67. Radiosensitization of Mouse L Cells by an Organic Nitroxyl Free Radical under an Extremely Hypoxic Condition

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The literature has pointed out that a largely developed tumor tissue contains hypoxic cells which are resistant to radiation (Thomlinson and Gray 1955). Organic nitroxyl free radicals have been known as the effective compounds which sensitize hypoxic cells (Emmerson *et al.* 1971).

The present study was undertaken to deal with radiosensitization of mouse L cells by one of nitroxyl free radicals, 2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl (N-oxyl), under an extremely hypoxic condition.

Materials and methods. Mouse L cells were grown in the monolayer culture in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (Gibco). Cell suspension was prepared from a log-phase culture by centrifugation following trypsinization.

An organic nitroxyl free radical, 2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl (N-oxyl), was synthesized according to the method of Rozantzev and Krinzkaya (1965). The drug was dissolved in Eagle's MEM without serum at a concentration of 1 mM, and pH was adjusted to 7.2 with dibasic sodium phosphate. The cell suspension was treated with the drug for 1 h before and during irradiation. The drug at this concentration was not cytotoxic under hypoxic and aerobic conditions.

Two kinds of gas-exchanging apparatuses were set up under an X-ray source. One of them was made with glass chambers (120 ml) and Teflon connecting tubes (apparatus A; Fig. 1A), and another was made with stainless steel (apparatus B; Fig. 1B). The latter consisted of two degassing-irradiation chambers (240 ml), a gas-humidifying chamber and a water-lock chamber, which were connected with stainless steel pipes and Teflon packings (Utsumi *et al.* 1979). In order to produce a hypoxic condition, ten ml of the cell suspension, 1×10^5 cells per ml, were stirred with the aid of a magnetic stirrer and flushed with high purity N₂ gas (O₂ < 0.2 ppm). N₂ gas was

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humidified, warmed and flowed for 30 min at a flow rate of 1 liter per min before and during irradiation.

Fig. 1. Photographs of two kinds of gas-exchanging apparatuses. A: Apparatus A made with glass. B: Apparatus B made with stainless steel.

X-irradiation was carried out with a Toshiba KXC-18 X-ray source operating at 188 or 200 kVp and 20 or 25 mA with 0.5 mm Cu and 1 mm Al filters for the apparatus A, and without filters for the apparatus B. The mean dose rate was 200 rad per min. An aerobic irradiation was carried out at the same condition as mentioned above without flowing N_2 gas. Immediately after irradiation, the cell suspension was washed with fresh medium, and 200 cells were plated in a plastic petri dish containing 5 ml of growth medium. The dishes

were incubated at 37°C in the humidified air containing 5% CO₂ to allow colony formation. On the 10th day, the formed colonies were fixed with 3 parts of methanol and 1 part of acetic acid, and stained with the Giemsa solution. Colony-forming ability was determined by counting the colonies containing more than 50 cells. In each experiment, 5 dishes were used per point. Each experiment was duplicated.

Results and discussion. Survival curves under aerobic and hypoxic conditions are shown in Fig. 2, and radiobiological parameters of the survival curves are presented in Table I. The extremely hypoxic condition was obtained with the apparatus B but not with the apparatus A. The presence of a small amount of oxygen in the apparatus A gave an appreciable shoulder of a survival curve. The largest shoulder was seen in the aerobic survival curve. On the other hand, under the extremely hypoxic condition, a survival curve showed no shoulder region, and was exponential at the dose range examined, having the extrapolation number of 1.05 and the mean lethal dose (D_0) value of 570 rad (Table I). The oxygen enhancement ratio was calculated as

Fig. 2. Survival curves of mouse L cells following X-irradiation under various conditions.

2.73 when the apparatus A was used, and 3.56 with the apparatus B (Table I).

Table I. Radiobiological parameters of the survival curves of mouse L cells following X-irradiation under various conditions

Irradiation condition	Extrapolation number	D ₀ (rad)	Enhancement ratio
Apparatus A			
N ₂	2.00	354	1.00
Air	7.00	130	2.73
Apparatus B			
N ₂	1.05	570	1.00
Air	4.20	160	3.56
N ₂ +1 mM N-oxyl	1.80	350	1.63

Using cell lines of a malignant origin, the similar results were obtained by Révész and Littbrand (1964) and Pettersen *et al.* (1973). On the contrary, some workers who employed normal cell-origin lines such as Chinese hamster cells as materials reported that oxygen acted as a dose modifier (Elkind *et al.* 1965; Chapman *et al.* 1971; Koch *et al.* 1973). The discrepancy seems to be due in part to the different modes of responses to irradiation between the normal cell line and the malignant line.

Commins and Dewey (1976) demonstrated that X-irradiation liberated oxygen from a glass container into water at the rate of 2.4×10^{-10} moles per liter per rad at 20°C. The present results seem to suggest that oxygen-liberation may take place in the experiment with the apparatus A. In contrast, the oxygen-liberation is minimized in the experiment with the apparatus B.

The sensitizing effect of N-oxyl under the extremely hypoxic condition with the use of the apparatus B was observed as shown in Table I. The presence of the drug increased the slope and the shoulder size of the survival curve with the extrapolation number of 1.80 and D₀ value of 350 rad. A significant sensitizing effect was observed at radiation doses more than 1,000 rad. The enhancement ratio was 1.63. Namely, the L cells treated with N-oxyl showed a high radio-sensitivity.

The evidence obtained suggests that N-oxyl acts as oxygen-mimic under the extremely hypoxic condition.

Summary. Mouse L cells are X-irradiated at various doses under aerobic and hypoxic conditions, and the colony-forming ability is examined. The survival curve is exponential under the extremely hypoxic condition, and the oxygen enhancement ratio is calculated as 3.56. The presence of an organic nitroxyl free radical (N-oxyl) in-

creases the slope and the shoulder size of the survival curve, with the enhancement ratio at 1.63. The evidence suggests that N-oxyl acts as oxygen-mimic.

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