Effect of a Hypoxic Cell Sensitizer Doranidazole on the Radiation-induced Apoptosis of Mouse L5178Y Lymphoma Cells

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We investigated the sensitizing effect of the 2-nitroimidazole analogue doranidazole, a new hypoxic radiosensitizer, on radiation-induced apoptosis in L5178Y cells. Apoptosis was assessed by checking DNA ladder formation, the presence of sub-G1 peaks in flow cytometry, and chromatin condensation. A radiosensitizing effect of doranidazole was also confirmed by a soft-agar colony assay of surviving cells. In the assay of DNA ladder formation, DNA fragmentation was observed following irradiation under an aerobic or hypoxic condition with or without doranidazole. The proportions of the cells at the sub-G1 peak in a flow cytometric measurement was not very different among the irradiations at 5 Gy under the aerobic condition, 15 Gy under hypoxia, and 10 Gy with 1 mM doranidazole under hypoxia. The fraction of cells with chromatin condensation was found to be significantly increased with doranidazole up to 3 mM when applied under hypoxic irradiation, but did not increase even at 10 mM. The sensitizer enhancement ratio was estimated to be about 1.7 with a concentration of 1 mM. This enhancement ratio was not different from that observed by assaying cell survivals. On the other hand, doranidazole showed no radiosensitizing effect under aerobic conditions with 1 mM. In conclusion, the radiation-induced apoptosis of L5178Y cells was enhanced by doranidazole under hypoxia.

INTRODUCTION

Numerous new hypoxic cell radiosensitizers have been synthesized and screened chemically since the introduction of misonidazole1–3, and several of them have been reported to be under clinical evaluation4–8. Although the clinical usefulness of the sensitizers remains open to discussion, a recent metanalysis by Overgaard9 showed a small, but significant, benefit of hypoxic cell sensitizers in conventional radiotherapy. While clinical studies of the 2-nitroimidazole sensitizers are ongoing, the mechanism of their radiosensitizing effects has not yet been completely clarified. It is generally understood that these radiosensitizers act to fix radiation-induced damage by reacting with macromolecular radicals and by inhibiting DNA repair. This would lead to an increase of reproductive cell death. On the other hand, there has been increasing interest in the role of apoptosis in radiation-induced cell death. The effects of these radiosensitizers on radiation-induced apoptosis have not yet been investigated.

A new 2-nitroimidazole nucleoside analogue, doranidazole (code number: PR-350, (±)-(2RS,3SR)-

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3-[2-nitro-imidazol-1-yl]-methoxy]butane-1,2,4-triol), has very low toxicity with a 50% lethal dose in mice exceeding 5 g/kg\textsuperscript{10–13}. It is now undergoing in Japan a phase II clinical test either in combination with intraoperative radiotherapy or in conventional radiotherapy\textsuperscript{14}. In the present study, we investigated the radiosensitizing effect of doranidazole on radiation-induced apoptosis of mouse L5178Y lymphoma cells, and compared the magnitude of radiosensitization with that determined by assaying cell survivals.

**MATERIALS AND METHODS**

**Compound, cells and radiation procedure**

Doranidazole (molecular weight 247) was supplied by Pola Chemical Industries, Inc. (Yokohama, Japan), and was dissolved in a culture medium for *in vitro* studies. L5178Y cells were maintained in a RPMI 1640 medium containing 10% fetal bovine serum (FBS). To put the cells into hypoxia\textsuperscript{15}, a mixture of 95% N\textsubscript{2} and 5% CO\textsubscript{2} gasses was flushed over the cell suspension for at least 45 min. For aerobic cells, a mixed gas of 95% air and 5% CO\textsubscript{2} was flushed. For assays of apoptosis, cell suspensions were centrifuged at 1,000 rpm for 3 min and cells were resuspended for up to 48 h of incubation. Following the incubation, cells were centrifuged at 1,000 rpm for 3 min to remove the supernatant, resuspended in phosphate buffered saline (PBS), and collected again by centrifugation. Then, the cell pellets were subjected to analysis. Details of the assays for apoptosis were described previously\textsuperscript{16}.

All irradiations were performed using an X-ray apparatus (200 kVp, 19 mA, 0.5 mm Cu + 0.5 mm Al filter) at a dose rate of 1.2 Gy/min.

**DNA ladder assay**

Cell pellets collected by centrifugation were treated with a 100 µl of cell-lysis buffer (10 mM EDTA, 0.5% Triton X-100, 10 mM Tris-HCl pH 8.0) for 10 min at 4°C. The lysate was centrifuged at 16,000 rpm at 4°C for 20 min to remove cell debris. The supernatant was then incubated with 2 µl of 20 mg/ml RNase A for 1 h at 37°C, and then with 1.6 µl of 25 mg/ml proteinase K for 1 h at 37°C. The extracted DNA was collected in 25 µl of 5 M NaCl and 125 µl isopropanol at –20°C overnight, and then suspended in a TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 7.4). The DNA samples and a DNA size marker, with a loading solution (0.25% bromophenolblue, 40% sucrose, [5:1]), were applied to a 2% agarose gel in TBE buffer (2 mM EDTA, 89 mM tris-borate). The samples were electrophoresed for 2 h in TBE buffer with 0.5 µg/ml ethidiumbromide using a Mupid II electrophoresis tank (Cosmo Bio Co., Tokyo) operated at 50 V.

**Flow cytometry**

The cell pellets were fixed with 200 µl of 70% ethanol at –20°C for at least 4 h. Following centrifugation (4°C, 2,500 rpm, 5 min) and removal of the supernatant, the cells were suspended in PBS, and then collected again by centrifugation. The cells were resuspended in 100 µl of PBS and then treated with 0.4 µl of 25 mg/ml RNase A for 30 min at 37°C. After the treatment and precipitation by centrifugation, the cells were resuspended in 1 ml of PBS, stained with 50 µl of 1 mM bis-benzamide (Hoechst 33342) and collected again by centrifugation. Then, the cell pellets were subjected to analysis. Details of the assays for apoptosis were described previously\textsuperscript{16}.

**Chromatin condensation**

The cell pellets were fixed with a 1% glutaraldehyde solution at 4°C overnight. After the suspensions were centrifuged at 1,000 rpm for 3 min, the cells were resuspended in PBS. One µl of 1 mM bis-benzamide (Hoechst 33342) was added to 10 µl of cell suspension, and mixed. One drop of the cell suspension was placed on a slide glass, and the number of cells with or without chromatin condensation was scored using a fluorescent microscope.

**Cell survival assay**

The survival of a cell was determined by a soft-agar colony formation method. The cells were irradiated either with or without 1 mM doranidazole, under...
aerobic or hypoxic conditions. After removing doranidazole by centrifugation, appropriate numbers of cells in 1 ml of RPMI 1640 containing 10% FBS and 0.25% agarose were plated onto 6 cm diameter culture dishes covered with 5 ml of RPMI 1640 containing 0.5% agarose and 10% FBS. After 10 days of incubation, the cell survivals were determined by scoring the number of surviving colonies.

RESULTS

DNA ladder assay

Figure 1 shows the formation of a DNA ladder of L5178Y cells at 48 h following 5 Gy of X-ray irradiation with or without 1 mM doranidazole treatments, under hypoxic or aerobic conditions. DNA ladder formation was induced in all of the cells irradiated by X-rays. On the other hand, either a hypoxic or a doranidazole treatment did not induce DNA ladder formation without irradiation. The efficiency of DNA ladder formation by irradiation was found to be lower under hypoxic than aerobic conditions. A small difference in ladder formation by the doranidazole treatment was found under hypoxia, but not under aerobic irradiation.

Flow cytometric analysis

Figure 2 shows the distributions of the DNA contents in L5178Y cells at 0 and 48 hours following X-ray irradiations under aerobic (5 Gy), hypoxic (15 Gy), or hypoxic conditions with 1 mM doranidazole (10 Gy). A decrease in the G1 peak was observed (data not shown) at 18 hours after irradiation, and a high peak in the sub-G1 phase as an induction of apoptosis was detected at 48 hours. The proportion of the peak area of the sub-G1 phase was not so different among the three groups. Under aerobic conditions, 1 mM doranidazole did not act to increase the number of apoptotic bodies (data not shown).

Chromatin condensation

The features of chromatin condensation in L5178Y cells were found to be similar to those reported for other cell lines. Figure 3 shows dose-
response curves for the chromatin condensation of L5178Y cells at 48 hours after irradiation. Under the aerobic condition, 1 mM doranidazole did not increase the fraction of chromatin condensation. As shown in Fig. 3, doranidazole apparently had a sensitizing effect under the hypoxic condition. At the 30% level of chromatin condensation, the oxygen enhancement ratio (OER) was approximately 2.5, and the sensitizer enhancement ratio (SER) was approximately 1.7.

Figure 4 shows the induction of chromatin condensation after 8 Gy of hypoxic irradiation as a function of the doranidazole dose. Although the number of the cells with chromatin condensation increased with the doranidazole concentration of up to 3 mM, no more increment was found at 10 mM. Up to a concentration of 10 mM, a doranidazole treatment alone did not increase the frequency of chromatin condensation without irradiation under both aerobic and hypoxic conditions.

Cell survival

Figure 5 shows cell-survival curves of L5178Y cells that were irradiated under hypoxia with a 1 mM doranidazole treatment together with those under aerobic irradiation or without any drug treatment. Doranidazole showed no sensitizing effect under aerobic conditions, but had a significant effect under hypoxic conditions. At the level of a cell survival fraction of 0.1, the OER was 2.75 and the SER was 1.75. At the level of a cell survival fraction of 0.01, the OER was 2.5.
was 2.75 and the SER was 1.55.

**DISCUSSION**

This study showed that apoptosis was induced in L5178Y cells following irradiation under both aerobic or hypoxic conditions, but not following a hypoxic or doranidazole treatment alone (Figs. 1 and 4). For a quantitative analysis of the DNA ladder formation assay, we simply checked the induction of apoptosis following 5 Gy of irradiation with or without doranidazole. We also attempted a flow cytometric study for apoptosis induction in L5178Y cells (Fig. 2) with two different gaseous conditions, drug treatments and radiation doses, and obtained similar conclusion to that in Fig. 3. The induction of sub-G1 fraction was almost the same among the cells irradiated at 5 Gy under aerobic conditions, those irradiated at 15 Gy under hypoxic conditions and those irradiated at 10 Gy with 1 mM doranidazole under hypoxic conditions. From this result, it can be estimated that the OER was approximately 3 and the SER was around 1.5. We made a more valid analysis by scoring chromatin condensation. Dose-response curves could be obtained (Fig. 3), and the OER was estimated to be 2.75 and the SER was 1.55–1.75. This OER value showed good agreement with a previous report. The fraction of chromatin condensation (Fig. 4) increased with an increase of the doranidazole concentration up to 3 mM, but no more increase was found at 10 mM. This result can be interpreted that the proportion of apoptotic cells reached the maximum between 0.6 and 0.7 with the concentration of doranidazole between 3 and 10 mM. From these results, it is concluded that doranidazole has a sensitizing effect on the radiation-induced apoptosis of L5178Y cells.

A nitroimidazole group of radiosensitizers are considered to selectively sensitize hypoxic cells by capturing macromolecular radicals, thereby acting to fix damage as well as to inhibit DNA repair. It is reasonable that such types of radiosensitizers enhance apoptotic cell death. Although little is known about the molecular target of radiation-induced apoptosis, DNA damage can trigger apoptosis. The enhancement of radiation-induced apoptosis by the nitroimidazole group of radiosensitizers may be a real phenomenon. The SER and OER obtained from the chromatin condensation assay (Fig. 3) were similar to those obtained from the surviving cell assay (Fig. 5). The SER was also similar to that observed in the other experiments with a doranidazole concentration of 1 mM. Since considerable proportions of death in L5178Y cells results from apoptosis when cells are treated with 1 mM doranidazole and irradiated under hypoxia, the agreement of SER values in terms of apoptosis and cell survival may be understandable. In addition, our previous study suggested that the molecular target for reproductive cell death and apoptosis could be, at least in part, the same (i.e., DNA break). The findings of the present study would be in favor of this hypothesis.

In summary, this study showed that the apoptosis of L5178Y cells was enhanced by an electron-affinic radiosensitizer doranidazole. Although this type of radiosensitizers has mostly been used for the squamous type of cell carcinomas or adenocarcinomas, the findings of this study indicate that doranidazole could be useful against bulky (and radioresistant) lymphoma and small cell carcinoma, which readily undergo apoptosis following irradiation.

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