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Augmentation in Chemosensitivity of Intratumor Quiescent Cells by Combined Treatment with Nicotinamide and Mild Hyperthermia

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C3H/He and Balb/c mice bearing SCC VII and EMT6/KU tumors, respectively, received continuous administration of 5-bromo-2′-deoxyuridine (BrdU) for 5 days using implanted mini-osmotic pumps to label all proliferating (P) cells. Nicotinamide was administered intraperitoneally before cisplatin injection and/or tumors were locally heated at 40°C for 60 min immediately after cisplatin injection. The tumors were then excised, minced and trypsinized. The tumor cell suspensions were incubated with cytochalasin-B (a cytokinesis-blocker), and the micronucleus (MN) frequency in cells without BrdU labeling (quiescent (Q) cells) was determined using immunofluorescence staining for BrdU. The MN frequency in total (P+Q) tumor cells was determined from tumors that had not been pretreated with BrdU labeling. The sensitivity to cisplatin was evaluated in terms of the frequency of induced micronuclei in binuclear tumor cells (MN frequency). In both tumor systems, the MN frequency in Q cells was lower than that in the total cell population. Nicotinamide treatment elevated the MN frequency in total SCC VII cells. Mild heating raised the MN frequency more markedly in Q cells than in total cells. The combination of nicotinamide and mild heat treatment increased the MN frequency more markedly than either treatment alone. In total SCC VII cells, nicotinamide increased ^{195m}Pt-cisplatin uptake. Mild heating elevated ^{195m}Pt-cisplatin uptake in total EMT6/KU cells. Cisplatin-sensitivity of Q cells was lower than that of total cells in both tumor systems. Nicotinamide sensitized tumor cells including a large acutely hypoxic fraction, such as those of SCC VII tumors, through inhibition of the fluctuations in tumor blood flow. Tumor cells including a large chronically hypoxic fraction such as Q cells were thought to be sensitized by mild heating through an increase in tumor blood flow.

Key words: Quiescent cell — *cis*-Diamminedichloroplatinum(II) — Nicotinamide — Hyperthermia — Hypoxic cells

It is known that many tumor cells in solid tumors are non-proliferating (quiescent, Q).[1] Over the last 25 years, the nature of Q cells has been extensively examined, but many of their characteristics are still poorly understood.[1] To improve the treatment of cancer, the responses of Q cells in solid tumors to various anticancer therapeutic modalities need to be determined, since many tumor cells are quiescent *in situ*, but are still clonogenic.[2] There have been reports that Q tumor cells are more resistant to irradiation than exponentially growing tumor cells because of their higher hypoxic fraction and greater potentially lethal damage repair capacity.[3] However, there have been few reports regarding Q cell sensitivity to chemotherapeutic agents.[1]

Tumor hypoxia is clearly an important problem, and improved patient responses to radiotherapy can be achieved with treatments which overcome tumor radiation resistance that results from the presence of hypoxic cells.[4] There are already numerous treatment protocols for dealing with diffusion-limited hypoxia (chronic hypoxia).[5] Subsequent studies showed that nicotinamide can prevent transient fluctuations in tumor blood flow that lead to the development of perfusion-limited hypoxia (acute hypoxia).[6]

On the other hand, the effectiveness of hyperthermia as an adjuvant modality to radiotherapy has been demonstrated.[7] Laboratory experiments using animal tumors showed that heating for 30 to 60 min at relatively high temperatures, i.e., >43 to 44°C, damages intratumor blood vessels and kills tumor cells.[8] In addition, hyperthermia causes direct cellular radiosensitization.[9] However, currently available hyperthermia devices have been ineffective in raising the temperature of human tumors sufficiently to cause vascular damage, kill tumor cells, and directly radiosensitize the tumor cells. Furthermore, correlations between clinical responses to hyperthermia and lowest temperatures in tumors have been reported, and the prognostically important temperatures have been <41°C.[10,11] Therefore, Oleson suggested that hyperthermia might have improved tumor oxygenation and thus indirectly radiosensitized tumors through an increase in

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tumor blood flow in previous clinical studies in which heat treatment was shown to improve the effectiveness of radiotherapy.[12]

In this study, to enhance the cisplatin-sensitivity of Q cell populations in murine solid tumors (SCC VII squamous cell carcinoma and EMT6/KU sarcoma), we investigated the effectiveness of combined treatment with nicotinamide and/or low-temperature heat treatment on chemotherapy, using our method for selectively detecting the responses of Q cells in solid tumors.[13] We also examined the influence of nicotinamide administration and mild heat treatment on the uptake of cisplatin by total (proliferating, P+Q) tumor cells, using ^{195m}Pt-radiolabeled cisplatin synthesized at our institute.

MATERIALS AND METHODS

Tumors, mice, and labeling with 5-bromo-2′-deoxyuridine (BrdU) SCC VII squamous cell carcinoma derived from C3H mice, and EMT6/KU sarcoma derived from Balb/c mice, were maintained *in vitro* in Eagle's minimum essential medium containing 12.5% fetal bovine serum. Cells were collected from monolayer cultures, and approximately 1.0×10^5 cells were inoculated subcutaneously into the left hind legs of 8- to 11-week-old syngeneic female C3H/He or Balb/c mice. Fourteen days after inoculation, the tumors reached approximately 1 cm in diameter. Nine days after inoculation, mini-osmotic pumps (Alzet model 2001, Palo Alto, CA) were implanted subcutaneously for 5 days of continuous labeling. Administration of BrdU did not change the tumor growth rate. The tumors were 1 cm in diameter at treatment. The labeling index after 5 days of continuous labeling with BrdU was 55.3±4.5% (mean±SD) for the SCC VII tumors and 74.6±5.4% for EMT6/KU tumors, and reached a plateau level at this stage. Therefore, in this study, we regarded tumor cells not incorporating BrdU after continuous labeling as Q cells.

Treatment After labeling with BrdU, the tumor-bearing C3H/He and Balb/c mice were divided into four groups. In group 1, cisplatin was administered intraperitoneally at doses of 1/20, 1/10, 1/4 and 1/2 LD_{50} (mean lethal dose, 17.7 mg/kg for both mouse strains). In group 2, the mice were injected with cisplatin 90 min after intraperitoneal injection of nicotinamide (1000 mg/kg) to obtain a sufficient effect of this drug.[14] In group 3, the tumors grown in the left hind legs of mice were heated at 40°C for 60 min in a water bath immediately after cisplatin injection. Since we used the same kind of tumor system and the same tumor size upon heating as reported by Nishimura *et al.*,[15] we employed the same heating method. In general, temperature at the tumor center equilibrated within 3 to 4 min after immersion in the water bath and remained 0.2–0.3°C below the water bath temperature. The temperature difference between the tumor center and the periphery was within 0.1°C. The water bath temperature was maintained 0.3°C above the desired tumor temperature and all temperatures mentioned in this paper refer to the tumor temperature. Group 4 mice received both nicotinamide and mild heat treatment.

Each treatment group included both C3H/He and Balb/c mice pretreated and not pretreated with BrdU. The tumors were excised 1 h after cisplatin injection.

Immunofluorescence staining of BrdU-labeled cells and observation of micronucleus formation These procedures have been described in detail elsewhere.[13] After the treatments described above, excised tumors from mice given BrdU were minced and trypsinized at 37°C for 15 min, using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid. Tumor cell suspensions were inoculated in 60-mm tissue culture dishes containing 5 ml of complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division. The proportion of binuclear cells reached a maximum 48 h after initiation of culture. The cultures were trypsinized and single-cell suspensions were fixed with 70% ethanol. After centrifugation, the cell pellets were resuspended in 0.4 ml of cold Carnoy's fixative. The suspensions (30 μl) were then placed on glass microscope slides using a dropper and dried at room temperature. The slides were treated with 2 *M* hydrochloric acid for 30 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma, St. Louis, MO). To observe double staining of tumor cells with FITC and propidium iodide (PI), cells on the slides were treated with 30 μl of PI (1–5 μg/ml in phosphate-buffered saline) while under the fluorescence microscope. When the intensity of the red fluorescence produced by PI became similar to that of the green fluorescence in nuclei prestained with FITC, the treatment was stopped by rinsing the slides with water. The micronucleus (MN) frequency in unlabeled Q cells could be examined by counting the micronuclei in those binuclear cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed.[16] The ratio obtained in tumors not pretreated with BrdU indicated the MN frequency of all phases of the total tumor (P+Q) cell populations.

The MN frequency of BrdU-labeled cells, which could be regarded as P cells upon treatment, was modified because the radiosensitization effect of the incorporated BrdU could potentially influence the frequency of micro-

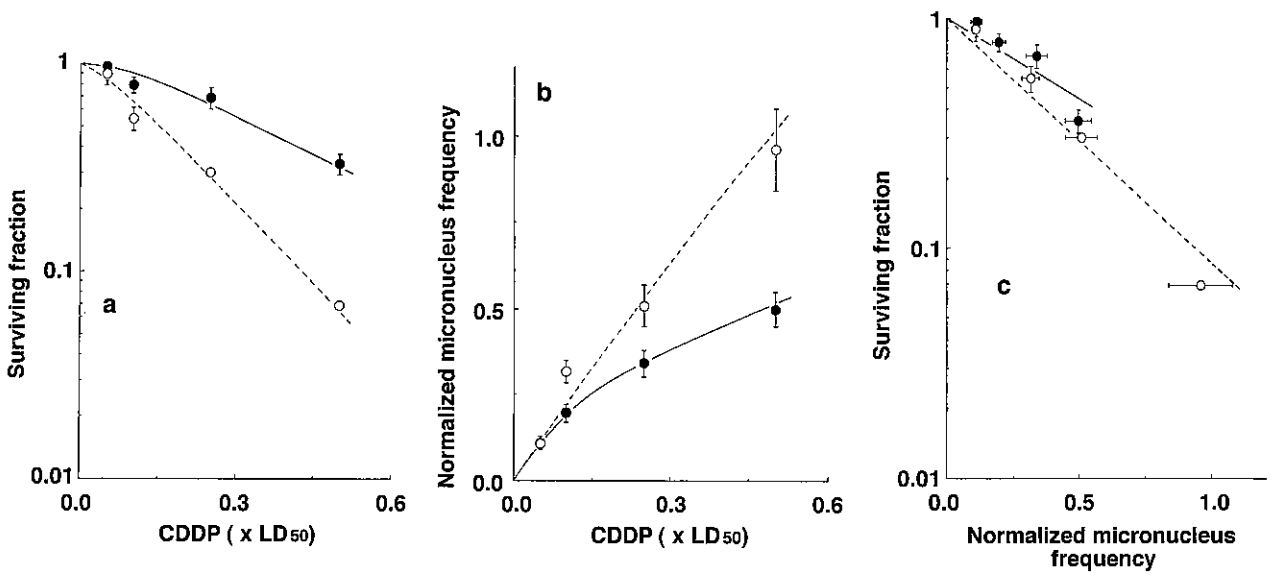

Fig. 1. MN assay combined with cytochalasin-B treatment and cell survival assay (*in vivo-in vitro* assay) were started 1 hour after intraperitoneal injection of cisplatin (CDDP) into tumor-bearing mice. Cell-survival curves and dose-response curves for normalized MN frequency (MN frequency-C, where C is the MN frequency in cells from tumors in animals not given cisplatin) in total cells in SCC VII and EMT6/KU tumors are shown in a and b. Correlations between normalized MN frequency and surviving fraction in each tumor system are shown in c. The calculated regression lines were $\ln Y = -2.10X$ and $\ln Y = -2.63X$ (X=normalized MN frequency, Y=surviving fraction), and significant positive correlations of $r = -0.92$ and $r = -0.98$ were observed (both $P < 0.001$). Thus, there is a close relationship between MN frequency and surviving fraction, and chemosensitivity to cisplatin can be expressed in terms of the MN frequency instead of surviving fraction. Bars represent SD. ● SCC VII, ○ EMT6/KU.

nuclei and binuclear cell appearance in BrdU-labeled cells.[17] Therefore, the correct MN frequency of P cells without the BrdU effect could not be obtained. In addition, during the continuous labeling with BrdU over 5 days, the shift of cells from the P to Q population can result in labeled Q cells. These cells were excluded when we scored micronuclei in binuclear cells showing only red fluorescence by PI, because these cells were stained with FITC.

Although the effects of cytochalasin-B on chromosome damage in cells have not been completely elucidated, a close relationship has been reported between cell survival and MN frequency obtained with cytochalasin-B treatment after irradiation with X-rays.[16, 18] As in the case of X-irradiation, DNA is considered to be the primary target for cell killing by cisplatin.[19] Furthermore, in the total tumor cell populations of both cell lines employed here, after intraperitoneal injection of cisplatin into tumor-bearing mice, there was a close relationship between MN frequency obtained with cytochalasin-B treatment and surviving fraction determined by the *in vivo-in vitro* assay method (Fig. 1). Therefore, the chemosensitivity of tumor cells can be expressed in terms of the frequency of micronuclei instead of the loss of clonogenicity.

Measurement of ^{195m}Pt uptake in tumor cells To examine the influence of intraperitoneal injection of nicotinamide and mild heat treatment on the uptake of cisplatin in the total number of tumor cells within the solid tumors, both C3H/He and Balb/c mice not given BrdU received intraperitoneal administration of ^{195m}Pt-radiolabeled cisplatin at doses of 1/20, 1/10, 1/4, and 1/2 LD_{50} of non-labeled cisplatin, 90 min after intraperitoneal injection (corresponding to groups 2 and 4) of nicotinamide (1000 mg/kg) or no injection (corresponding to groups 1 and 3). In addition, the tumors grown in the left hind legs of mice that corresponded to those in groups 3 and 4 were heated at 40°C for 60 min in a water bath immediately after the injection of ^{195m}Pt-radiolabeled cisplatin. ^{195m}Pt was produced by irradiating 95%-enriched ^{194}Pt (purchased from Oak Ridge National Laboratory, USA) in the hydraulic conveyer of the Kyoto University Reactor at a thermal neutron flux of approximately 8.15×10^{13} $n \cdot cm^{-2} \cdot s^{-1}$ for 75 h. ^{195m}Pt-labeled cisplatin was synthesized using ^{195m}Pt by the conventional method, and was then separated and purified by HPLC (column: Shodex 2004).[20]

Tumors from mice given ^{195m}Pt-labeled cisplatin were excised, minced, and trypsinized 1 h after intraperitoneal

administration of cisplatin. Tumor cell suspensions were centrifuged, and the radioactivity of precipitated cell fractions taking up ^{195m}Pt-cisplatin was measured with an NaI(Tl)-scintillation counter (Aloka, RLC-551).

Four mice were used for each set of conditions and each experiment was repeated 3 times. To examine the differences between pairs of values, Student's t test was used when the variances of the two groups could be assumed to be equal; otherwise, Welch's t test was used.

RESULTS

Table I shows the MN frequencies for total tumor cells and for Q cells in both tumor systems. There were no significant differences in MN frequency between no treatment and mild heating for each cell population in both tumor systems.

Fig. 2 shows the dose-response curves for the normalized micronucleus frequency (MN frequency-C, where C is the MN frequency in cells from tumors of animals not given cisplatin) in total and Q tumor cells in SCC VII (a) and EMT6/KU (b) tumors. The normalized micronucleus frequency for Q cells was lower than that for total tumor cells in both tumor systems. The normalized MN frequencies for Q cells of SCC VII tumors and for total and Q cells of EMT6/KU tumors increased in the following order: no treatment < nicotinamide < mild heating < nicotinamide + mild heating. However, the normalized MN frequencies for total cells of SCC VII tumors increased in the following order: no treatment < mild heating < nicotinamide < nicotinamide + mild heating. Treatment with nicotinamide raised the normalized MN frequency more markedly than mild heat treatment for total cells of SCC VII tumors, and the difference compared with treatment without nicotinamide or mild heating was significant. The combined treatment with nicotinamide and mild heating elevated the normalized MN frequency more markedly than treatment with either nicotinamide or mild heating alone. The increase in normalized MN

Table I. Micronucleus Frequencies without Cisplatin Treatment

	SCC VII	EMT6/KU
Total tumor cells		
No treatment	0.056±0.005[a)]	0.074±0.008
Nicotinamide	0.059±0.005	0.125±0.004
Mild heating	0.057±0.006	0.077±0.011
Nicotinamide+Mild heating	0.060±0.008	0.133±0.006
Quiescent cells		
No treatment	0.113±0.006	0.090±0.004
Nicotinamide	0.108±0.005	0.128±0.006
Mild heating	0.111±0.006	0.093±0.003
Nicotinamide+Mild heating	0.111±0.007	0.145±0.005

a) Average±1 SD.

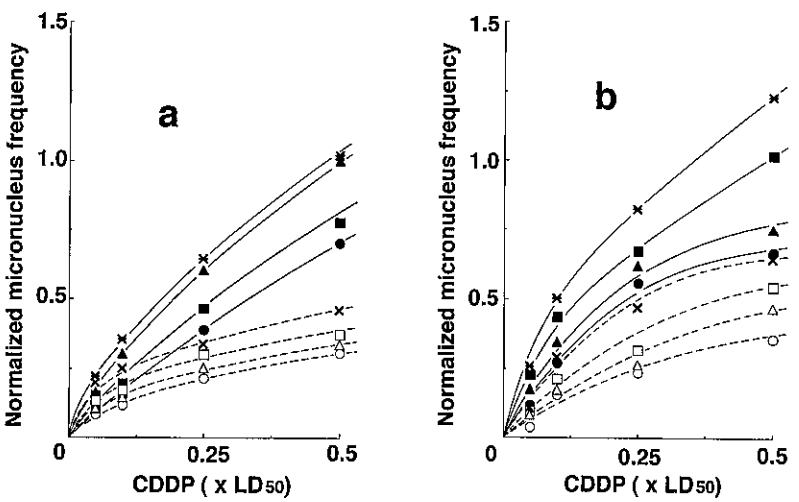

Fig. 2. Dose-response curves for normalized MN frequency (MN frequency-C, where C is the MN frequency in cells from tumors in animals not given cisplatin (CDDP)) in total cells and Q cells in SCC VII (a) and EMT6/KU (b) tumors. To avoid confusion, only mean values are shown. In both tumor systems, there were significant differences between total tumor cells and Q cells ($P<0.05$). In the total cells of SCC VII tumors, nicotinamide (NA) treatment, and in each cell population of both tumor systems, combined treatment with nicotinamide and mild heating produced significant increases in normalized MN frequencies ($P<0.05$). Total cells: ●, ■ NA (−); ▲, ✖ NA (+); ●, ▲ heat (−); ■, ✖ heat (+). Q cells: ○, □ NA (−); △, × NA(+); ○, △ heat (−); □, × heat (+).

Table II. Dose-modifying Factors for Quiescent Cells Relative to Total Cells in Solid Tumors

Normalized micronucleus frequency	Dose-modifying factor[a)]	
	SCC VII	EMT6/KU
0.2	1.83	2.69
0.25	2.24	2.78
0.3	2.51	2.93
0.35	—	3.26

a) The dose-modifying factor is the cisplatin dose needed to obtain the normalized micronucleus frequencies of 0.2, 0.25, 0.3 and 0.35 in quiescent cells/the cisplatin dose needed to obtain the normalized micronucleus frequencies of 0.2, 0.25, 0.3 and 0.35 in total cells.

frequency produced by the combined treatment compared with those observed without nicotinamide or mild heat treatment was significant in each cell population of both tumor systems.

The dose-modifying factors (DMF) in both tumors treated without nicotinamide or mild heating, which compare the cisplatin doses needed to obtain various normalized MN frequencies in Q cells with those in total tumor cells, were calculated using the mean values shown in Fig. 2 (Table II). The DMF of Q cells were significantly greater than 1.00.

To evaluate the effects of nicotinamide and mild heating on MN frequency in total and Q tumor cells, we calculated the enhancement ratio (ER) at various normalized MN frequencies, using the mean values for the data given in Fig. 2 (Table III). ER was defined as the ratio of the cisplatin doses needed to obtain equivalent normalized MN frequencies without and with treatment. Nicotinamide treatment produced higher ERs for total cells than those for Q cells in SCC VII tumors. The ERs for Q cells were higher than those for total tumor cells with mild heat treatment in both tumors. The ERs for combined treatment with nicotinamide and mild heating were higher than those for either treatment alone in each cell population of both tumor systems.

Fig. 3 shows the distribution of ^{195m}Pt as a function of the concentration of ^{195m}Pt-cisplatin in total tumor cells in both tumor systems for each treatment. To assess the effects of the combination of nicotinamide or mild heating on the uptake of ^{195m}Pt-cisplatin into each tumor cell, the increased uptake ratio was calculated at each concentration of ^{195m}Pt-cisplatin used, employing the mean values for the data shown in Fig. 3 (Table IV). The greater the dose of ^{195m}Pt-cisplatin administered, the more ^{195m}Pt-cisplatin was taken into each tumor cell even when neither nicotinamide nor mild heat treatment was combined. Therefore, no apparent dose-responsiveness between administered ^{195m}Pt-cisplatin dose and the uptake ratio was shown in Table IV. However, at equivalent administered doses of ^{195m}Pt-cisplatin, nicotinamide treatment increased the uptake of ^{195m}Pt-cisplatin into the tumor cells more markedly than mild heat treatment in SCC VII tumors. In contrast, mild heating elevated the uptake more markedly than nicotinamide in EMT6/KU tumors. For both tumors, the combined treatment with nicotinamide and mild heating raised the uptake ratios

Table III. Enhancement Ratios

Cell line system Treatment	Normalized micronucleus frequency	Enhancement ratio[a)]	
		Total tumor cells	Quiescent cells
SCC VII			
Nicotinamide			
	0.2	2.00	1.30
	0.25	1.96	1.26
	0.3	1.86	1.19
	0.5	1.72	—
	0.7	1.60	—
Mild heating			
	0.2	1.33	1.92
	0.25	1.32	1.60
	0.3	1.31	1.66
	0.5	1.26	—
	0.7	1.22	—
Nicotinamide+Mild heating			
	0.2	2.90	3.48
	0.25	2.75	3.29
	0.3	2.50	2.85
	0.5	2.00	—
	0.7	1.76	—
EMT6/KU			
Nicotinamide			
	0.2	1.21	1.25
	0.25	1.30	1.28
	0.3	1.20	1.32
	0.35	1.19	1.45
	0.5	1.19	—
	0.7	1.49	—
Mild heating			
	0.2	1.78	1.69
	0.25	1.68	1.73
	0.3	1.71	1.81
	0.35	1.68	2.00
	0.5	1.62	—
	0.7	2.11	—
Nicotinamide+Mild heating			
	0.2	2.42	2.48
	0.25	2.29	2.59
	0.3	2.36	2.65
	0.35	2.32	2.96
	0.5	2.28	—
	0.7	2.99	—

a) The ratio of the cisplatin dose needed to obtain each normalized micronucleus frequency in tumor cells without, and with, treatment.

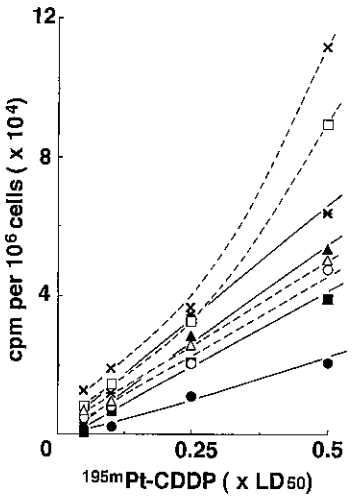

Fig. 3. Distribution of ^{195m}Pt as a function of the concentration of ^{195m}Pt-cisplatin (CDDP) in total tumor cells within SCC VII and EMT6/KU tumors. To avoid confusion, only mean values are shown. In SCC VII tumors, nicotinamide (NA) treatment and combined treatment with nicotinamide and mild heating produced significant increases in ^{195m}Pt-cisplatin uptake ($P<0.05$). In EMT6/KU tumors, mild heat treatment and combined treatment caused significant increases in ^{195m}Pt-cisplatin uptake ($P<0.05$). SCC VII: ●, ■ NA (−); ▲, ✖ NA (+); ●, ▲ heat (−); ■, ✖ heat (+). EMT6/KU: ○, □ NA (−); △, × NA(+); ○, △ heat (−); □, × heat (+).

Table IV. Increased Uptake Ratios Produced by Treatment with Nicotinamide and/or Mild Heating

Treatment ^{195m}Pt-cisplatin dose	Uptake ratio[a] SCC VII	EMT6/KU
Nicotinamide		
$1/20LD_{50}$	1.85	1.83
$1/10LD_{50}$	2.97	1.24
$1/4LD_{50}$	2.73	1.13
$1/2LD_{50}$	2.43	1.10
Mild heating		
$1/20LD_{50}$	1.33	2.71
$1/10LD_{50}$	1.73	1.75
$1/4LD_{50}$	1.64	1.69
$1/2LD_{50}$	1.84	1.96
Nicotinamide+Mild heating		
$1/20LD_{50}$	3.50	3.50
$1/10LD_{50}$	4.40	2.14
$1/4LD_{50}$	3.46	2.13
$1/2LD_{50}$	2.96	2.45

a) Values are ratios of the uptake of ^{195m}Pt-cisplatin with treatment to that without treatment.

more markedly than either treatment alone, and the difference compared with treatment without nicotinamide or mild heating was significant.

DISCUSSION

The presence of Q cells in a tumor is thought to influence the sensitivity to various treatments.[1] Q cells in solid tumors are thought to be in this state partly because of oxygen and nutrient deprivation.[3,21] However, characterization of Q cells in solid tumors and analysis of their sensitivity to various treatments have been greatly hampered by the lack of adequate techniques to identify such cells and to obtain them in large homogeneous populations. Thus, we investigated Q cells using our previously developed method for the selective determination of responses of such cells in solid tumors.[13]

It has been shown that solid tumors contain hypoxic cells produced by the limitations of the diffusion of oxygen (chronic hypoxia) and the temporary occlusion of vessels or the transient slowing of blood flow (limitations of perfusion, or acute hypoxia).[6] Subsequently, it was found that nicotinamide has the potential to decrease the radioresistance conferred by acute hypoxia, probably by reducing the interstitial pressure in tumor tissue.[22] In addition, it has recently been clarified that modest hyperthermia causes an increase in tumor pO_2, probably resulting from improvement in the supply of oxygen via an increase in tumor blood flow.[23–25] We therefore examined the usefulness of combined treatment with nicotinamide and/or low-temperature heat treatment on chemotherapy with cisplatin.

Similarly to the results of our previous study using X-ray irradiation to treat solid tumors,[26] we confirmed that the chemosensitivity of Q cells to cisplatin was lower than that of the total tumor cell population (Table II). However, when cisplatin was used, the DMF for Q cells was higher than the values we obtained using X-ray irradiation.[26] This difference could probably be attributed to the uneven distribution of cisplatin in the Q cells, due to the heterogeneity of tumor vasculature. A decrease in the average blood flow with tumor growth can lead to randomly distributed regions with altered microenvironments.[27] One physiological consequence of poor tumor perfusion is the occurrence of regions of hypoxia within the tumor mass. In addition, it is known that this poor tumor perfusion may also limit the clinical efficacy of chemotherapeutic agents as a result of reduced cell proliferation in areas receiving less nutrients, inadequate drug uptake, and the non-optimal distribution of the drug in the tumor tissue.[28] Our findings in Q cells are consistent with this hypothesis, since our previous report showed that the Q cell population has a larger hypoxic fraction than the total cell population in solid tumors.[26]

Previously, we found that Q cells within SCC VII and EMT6/KU tumors and the total cells within EMT6/KU tumors included a large chronically hypoxic fraction, and that the total cells within the SCC VII tumors contain a large acutely hypoxic fraction.[26] It has been shown that nicotinamide can prevent the transient fluctuations in blood flow that lead to the development of acute hypoxia,[6] although another recent report showed that with regard to tumor intravascular HbO_2 saturation, nicotinamide had only minor effects on oxygenation, whereas a combination of nicotinamide and carbogen produced marked and equivalent improvements in oxygen availability.[29] Taking into consideration the ERs and the increased uptake ratios shown in Tables III and IV, the administration of nicotinamide before chemotherapy was effective in tumor control to some extent, especially in tumors that included large acutely hypoxic fractions, such as SCC VII tumors.

The MN frequencies of cells from animals not treated with cisplatin (Table I) showed that the mild hyperthermia employed here could not induce direct thermal cytotoxicity. In addition, it has been reported that this level of mild hyperthermia cannot delay tumor growth or cause direct thermal radiosensitization.[9, 30]

As shown in Tables III and IV, mild heat treatment sensitized the Q cells of both tumors and the total cells of the EMT6/KU tumor to cisplatin to varying degrees, and significantly increased the uptake ratio into the total cells of EMT6/KU tumors. These observations suggested that mild heat treatment might release some hypoxic regions from chronic hypoxia through an increase in tumor blood flow, probably resulting in distribution of higher doses of cisplatin and sensitization of tumor cells. Further, in our previous study, we also found that EMT6/KU tumors as a whole include a larger hypoxic fraction, especially chronically hypoxic fraction, than SCC VII tumors.[26] Therefore, mild heat treatment produced higher ERs for EMT6/KU tumor cells than those for SCC VII tumor cells. To clarify the hypoxia-releasing effect of mild hyperthermia, we are currently involved in studies to examine which hypoxic fraction is preferentially influenced by mild hyperthermia, using our method for detecting the Q cell sensitivity selectively *in vivo*.

As shown in Tables III and IV, in each cell population of both tumors the ERs and the increased uptake ratios for combined treatment with nicotinamide and mild heating were higher than those with either treatment alone. Thus, from the viewpoint of controlling not only the total cells, but also the Q cells in solid tumors, combined treatment with nicotinamide and mild heating is potentially useful for producing higher ERs than conventional chemotherapy alone. Agents that can prevent transient microregional alterations in tumor blood flow constitute another class of agents with a defined use in cancer chemotherapy, and further, the release of chronic hypoxia constitutes an effective part of any combined treatment in cancer chemotherapy.

The Q cell assay method employed here appears to be useful for detecting the sensitivity to chemotherapy of Q cells in solid tumors. Using this method, we plan to investigate the responses of Q cells to treatment with radiation and chemotherapeutic agents and/or hypoxic cell sensitizers as well as their responses to high linear energy transfer radiation, including thermal and/or epithermal neutrons, which are available at our institute.

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