Modification of Radiosensitivity of Ehrlich Ascites Carcinoma Cells "in vivo" Part II. Sensitizing Ability of Sulfhydryl-binding Agents to Hypoxic Tumor Cells

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生体内におけるエールリッヒ腹水癌細胞の放射線感受性の修飾について II. Hypoxic な腫瘍細胞に対する SH 基結合物質の放射線増感効果

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悪性腫瘍の放射線治療に抗癌剤もしくは増感剤を併用する目的は，これら薬剤が放射線抵抗性の細胞—特に hypoxic な細胞—に作用し，放射線に対する感受性を高めるか，あるいは直接その増殖能を阻止することを期待することにある．

われわれはこれらSH基結合物質としてIodoacetamide(IA) および N-ethylmaleimide (NEM) を用いた．動物は8～12週令の ddYF 雌マウスを実験に供し，用いた腫瘍細胞は我々の教室にて同系マウスに継代移植をしている Ehrlich ascites carcinoma cell (E.a.c) である．われわれの定例の実験方法により TD_{50} 分析を行い，結果を検討した．ＩＡの腫瘍細胞に対する Cytotoxicity をしらべ，3 μg/ グラム体重の濃度までは Cell Killing が示されなかつたので実験には2 μg/グラム体重を用いた．in vivo の実験には 10^6ケの E.a.c 細胞移植後7日目の腫瘍細胞を用い，in vitro にては腹腔内より E.a.c 細胞を採出し，細胞浮游液を37℃で5分間薬剤と共に処理し，N_2 ガスをX線照射前20分間及び照射中通気させた．一方，ＮＥＭは in vitro についてのみ実験を行い，濃度は5 μg/ 5 $\times 10^8$生細胞/ml で放射線増感作用を検討した．

以上の濃度にてこれらの薬剤は hypoxic な腫瘍細胞に対し放射線増感効果を示した．すなわち，DMF(hypoxic 細胞にて，照射そして薬剤処理の D_o に対する薬剤非処理で照射 hypoxic 細胞の D_o の比）がＩＡで1.66 (in vivo),1.70 (in vitro)，ＮＥＭで1.86 (in vitro) の結果を得たのでこれについて論議した．

It is well-established that radiation sensitivity of a malignant tumor largely depends on the sensitivity of oxygen-deficient, i.e., hypoxic tumor cells in the tumor. Therefore, radiation therapy combined with radiosensitizing agents intends that such oxygen-deficient cells in the tumor could be sensitized by the agents. Sulfhydryl-binding agents are of particular interest in this point of view. It is reported that anoxic bacterial cells were specifically sensitized by some of this agents while the well-oxygenized cells were not sensitized or a little sensitizing ability of the agents was demonstrated in the presence of oxygen.

The ability of two of the sulfhydryl-binding agents to sensitize hypoxic mammalian tumor cells was investigated using an animal-tumor system and is presented in this paper.

Material and Methods

Animal-tumor system: Eight to twelve-week-old female mice of the ddYF strain supplied by Funabashi-nojo, Chiba were used in all the experiments. They were kept in small animal facilities at a constant temperature. Animals were housed in groups of 8–12 in a mouse cage and provided with standard purina pellets and water ad libitum. The tumor cells used were Ehrlich ascites carcinoma cells propagated in our laboratory by weekly transplantation of 10^6 viable tumor cells.

Drug treatment of tumor cells: Test agents were Iodoacetamide (IA) and N-ethylmaleimide (NEM), both of which are known as the sulfhydryl-binding agents. Tumor cells were treated by one of the agents "in vivo" or "in vitro". However, all the assays were performed "in vivo".

1) Treatment "in vivo"; Donor animals carrining 7 day-old ascites carcinoma received one of the agents intraperitoneally. Five minutes later, 1 ml blood was taken out through the optic sinus and the animals were sacrificed by cervical dislocation. They were irradiated with avarious dose in supine position 15 minutes after their death and ascites fluid was removed for the transplantation.

2) Treatment "in vitro"; Ascites tumors were removed from peritoneal cavities of animals carrining 7 day-old tumor to a petri dish and number of cells were adjusted to 5×10^8/ml by use of Hanks media which contains 5% fetal calf serum. The cell suspension was incubated with one of the agents for 5 minutes at 37°C and again removed to a small plastic container for X-ray irradiation. Nitrogen gas (containing 20 ppm oxygen) was flowed for 20 minutes before the start of irradiation to obtain hypoxia.

X-irradiation: Detailed methods are described in a previous paper[14]. Physical factors employed for both of "in vivo" and "in vitro" treatments were: The half value layer = 0.8 mmCu., target-surface distance = 25 cm, and dose-rate = 512 rads/min.

Transplantation and experimental assay method: (see ref. 14 for details) All the experiments were based on assays of TD_{50} (number of viable tumor cells expected to transplant a tumor in half of the inoculated sites). Trypan-blue staining method and Hanks media containing 5% fetal calf serum were used for viable cell count and for serial cell dilution respectively. Lethally irradiated tumor cells which had received 10,000 rads were mixed with viable cells in the proportion of 10^4 : 1, because in this case a few viable cells were expected to produce a tumor in half of the transplanted sites. This admixtured cell suspension were diluted serially for TD_{50} assays. For each assay six to eight different cell concentrations in 1:1 or 1:2 dilution were employed. The cell suspension containing a fixed number of cells in 3 μl were injected intracutaneously in six sites on the dorsal skin of mouse. Test tubes containing these cell suspensions were stood in iced water until termination of the transplantation. The recipient animals were delivered whole body irradiation of 400 rads 24 hours before the challenge and were assigned by a random number schema into one of the dose levels in one of the assays. For each assay 12–15 mice were used.

Scoring tumor takes and analysis of TD_{50}: The transplanted sites were palpated for possible tumor growth every five or seven days after the inoculation. This examination was started on the seventh day and was continued for 30 days after the inoculation. If a tumor grew up to more than 5 mm in diameter, it was scored as a "tumor take". If an animal died before the termination of scoring, it was excluded from

the TD_{50} assay unless it had tumors in all the inoculated sites.

The TD_{50} was computed from tumor take frequency by logit analysis. The survival fraction of tumor cells after the irradiation was calculated from the ratio: TD_{50} (control)/TD_{50} (irradiated).

Results

=Iodoacetamide (IA)=The first study was attempted to test cytotoxicity of IA on the tumor cells. Animals carrinig 7 day-old ascites tumor received a different dose of IA. Thirty minutes later the ascites was removed and served for TD_{50} assays. The experimental methods were the same as mentioned above except lethally irradiated tumor cells were not added in this study. The results demonstrated that IA did not affect the reproductive integrity of the tumor cells up to the dose of 3 μg/g (a gram of mouse body weight) as shown in table 1. Therefore, IA was administered at non-toxic level for sensitization experiments.

Strong sensitizing ability of IA was observed in both of "in vivo" and 'in vitro" treatments (see Fig. 1).

Table 1. Effect of Intraperitoneal Treatment of IA for 30 minutes on Viability of Ehrlich Ascites Tumor Cells

Dose of IA (μg/g)	$TD_{50} \pm 95\%$ Confidence Limit (viable cells)
0	2.3×10^2 ($1.1 \times 10^2 - 4.8 \times 10^2$)
1.5	3.2×10^2 ($1.4 \times 10^2 - 7.0 \times 10^2$)
3.0	2.9×10^2 ($1.4 \times 10^2 - 6.0 \times 10^2$)
4.5	9.6×10^2 ($3.7 \times 10^2 - 2.5 \times 10^3$)
6.0	7.5×10^2 ($3.3 \times 10^2 - 1.7 \times 10^3$)

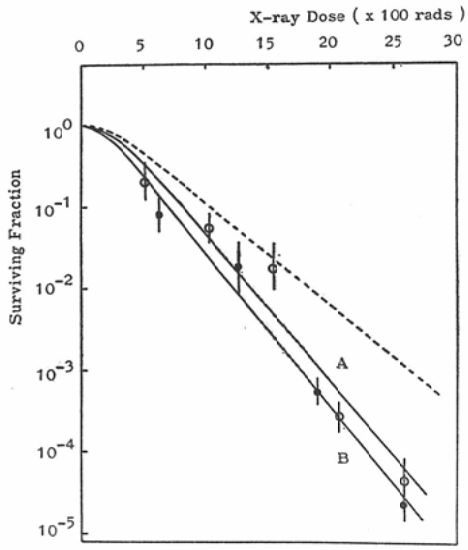

Figure 1: Dose-Survival Curve for IA-treated Ehrlich Ascites Carcinoma Cells after a Single Dose given under Hypoxic Condition.
Open circles (curve A) and solid circles (curve B) indicate "in vivo" and "in vitro" treatments respectively. Dotted line shows x-ray dose-response curve of the same hypoxic cells.

Table 2. Effectiveness of IA and NEM on Radiationsensitivity of Hypoxic Ehrlich Ascites Carcinoma Cells Evaluated in Terms of m (extrapolation number), D_0 (radiation dose to reduce survival fraction from 1 to 1/e in the straight portion of the cell-survival curve) and D.M.F. (dose modifining factor)

Drug Treatment	m	D_0 (rads)	D.M.F.
IA "in vivo"	2.8	235	1.66
IA "in vitro"	2.5	230	1.70
NEM "in vitro"	3.0	210	1.86
No Treatment	2.0	390	—

Amount of IA used were 2 μg/g "in vivo" and 2 μg/5 × 10^8 viable cells/ml. "in vitro". Chi-square method was fitted for the calculation of m (extrapolation number) and D_0 (radiation dose to reduce surviving fraction from 1 to 1/e on the straight portion of the dose-survival curve). They are presented in table 2. Dose modifining factor (D.M.F.)* were 1.66 and 1.70 for "in vivo" and "in vitro" treatments respectively.

=N-ethylenamide (NEM) = NEM was tested only by "in vitro" treatment. The amount employed was 5 μg/5 × 10^8 viable cells/ml. Cytotoxicity of the agent was examined in the same experiment to investigate the sensitizing ability. Hypoxic tumor cells were also sensitized by this amount of NEM (Fig. 2)

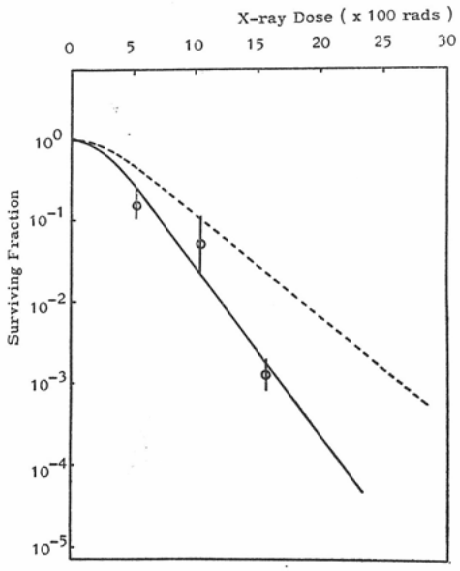

Figure 2: Dose-Survival Curve for NEM-treated Ehrlich Ascites Carcinoma Cells after a Single X-ray Irradiation under Hypoxic Condition. Dotted line indicates x-ray dose-survival curve of the same hypoxic cells.

by which the reproductive capability of tumor cells was not impaired. D_0 was reduced to 210 rads from 390 rads of hypoxic tumor cells received irradiation alone. D.M.F. was 1.86, i.e., very cross to that of IA.

Discussion

Several chemical compounds are reported as radiation sensitizers; some of them are already in clinical

*D.M.F. = a ratio of D_0 of hypoxic tumor cells irradiated without drug treatment to D_0 of those cells received both of irradiation and drug treatment.

trials, e.g., halogenated pyrimidines[1)8)9)] or methotrexate[2)8)], and the others[5)7)10)12)] are still under investigations. The sulfhydryl-binding agents are of particular interest because some of them could sensitize hypoxic or oxygen-deficient bacterial cells specifically. Bridges[4)] reported that the survival curve was about twice as steep in the presence of 10^{-3}M NEM when irradiation was given under anoxic condition, while in the presence of oxygen the sensitizing effect of NEM was much less than under anoxia. Dean and Alexander[6)] showed that radiosensitive E. coli was not or only little sensitized by IA in aerated condition, while radioresistant Micrococcus radiodurans was remarkably effected by IA even in oxygenated condition.

Several hypothesis for the sensitization mechanism of the sulfhydryl-binding agents are extensively discussed by Bridges[4)]. One of the hypothesis is that the agent could remove intracellular sulfhydryl radioprotective agent by the effective neutralization and so on. However there is no explanation available why the anoxic cells are preferencially sensitized by some of the agents.

In clinical point of view, the use of radiosensitizing agents would be expected to sensitize radioresistant or oxygen-deficient cells and to increase the therapeutic ratio, i.e., the ratio of normal tissue tollerance dose to the tumor cure dose. Present studies demonstrated that hypoxic "mammalian" tumor cells were also sensitized by IA and by NEM as anoxic bacterial cells. However to evaluate the therapeutic ratio, this study should be followed by another research to investigate the effect of these agents on normal tissue response.

Before the clinical trial of these agents, following two questions should be answered in addition to the therapeutic ratio. One is whether the agents could be incorporated in hypoxic cell population in a solid tumor. The other is toxicity of the agents to the tumor host.

It is very disappointing that very few dosage of NEM are toxic to Ha/ICR mice[11)]. Moroson reported that 15 μg/g would correspond to LD_{100} of this strain of mice. However, IA was not. He also demonstrated that 40 μg/g of IA did not sacrifice Ha/ICR mouse, while this dose killed 65% of BDF_1 mice. Present study indicates that 2 μg/g of IA has remarkable sensitizing effect on hypoxic Ehrlich ascites tumor cells.

The former question whether the agent could be incorporated in hypoxic tumor cells is not answered at the moment, especially for this particular agent(s). Suit, Urano and Hewitt[13)] demonstrated that hypoxic cell population of a C3H mouse mammary carcinoma was distinctly sensitized by 5-iododeoxy-uridine. Incorporation of cyclophosphamide into the hypoxic cells was also suggested in solid Ehrlich ascites tumors by us[15)]. These data might suggest that the incorporation of chemicals into oxygen-deficient tumor cells in a solid tumor is not the same extent as the oxygen diffusion from blood capillaries to tumor cells and that there is a possibility of IA incorporation into hypoxic tumor cells.

Summary

Sensitizing ability of two of the sulfhydryl-binding agents: IA (iodoacetamide) and NEM (N-ethylmaleimide) was studied in an animal-tumor system. Ehrlich ascites tumor cells were treated by one of the agents "in vivo" or "in vitro". Cell irradiation was carried out in the presence of one of the agents. Results were assayed by TD_{50} or determination of number of cells to transplant a tumor in half of the inoculated sites. Radiation response of the hypoxic tumor cells was sensitized by both of the agents. Dose

modifining factors for "in vivo" treatment of IA, 'in vitro" treatment of IA and for "in vitro" treatment of NEM were 1.66, 1.70 and 1.86 respectively. Possibility of the agents to use in clinical radiotherapy was discussed but more data are requested before the trial.

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