J. RADIAT. RES., 34, 195–203 (1993)

Radiosensitization of Human Lung Fibroblasts by Chemicals that Decrease ATP Levels

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(*Received, October 7, 1993*)
(*Revision received, May 28, 1993*)
(*Accepted, July 2, 1993*)

Radiosensitization/human lung fibroblasts/ATP content/pyruvate/novobiocin

Radiosensitization by lactate, pyruvate, nalidixic acid and novobiocin was studied in exponentially growing SH-18L human lung fibroblasts. All the chemicals had a slight radiosensitizing effect at a low concentration and a definite effect at a higher one. Decreases in the D_0 and/or Dq values were present in each dose survival curve. Fibroblasts incubated with the low concentration of each chemical for 24 hrs after X irradiation showed no reduction in intracellular ATP content, whereas, the higher concentration produced a significant decrease. These observations suggest that the decrease in the ATP content may be involved in the radiosensitization of human fibroblasts at high concentrations of these chemicals. In contrast, radiosensitization at a low concentration is not explained by a relationship to ATP content. Different mechanisms may be involved in radiosensitization at low and high concentrations of these chemicals.

INTRODUCTION

Ionizing radiations are widely used in cancer therapy. To be successful, such treatment requires that normal and cancer cells respond differentially to the irradiation given. Numerous attempts have been made to identify specific physical or chemical factors which might selectively modify the survival of irradiated cells.

Intracellular changes in the intermediary metabolism are important aspects in cell death. Kumar *et al*[1] reported that intermediate metabolites such as lactate and pyruvate sensitize V79 cells dose dependently. Accumulations of lactate and pyruvate also lower the ATP concentration, thereby inhibiting DNA repair. Increases in the contents of lactate and pyruvate also may result in metabolic acidosis[2], therby affecting cellular functions.

Nalidixic acid and novobiocin, inhibitors of the bacterial enzyme DNA gyrase[3], inhibit DNA, RNA and protein synthesis in several human and rodent cell lines[4,5]. More recently these

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chemicals have been shown to inhibit topoisomerase II activity in Hela cells[6]. The actual action of novobiocin/nalidixic acid, however, is still not clear[7,8]. Instead of acting on the topoisomerase these may act at the mitochondrial level thereby affecting ATP production. Rowley[9] has shown that novobiocin and nalidixic acid block progression of the cell cycle in the G_2 stage.

We investigated the role of cellular ATP in the radiosensitization of human lung fibroblasts by measuring cell survival and the ATP contents of cells incubated with the intermediate metabolites (lactate or pyruvate), nalidixic acid or novobiocin after irradiation.

MATERIALS AND METHODS

Chemicals

Sodium pyruvate and sodium DL-lactate were purchased from Nakalai Tesque, Japan and novobiocin and nalidixic acid from Sigma Chemical Company, USA. For the ATP measurements, an ATP bioluminescence CLS kit from Boehringer Mannheim Biochemica, FRG was used.

Cell culture

An SH-18L human lung fibroblast cell line that had been established in this laboratory[10] was used after a PDL (population doubling level) of 20. The cells were grown as monolayers in plastic tissue culture dishes (10 cm diameter) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The nutrient medium used was Eagle's modified minimum essential medium (MEM) (Nissui, Japan) supplemented with 10% fetal calf serum (FCS) (Flow).

X-irradiation and exposure to the chemicals

Exponentially growing asynchronous human lung fibroblast cells were irradiated in air with soft X-rays at room temperature (Softex CMB-2, 60 kVp, 10 mA with a 0.3 mm Al filter) at the dose rate of 2.4 Gy/min. Sodium DL-lactate, pyruvate (10 mM, 20 mM), nalidixic acid (1 mM, 2 mM) or novobiocin (0.25 mM, 0.5 mM was added immediately after administration of the various doses of X-irradiation. After 24 hrs of incubation in these chemicals, cells were washed twice with MEM then incubated in Eagle's MEM plus 10% FCS for 15 days to assess colony formation and estimate cell survival.

ATP-assay

The ATP content of the cells incubated with or without the chemicals for 2, 6, 16 and 24 hrs after 8 Gy of X-irradiation was determined in a luciferin-luciferase enzyme system[11]. The cells ($1-2\times10^5$ cells/6 cm plate) were scraped from the dishes with a rubber spatula in cold trichloroacetic acid (TCA) after several washes with ice-cold buffered saline (PBS). They then were transferred to test tubes and centrifuged for 5 minutes at 2,500 rpm. The supernatant was washed three times with water-saturated ether to remove TCA. An portion of the supernatant was used for the ATP assay with ATP-bioluminescence CLS, Boehringer, using a Packard 1,500

with a single photon count (SPC) channel. A standard curve was plotted for each experiment. Protein was assayed with the Bio-Rad D_c protein assay reagent.

Flow cytometry

Flow cytometry was done with an EPICS-C (Coulter) flow cytofluorometer, as described elsewhere[11,12]. Briefly, fibroblasts collected by trypsinization were washed twice with phosphate-buffered saline (PBS) and fixed with ethanol-PBS (1 : 1). These fixed cells were treated with 0.5 ml RNase A (1 mg/ml) at 37°C for 45 min then stained with 0.005% propidium iodide at room temperature for 20 min.

RESULTS

Effect of chemicals on the survival of X-irradiated cells

We first examined the toxic effect of each chemical in terms of its effect on the colony-forming ability of the non-irradiated fibroblasts. The cells were incubated with a chemical for 24 hrs, after which an appropriate number of cells was replated into culture dishes for colony

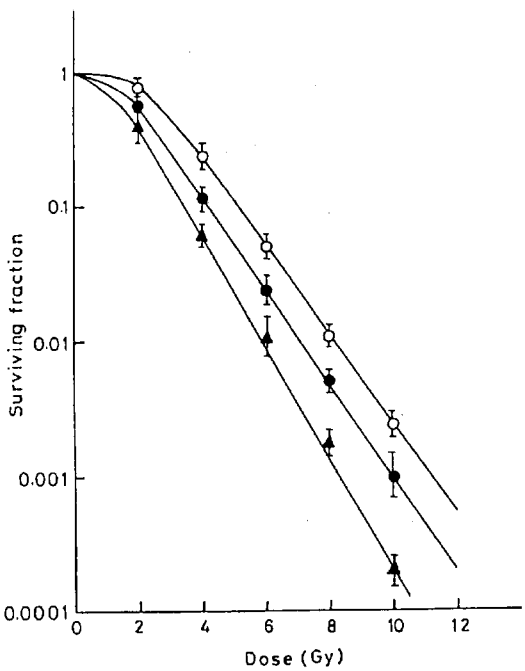

Fig. 1. Survival of X-irradiated SH-18L human lung fibroblast cells treated with sodium lactate for 24 hrs after irradiation. Each point represents the mean of triplicate samples together with the standard deviation (vertical bar). ○: control, ●: 10 mM lactate, ▲: 20 mM lactate.

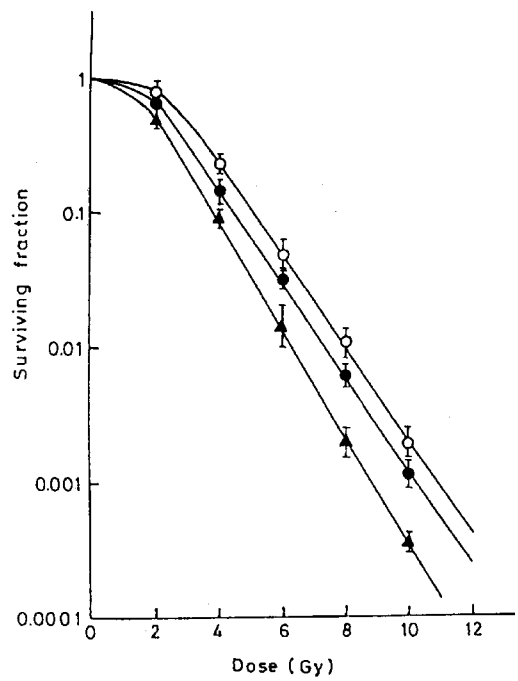

Fig. 2. Survival of X-irradiated SH-18L human lung fibroblast cells treated with sodium pyruvate for 24 hrs after irradiation. Each point represents the mean of triplicate samples together with the standard deviation (vertical bar). ○: control, ●: 10 mM pyruvate, ▲: 20 mM pyruvate.

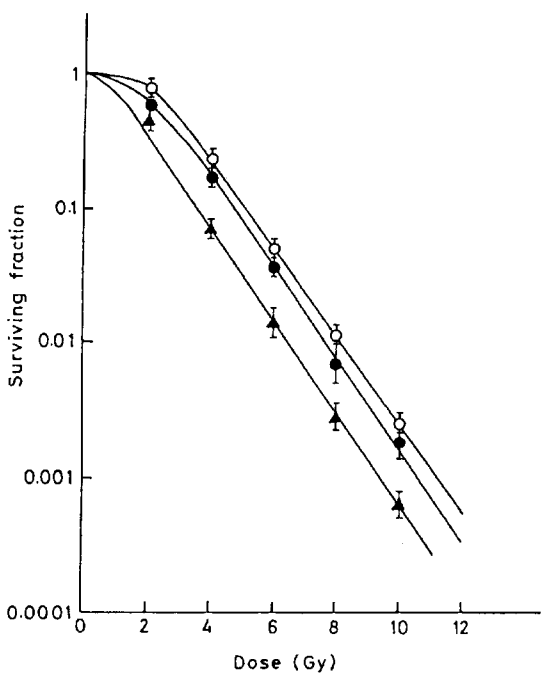

Fig. 3. Survival of X-irradiated SH-18L human lung fibroblast cells treated with novobiocin for 24 hrs after irradiation. Each point represents the mean of triplicate samples together with the standard deviation (vertical bar). ○: control, ●: 0.25 mM novobiocin, ▲: 0.5 mM novobiocin.

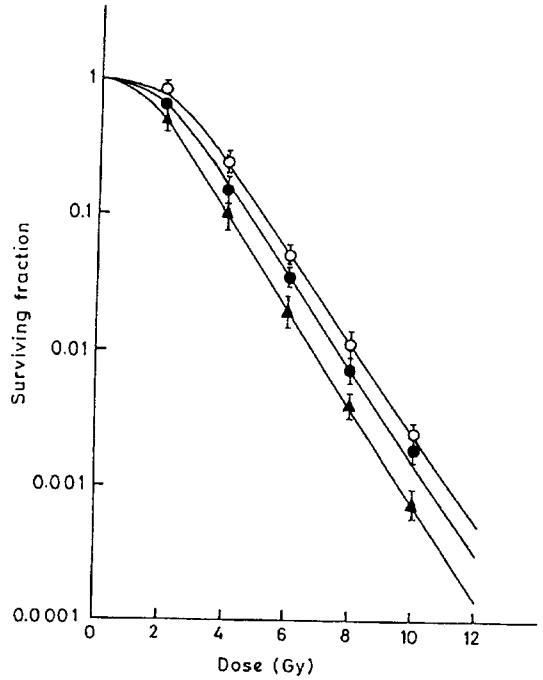

Fig. 4. Survival of X-irradiated SH-18L human lung fibroblast cells treated with nalidixic acid for 24 hrs after irradiation. Each point represents the mean of triplicate samples together with the standard deviation (vertical bar). ○: control, ●: 1 mM nalidixic acid, ▲: 2 mM nalidixic acid.

formation. All the chemicals except novobiocin, were non-toxic at the doses used in the subsequent experiments, plating efficiencies being between 25.2 and 30.9%. Treatment with novobiocin at 0.25 mM for 24 hrs caused no toxicity (26.3%), but at 0.5 mM a reduction in colony-forming ability (plating efficiency=7.4%) occurred.

In cells irradiated and exposed to lactate, pyruvate (10 mM), nalidixic acid (1 mM) or novobiocin (0.25 mM) for 24 hrs, there was a decrease in the shoulder width (Dq) but little change in the final slope ($1/D_0$) of the curve (Figures 1–4). At a high concentration of lactate or pyruvate (20 mM), there was a decrease in the D_0 (Control cells D_0, 1.31 Gy, Cells incubated with lactate D_0, 1.04 Gy and Cells incubated with pyruvate D_0, 1.09 Gy) together with a decrease in Dq (Control cells Dq, 2.14 Gy, Cells incubated with lactate Dq, 1.02 Gy, and Cells incubated with pyruvate Dq, 1.33 Gy). The D_0 change in cells exposed to novobiocin at 0.5 mM and to nalidixic acid at 2 mM was not significant (Control cells D_0, 1.31 Gy, Cells incubated with novobiocin D_0, 1.23 Gy, and Cells incubated with nalidixic acid D_0, 1.20 Gy). The Dq, however, was significantly lower with both chemicals (Control cells Dq, 2.11 Gy, Cells incubated with novobiocin Dq, 0.89 Gy, and Cells treated with nalidixic acid Dq, 1.16 Gy).

Effect of chemicals on ATP content in the cell

Fig. 5 shows changes in intracellular ATP contents after irradiation. When cells were irradiated with X-rays then plated after various intervals (2, 6, 16 and 24 hrs) without any chemical, only a small changes in ATP content took place. However, cells incubated with the high concentration of a chemical after irradiation for various intervals, there was a reduction in the ATP content between 2 and 24 hrs of incubation. The ATP content then was measured after

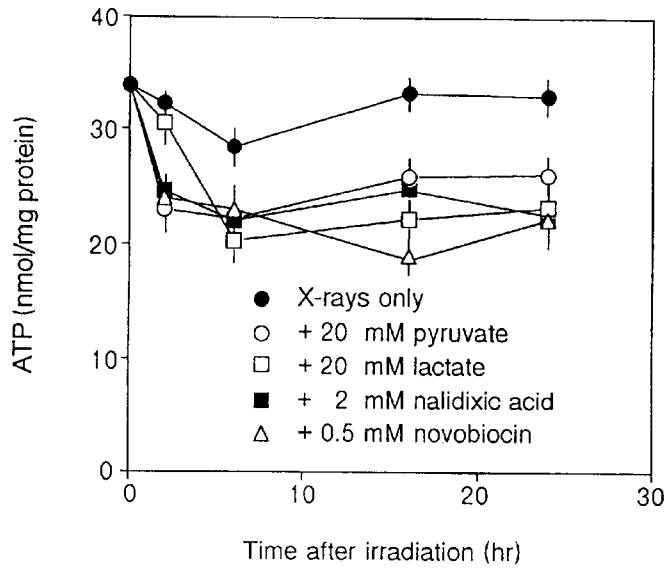

Fig. 5. Intracellular ATP contents in SH18L cells incubated with or without a chemical after X-irradiation. Each point represents the mean of triplicate samples together with the standard deviation (vertical bar). ●: X-rays (8 Gy), □: X-rays+20 mM lactate, ○: X-rays +20 mM pyruvate, ■: X-rays+20 mM nalidixic acid, △: X-rays+0.5 mM novobiocin.

Table 1. Effects of low and high concentrations of the chemicals assayed on ATP contents in X-irradiated human fibroblasts

Chemicals		ATP (nmol/mg protein)
(X-rays only)		32.9±1.9[a]
Pyruvate	(10 mM)	30.2±1.8
	(20 mM)	26.1±2.2*
lactate	(10 mM)	29.6±0.9
	(20 mM)	22.9±1.5*
nalidixic acid	(1 mM)	32.3±4.2
	(2 mM)	22.1±1.9*
novobiocin	(0.25 mM)	31.1±3.3
	(0.5 mM)	22.7±1.2*

[a] mean±standard deviation (n=6)

* $p<0.05$ by Student's t test

24 hrs of incubation with the chemical at both the high and the low concentrations (Table 1). Eight Gy of X-irradiation was given just before chemical treatment. There were no significant changes in ATP contents when the low concentration of a chemical was used. In contrast, the high dose of any of the chemicals produced a statistically significant reduction in the ATP content (Table 1).

Changes in cell cycle distribution due to chemical treatment

The distribution profiles in the cell cycle are shown in Fig. 6. Non-irradiated and non-chemical-treated exponentially growing cells had a typical distribution profile (Fig. 6a). When fibroblasts were incubated for 24 hrs after 8 Gy of irradiation, the fraction of cells in the G_2 phase increased, whereas cells in the G_1 and S phases decreased (Fig. 6b). This increase may reflect a G_2 delay produced by X-irradiation. At the high concentration none of the chemicals, except novobiocin, altered the profile of the irradiated cells, evidence that these chemicals did not disturb the progression of the cell cycle. Cells treated with novobiocin at 0.5 mM after irradiation have a profile similar to that of the non-irradiated cells (compare Fig. 6a and 6f). This similarity may be the result of novobiocin's inhibition of the progression of the cell cycle. Other evidence which supports this is that the cell number did not increase after novobiocin treatment (data not shown).

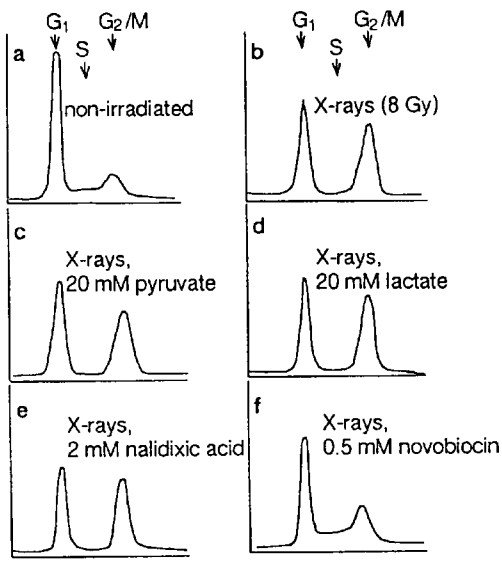

Fig. 6. Effects of the chemicals assayed on the cell cycle distribution of irradiated cells. An EPICS-C flow cytofluorometer was used. More than 30,000 cells were counted.

DISCUSSION

We found that a high concentration of lactate or pyruvate (20 mM) sensitizes human fibroblasts in terms of enhanced cell killing. There was a reduction both in the shoulder width (Dq) and the D_0. The effects of nalidixic acid and novobiocin appear to be mainly on the

shoulder, the final slope of the survival curve not being affected.

Several hypotheses may be put forward as to the role of the intermediate metabolites pyruvate and lactate in radiosensitization. High concentrations of lactate and pyruvate are known to lower the intracellular pH to a level at which the activities of pH-dependent enzymes, particularly phosphofructokinase, are inhibited. The glycolysis that depends on this enzyme may be greatly inhibited, resulting in a decrease in the ATP concentration. Our observation that cells treated with 20 mM lactate or pyruvate for 24 hrs after irradiation had statistically significant low ATP contents supports this hypothesis.

The radiosensitizing effects of novobiocin and nalidixic acid have been thought to be due to their effects on topoisomerase II because that enzyme has a role in DNA repair[13,14] and novobiocin is an inhibitor of the enzyme. The actual role of novobiocin in inhibiting excision repair has, however, become less clear of late[7,8]; it may act at the level of ATP production rather than on the topoisomerase. We found that novobiocin at 0.5 mM and nalidixic acid at 2 mM decreased the ATP content and enhanced the killing of fibroblasts which is consistent with this role. The decrease in the ATP content therefore may have a role in the radiosensitization produced by these chemicals.

Utsumi *et al* (1990)[15] showed that novobiocin inhibits the repair of PLD but not the repair of sublethal damage. They examined amsacrine, teniposide, etoposide, and novobiocin, well known inhibitors of replicative DNA synthesis. Only the last chemical, novobiocin, was effectively inhibited PLD repair. They suggested that when novobiocin is present in conditioned medium it may reduce the ATP/protein levels, and that this may be critical for its acting as a metabolic inhibitor of replicative DNA synthesis in the repair of PLD as opposed to the repair of SLD. Similar observations have been reported by Kumar *et al*[1]. Downes *et al*[8] showed that 1 mM of novobiocin significantly inhibited the ATP/ADP ratio in Hela cells incubated in fresh medium and thereby reduced the energy reserves of cells. Our observations support these hypotheses.

The repair of DNA is reported to require a continuous flow of metabolic energy in the form of adenosinetriphosphate (ATP). If this energy flow falls below a critical threshold value, DNA repair processes are completely inhibited[16,17]. Recently, in a respiratory-deficient mutant strain of yeast cells, 2-deoxyglucose has been shown to decrease the ATP concentration and thus inhibits the rejoining of DNA double strand breaks[18]. Waldstein *et al* (1974)[19] also showed that ATP is required for incision repair, or for a preceding enzymatic reaction, in *E. Coli.*

However, it is clear from the results shown in Figs. 1–4 and Table 1 that there are mechanisms other than that which reduces the ATP content. At the low concentration each chemical decreased cellular sensitivity to X rays but had no effect on ATP content. Because treatment with these chemicals was performed for a long period, the cell cycle traverse may have been affected. As jedged from the cytometric results (Fig. 6), however, none of these chemicals appear to have affected the progression of the cells through the cell cycle, except for novobiocin at 0.5 mM which was highly cytotoxic and therefore affected the cell cycle traverse. This may be related to the radiosensitizing effects of novobiocin; a more detailed investigation is required with respect to cell cycle progression. At present there is no definite answer as to how low concentrations of these chemicals enhance the radiosensitivity. The accumulation of lactate and

pyruvate causes activation of lysosomal enzymes[2], and novobiocin and nalidixic acid also have various other effects[20]. Therefore the effects of these chemicals on the sensitivity of human fibroblasts to ionizing radiation can not be simply explained. What is apparent is that the cellular ATP content appears to have a role in the radiosensitivity of mammalian cells.

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