoxic cells with platinum present during radiation and potentiation of cell kill with platinum complexes administrated after irradiation. Both these effects are expected to result in an improved therapeutic ratio. The later effect may include inhibition of recovery from radiation-induced potentially lethal damage an sublethal damage.

In the present experiments the Co value of spheroids was 15.0 μM and the ratio with the Co of monolayers (32.5 μM) was 0.46, thus the spheroids of HeLa cells had a greater sensitivity to carboplatin than monolayer cells. Therefore the effectiveness of carboplatin is mainly in the hypoxic cells of spheroids acting as hypoxic cell sensitizer. The enhanced effect was obtained for monolayers of HeLa cells using combined X-ray and carboplatin treatment, it was truely supra-additive effect as interaction of the two agents by isobologram analysis[8]. These result are encouraging in that they demonstrate a therapeutic potentiation when single dose of carboplatin is combined with a single dose of radiation. Ziegler[33] has reported a comparative radiosensitization study using cisplatin on two human and two rodent cell lines in which radiosensitization was seen only under oxic conditions in two cell lines, only under hypoxic conditions in another line, and under neither condition in the fourth line.

It must therefore be concluded that platinum compounds are not neccessarily dependent on hypoxia for their radiosensitization effects in mammalian cells. Further studies are required to investigate the use of more clinically relevant multiple dose fractionation of drug and radiation protocols, to examine carboplatin pharmacokinetics in the tumors and to evaluate the influence of timing between administration of the drug and radiation. Interaction between carboplatin and radiation might play an important role in meeting the challenging need for more effective local tumor control.

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=국문초록=

HeLa 세포의 Spheroid에 대한 방사선과 Platinum 유사체의 치사 효과

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HeLa 세포의 spheroid를 배양시켜 cis-platinum과 carboplatin으로 처리한 후 그 반응을 세포의 생존분획으로 분석하였다. 체외실험 model인 spheroid를 사용하여 platinum 유사체의 약효와 방사선 감수성을 평가하고 약제에 대한 단층 세포와 spheroid의 감수성 차이를 세포-생존곡선에서 규명하기 위하여 본 실험을 시행하였다. Cis-platinum 농도-곡선에서 spheroid의 Cq=3.4 μM이고 Co=1.2 μM이었다. 이것은 단층세포에 비하여 Co는 큰 변화가 없으나 Cq가 증가되어 cis-platinum이 저산소층 세포보다 활동적으로 분화하는 표면세포에 주로 작용하였으며, 반대로 carboplatin의 효과는 spheroid에 대한 (Co=15.0 μM) 감수성이 단층세포(Co=32.5 μM)에 비하여 크게 증가되어, spheroid의 심층 세포에 주로 작용하였다. 방사선과 carboplation의 병용효과를 세포 생존분획이 0.01 수준에서 isobologram으로 분석한 결과 상호작용으로 supra-additive 효과를 나타내었다. 따라서, carboplatin은 cis-platinum에 비하여 신장과 위장에 대한 독성작용이 적고, 방사선과 병용함으로써 향후 더욱 효과적인 종양 치료에 중요한 역할을 할 것으로 기대한다.