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UTILITY OF THE CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY TO ASSESS COMBINED EFFECTS OF IRRADIATION AND CISPLATIN

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Abstract To investigate the applicability of the cytokinesis-block micronucleus (MN) assay to assess combined effects of irradiation and cisplatin, the quantitative relationship between the induced MN frequency and clonogenic survival by colony forming assay was estimated using the human squamous cell carcinoma cell line PECA 4197. First, the MN frequencies induced by cisplatin treatment alone, at various concentrations, were examined and compared with the corresponding clonogenic survival. Secondly, cells were irradiated with and without cisplatin pretreatment and compared. Treatment with cisplatin alone exhibited a dose-dependent increase of induced MN frequency, at concentrations of 0-1.0 μg/ml and a treatment time of 180 min, with a significant relation to dose-dependently decreased clonogenic survival. Irradiation after cisplatin pretreatment (0.25 μg/ml, 180 min) resulted in a higher MN frequency than either irradiation or cisplatin alone and also than simple addition of the single effects after 2 and 4 Gy X-rays, suggesting a chemoradiosensitization. There was a significant correlation of the induced MN frequency with clonogenic survival for the combined effects of irradiation and cisplatin as well as for cisplatin or irradiation treatment alone. These results suggest that the induced MN frequency is a reliable quantitative measure to assess cell damage due to single or combined treatment with irradiation and cisplatin, providing encouragement for clinical use of the cytokinesis-block MN assay as a predictive tool to optimize and individualize multidisciplinary tumor therapy.

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Key words : cisplatin ; irradiation ; chemoradiosensitization ; micronucleus assay ; clonogenic survival.

放射線およびシスプラチン併用効果の評価における細胞分裂阻害微小核形成試験の有用性

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抄録　放射線およびシスプラチン併用効果の評価法としての細胞分裂阻害微小核形成試験の有用性を検討するために，微小核形成頻度とコロニー法による細胞生存率との量的相関について，ヒト扁平上皮癌培養細胞株 PECA 4197 を用いて検討した．まず，様々な濃度のシスプラチン単独処理による微小核形成頻度を求め，細胞生存率との相関を検討した．次いで，シスプラチン前処理と放射線照射を併用した場合の微小核形成頻度と細胞生存率を求め，同様の検討を加えた．この結果，0～1.0 μg/ml の濃度で180分間シスプラチン単独処理を加えた場合の微小核形成頻度は，濃度依存性に増加したが，同時に，濃度依存性に減少する細胞生存率と有意の量的相関を示した．放射線照射（2および 4 Gy）にシスプラチン前処理（0.25 μg/ml, 180分）を併用した場合，微小核形成頻度は，シスプラチン処理単独と放射線照射単独を単純に加算した値よりも高く，シスプラチン前処理による増感効果が示唆された．この場合にも，前処理の有無に関わらず微小核形成頻度と細胞生存率の量的相関が認められた．この量的相関は，シスプラチン単独処理，放射線照射単独，シスプラチン前処理後照射のいずれの場合にも良好に保たれており，これらの単独処理あるいは併用による細胞障害を定量的に評価する上で，微小核形成試験は高い信頼性を有すると考えられた．癌集学的治療の最適化・個別化における，治療効果予測法としての本法の臨床的有用性が示唆された．

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Introduction

Radiotherapy and chemotherapy both play important roles in the management of advanced solid tumors. However, not all tumors respond well and therefore, for each patient, an individualized and optimal regimen should be prescribed, if possible before starting treatment, by *in vitro* testing of tumor material[1~13]. Among several assays introduced for assessing radiosensitivity of human solid tumors[5~13], the micronucleus (MN) assay has been suggested as one of the most predictive tools applicable for the clinic[6,11~13]. Using human tumors in primary culture, Shibamoto et al.[12] have demonstrated the usefulness of the cytokinesis-block MN assay to predict tumor response to radiotherapy. In the same vein, Zölzer et al.[13], using serially biopsied samples and a conventional assay, reported that the MN frequency in response to radiotherapy allows good prediction of outcome for cervical carcinomas.

If the MN assay can also be applied for prediction of chemosensitivity and combined effects of irradiation and chemotherapeutic agents, this would facilitate individualizing and optimizing multidisciplinary treatment for each patient, especially for those with advanced solid tumors. Recently, Jeremic et al.[14] investigated MN induction in a murine tumor cell line employing 11 chemotherapeutic agents, and showed a general correlation in a quantitative way between the induced MN frequency and clonogenic survival. The correlation was independent of the mechanisms of action of the agents used, suggesting the applicability of this assay as a predictive tool to estimate chemosensitivity. In the present study, we further examined its applicability for assessing combined effects of irradiation and cisplatin, a potentiator of irradiation effects[15,16] and an important component of multidrug or multidisciplinary management of a variety of human solid tumors[17,18].

Materials and Methods

Cell line and agent preparation

The tumor cell line, PECA4197, derived from a human squamous cell carcinoma[19], was grown as monolayers in Eagle's minimum essential medium (Gibco) supplemented with 20% fetal calf serum (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cisplatin was kindly provided by Nihon Kayaku (Tokyo), kept in vials at room temperature, and diluted with culture medium to the desired concentrations immediately before use.

Treatment with cisplatin

Clonogenic survival assay

For 24 h before treatment 5×10^5 cells were incubated in 25 cm^2 flasks in the above-described Eagle's medium. The cells were treated with cisplatin in the desired final concentrations, as listed in the "results", for 180 min, washed twice with agent-free medium, and then immediately trypsinized, counted, and plated in appropriate numbers for colony formation into 19.6 cm^2 tissue culture dishes (Becton-Dickinson, USA). Eight to nine days later, colonies were fixed with 96% methanol, stained with 1% crystal violet and counted

to assess the clonogenic survival. Colonies containing ≧ 50 cells were counted. Two independent experiments, each performed in triplicate, were carried out.

MN assay

Cells (5×10^4) were plated into 19.6 cm^2 tissue culture dishes and treated with cisplatin in the manner described above. Immediately after the treatment and washing, Cytochalasin-B (CB) (Sigma, Germany) was added. For this purpose, CB was dissolved (1 mg/ml) in DMSO and frozen in aliquots. The stock solution was diluted with culture medium to a final concentration of 1.5 μg/ml[19, 20]. The CB incubation time was 48 h, chosen as appropriate from preliminary studies to obtain a high proportion of binucleated cells[20]. After rinsing with 0.9% NaCl, cells were fixed for 20 min with 96% methanol at room temperature, air-dried overnight, and stained with 4, 6-diamidino-2-phenylindole (DAPI ; 100 ng/ml in Tris buffer, pH 7.0 ; Serva, Germany). Scoring was performed at a magnification of 400 x using a microscope equipped for fluorescence (excitation maximum 344 nm, emission maximum 449 nm) and phase-contrast. At least 200 binucleated cells per dish (resulting in 1,200 binucleated cells for each observation point) were assessed and the MN frequency per single binucleated cell (MN/BNC) was measured. Only micronuclei fulfilling certain criteria[20] were scored. Two independent experiments, each performed in triplicate, were carried out.

Combined treatment with irradiation and cisplatin

Clonogenic survival assay

For 24 h prior to treatment 5×10^5 cells were incubated in 25 cm^2 flasks in the culture medium. The cells were then pretreated with cisplatin for 180 min at a final concentration of 0.25 μg/ml, which was decided from the clonogenic survival curve after treatment with cisplatin, and washed twice with agent-free medium. They were then irradiated using an X-ray machine (Stabilipan, Siemens, Germany ; 240 kV, 15 mA, 0.5 mm Cu filter, and a dose rate of 1 Gy/min) with doses of 1, 2, 4, 6 or 8 Gy, applied at room temperature. Control cells received sham irradiation. To obtain a survival curve for treatment with irradiation only, cells without cisplatin pretreatment were irradiated with doses of 1, 2, 3, 4, 6 or 8 Gy. Eight to nine days later, colonies were fixed, stained and counted. Two and four independent experiments for series with and without cisplatin pretreatment, respectively, each performed in triplicate, were carried out.

MN assay

Cells (5×10^4) were plated into 19.6 cm^2 tissue culture dishes and pretreated with cisplatin as described above. Immediately after washing, the dishes were irradiated with doses of 2 or 4 Gy. Dishes without cisplatin pretreatment were also irradiated. Control cells received sham irradiation. CB was then added and the cells were incubated for 48 h. Further methods were performed as described before. Two independent experiments, each performed in triplicate, were carried out.

Statistics

Kendall rank correlation was used for examining the statistical significance of correlation between the parameters, clonogenic survival and MN frequency.

Results

Treatment with cisplatin

Figures 1 a and 1 b show dose responses for clonogenic survival and the induced MN frequency (normalized after subtraction of the control value), respectively, after cisplatin treatment. As the concentration of cisplatin increased, the survival rate decreased while the induced MN frequency was elevated. In Fig. 1 c, induced MN frequencies are plotted as a function of the corresponding clonogenic survival after cisplatin treatment at various concentrations (0, 0.25, 0.5 and 1.0 μg/ml). The data were fitted with the Sigmaplot program (Jandel) and an inverse correlation was obtained (correlation coefficient, $r=-1.00$, $p=0.0415$: Kendall rank correlation).

Combined treatment with irradiation and cisplatin

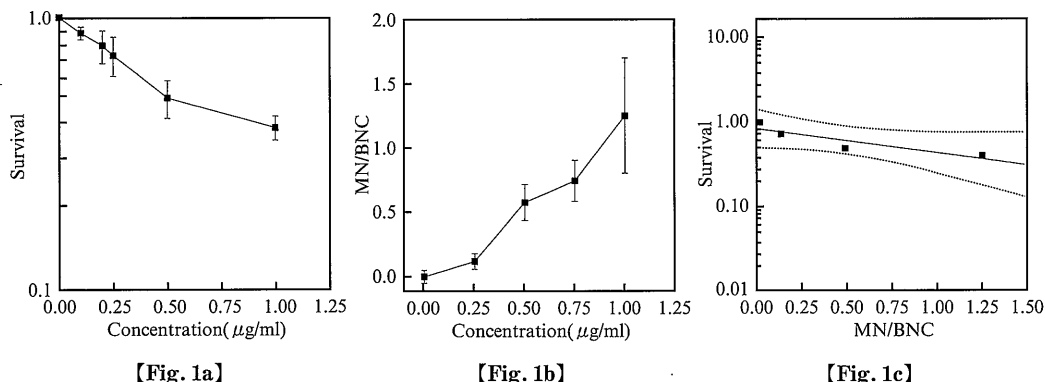
Clonogenic survival curves after irradiation, with and without pretreatment by cisplatin (0.25 μg/ml, 180 min), are demonstrated in Fig. 2 a. These data show reduction of D_0 and the "shoulder" part of the survival curve when pretreated with cisplatin (Table 1). The induced MN frequency increased with increasing radiation dose when irradiation alone was delivered to the cells, and irradiation after pretreatment with cisplatin resulted in a higher MN frequency than irradiation alone, with radiation doses of 2 and 4 Gy (Fig. 2 b). These values were also higher than those expected from the addition of the respective values for irradiation alone and cisplatin treatment alone [2 Gy : $1.01\pm0.12>(0.44\pm0.10)+(0.12\pm0.06)$, 4 Gy : $1.61\pm0.19>(1.01\pm0.19)+(0.12\pm0.06)$; the values were the means $\pm$ standard deviations for irradiation after pretreatment with cisplatin, irradiation alone, and cisplatin alone, respectively]. Fig. 2 c shows the inverse correlation between the induced MN frequency and clonogenic survival, regardless of the pretreatment (correlation coefficient, $r=-0.966$, $p=0.0085$: Kendall rank correlation).

General correlation between the induced MN frequency and clonogenic survival

When combining all data from Fig. 1 c and 2 c, a general correlation between the induced MN frequency and clonogenic survival was demonstrated independent of treatment, either cisplatin treatment alone, irradiation alone or irradiation after cisplatin pretreatment (correlation coefficient, $r=-0.844$, $p=0.0007$: Kendall rank correlation).

Discussion

It is well known that many chemotherapeutic agents induce micronuclei regardless of their mechanisms of cytotoxicity[11, 14, 21~23]. Cisplatin, which damages DNA mainly through intra- and interstrand crosslinks[18, 21, 23], is known to produce chromosomal fragments and subsequent MN formation[11,14,23]. Also in the present study, MN formation was observed after treatment with cisplatin with a dose-dependent increase in frequency, in the concentration range of 0-1.0 μg/ml and 180



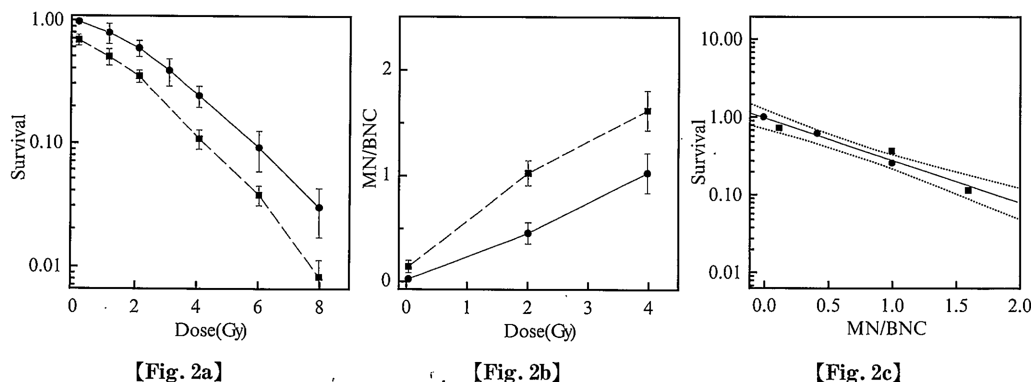
【Fig. 1a】　【Fig. 1b】　【Fig. 1c】

Fig. 1 Clonogenic survival (a) and the induced MN frequency (b) for cells treated with cisplatin. Symbols and bars represent the mean values and standard deviations for two independent experiments performed in triplicate. Figure (c) illustrates the correlation between the two parameters. The solid and dashed lines represent fitted regression and 95% confidence intervals, respectively. The concentrations of cisplatin (μg/ml) were 0, 0.25, 0.5 and 1.0. MN/BNC : induced MN frequency per single binucleated cell.

Table 1 Influences of cisplatin pretreatment on the parameters for clonogenic survival

Pretreatment	D_0	Dq	n[a]
None (irradiation alone)	1.9 Gy	1.5 Gy	2.3
Cisplatin[b] (0.25 μg/ml, for 180 min)	1.6 Gy	0.8 Gy	1.6

[a] Extrapolation number.
[b] The values for Dq and n were corrected for cytotoxicity predicted for the agent alone.



【Fig. 2a】　【Fig. 2b】　【Fig. 2c】

Fig. 2 (a) Clonogenic survival curves for cells treated with irradiation alone (●) and irradiation after pretreatment with cisplatin (0.25 μg/ml, 180 min ; ■). Symbols and bars represent the mean values and standard deviations for four and two independent experiments, respectively, each performed in triplicate. (b) Induced MN frequencies for cells treated with irradiation alone (●) and irradiation after cisplatin pretreatment (0.25 μg/ml, 180 min ; ■). Symbols and bars represent the mean values and standard deviations, respectively, for two independent experiments performed in triplicate. (c) Correlation between clonogenic survival and induced MN frequency : irradiation alone (●) and irradiation after cisplatin pretreatment (■). The solid and dashed lines represent fitted regression and 95% confidence intervals, respectively. MN/BNC : induced MN frequency per single binucleated cell.

min treatment time. In addition, we investigated whether the MN frequency correlated with clonogenic survival after the combination of cisplatin and irradiation. Because cisplatin is presently one of the most important components of multidrug and multidisciplinary treatment, the concentration (0.25 μg/ml) was selected in the range of plasma levels when administered to patients[21], assuming clinically-possible conditions of combined treatment. At each radiation dose, 2 and 4 Gy, the induced MN frequency was elevated by pretreatment with cisplatin, and an inverse correlation between the induced MN frequency and clonogenic survival was obtained.

Moreover, this correlation in a quantitative way between the induced MN frequency and clonogenic survival was observed independent of treatment, either cisplatin treatment alone, irradiation alone or irradiation after cisplatin pretreatment. As previously stated[20], it is necessary for the radiation-induced MN frequency to quantitatively correlate with corresponding clonogenic survival if the MN assay can be used as a predictive tool to estimate the intrinsic radiosensitivity. This is the case for its use to estimate chemosensitivity or combined treatment effects with irradiation and chemotherapeutic agent. As to chemosensitivity, Jeremic et al.[14] showed a general correlation between the induced MN frequency and clonogenic survival, independent of the agents used. In the present study, we further demonstrated a general correlation between the two about irradiation and/or cisplatin treatment. This indicates a broad applicability of MN assay to quantitatively estimate cytolethal cell damages induced by various types of agents, including those for combined treatment with irradiation and chemotherapeutic agents.

Regarding chemical agent-radiation interactions, it has been reported that cisplatin removes the "shoulder" and/or reduces the D_0 of the cell survival curve[15,18]. Its repair-inhibitory effects may result from agent-induced intrastrand crosslinking and radiation-induced single strand breaks on complementary strands of DNA, so as to fix damage that otherwise would be repaired[16]. For the aerobic cell radiosensitization with cisplatin, the mechanism that the Pt (I) intermediates, the metabolites of cisplatin [$(NH_3)_2$ Pt (II) Cl_2], react with O_2 to form $(NH_3)_2$ Pt (I) ClO_2, which is likely to react with peroxy radicals to give peroxy· complexes, may account for the effects[18,24]. Depletion of endogenous radioprotectors like non-protein bound thiols caused by cisplatin may also increase the radiosensitivity[15,18]. The reduction of the D_0 and "shoulder" of the cell survival curve after cisplatin pretreatment in our experiments is therefore in the line with the literature. The MN frequency induced by the combined treatment of irradiation and cisplatin was higher than that expected from simple addition of single effects. This suggests that the combined treatments were "supra-additive", consistent with the above-described changes in the cell survival curve after cisplatin pretreatment, and that this could be detected by the MN assay. There have been several reports on modification of radiation-induced MN frequency with different agents[11,25~29]. Masunaga et al[25].

investigated *in vivo* the modification of radiation-induced MN frequency with several chemicals, including cisplatin, in quiescent cell populations within murine solid tumors, and demonstrated the utility of the MN assay for detecting responses to the combined treatments. Catena et al.[26] showed, using human lymphocytes, that 3-aminobenzamide (3-AB), an inhibitor of poly (ADP-ribose) polymerase, increased the radiation-induced MN yields, although 3-AB alone was without influence. Furthermore, George et al.[27] demonstrated marked sensitization, using a murine tumor and the MN assay, with X-rays, misonidazole and hyperthermia in combination, as compared with X-rays alone. However, the earlier studies did not assess the quantitative relationship between the induced MN frequency and the corresponding clonogenic survival[20] resulting from the combined treatments. The significant correlation between the two, shown in the present study, has an impact upon the quantitative reliability of the MN assay as a measure of beneficial effects.

Although further investigations using chemotherapeutic agents other than cisplatin and primary or short term cultured cells of human tumors are still necessary, the present results are encouraging, suggesting utility of the MN assay in optimizing individual multidisciplinary cancer treatments with irradiation and chemotherapeutic agents.

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