Enhanced Cytotoxicity in Simultaneous and Sequential Drug–Heat Treatments of Cultured Chinese Hamster V79 Cells

Takayoshi SUZUKI, Kohfuku KOHDA, and Yutaka KAWAZOE*

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabedori, Mizuho-ku, Nagoya 467, Japan. *Received May 6, 1989*

Under hyperthermal conditions, some genotoxic drugs such as bleomycin, paraquat, and *N*-alkyl-*N*-nitrosoureas exhibit increased cytotoxicity toward cultured Chinese hamster V79 cells. Sequential combinations of heat and drug treatments, regardless of whether drug-exposure precedes or follows hyperthermia, also induce synergistic cytotoxicity to some extent. This may be attributed not only to the relationship of temperature and chemical injury as defined by the Arrhenius law, but also to a lethal interaction between the biological consequences of chemical injury and thermal damage. Ethanol, dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), and urea, which are known to affect cell membrane and protein, also exert synergistic cytotoxicity at 43 °C at a dose range that is nontoxic at 37 °C. When used sequentially with thermal treatment, they also proved to be synergistic. Glycerol, however, protected cells against thermal damage when used in a simultaneous chemical-thermal combination. But when treatments were carried out sequentially, glycerol proved destructive.

Keywords hyperthermia; bleomycin; ACNU; paraquat; Chinese hamster V79

It has been reported that hyperthermia sometimes enhances the cytotoxicity of anticancer agents.[1-5] With regard to the mechanism operating in thermochemotherapy, Lin *et al.* studied the enhanced cytotoxicity of bleomycin (BLM) under hyperthermal conditions and proposed a mechanism that involves the inhibitory effects of heat on enzymic BLM degradation and also on protein synthesis related to the repair of BLM-induced damage.[3] Wallner and Li reported on the effect of sequencing on the hyperthermal potentiation of mitomycin C and cisplatin and suggested the optimal timing of a sequential combination of chemotherapy with hyperthermia.[4] Drug–heat synergism has also been discussed in connection with membrane fluidity and protein denaturation. Ethanol,[6,8] glycerol,[9-11] and dimethyl sulfoxide (DMSO)[12,13] are representative chemicals often used for these studies. In spite of a number of papers previously published on this topic, more fundamental studies on drug cytotoxicity under hyperthermal conditions are required. With this in mind, the present paper describes the synergistic effect of drugs under hyperthermal conditions in cultured Chinese hamster V79 cells. The chemicals examined include the genotoxic drugs, BLM, paraquat (PQ), *N*-methyl-*N*-nitrosourea (MNU), and *N*-(2-chloroethyl)-*N'*-(4-amino-2-methylpyrimidin-5-yl)methyl-*N*-nitrosourea (ACNU). Several chemicals thought to affect membrane fluidity or protein denaturation under hyperthermal conditions are also included, such as ethanol, DMSO,[13] glycerol, ethylenediaminetetraacetic acid (EDTA), and urea. The present study reveals that all the chemicals examined, except for glycerol, exhibit synergistic cytotoxicity to a greater or lesser extent in both simultaneous and sequential combinations with hyperthermia. Glycerol protects the cells against thermal damage in simultaneous treatments, while it aggravates thermal damage in sequential treatment, regardless of whether thermal treatment preceded or followed glycerol exposure.

Materials and Methods

BLM (bleomycin A_2) was purchased from Nippon Kayaku Co., Ltd. (Tokyo). MNU, PQ, ethanol, DMSO, EDTA, and urea were obtained from Wako Pure Chemical Industry, Ltd. (Tokyo). ACNU was donated by Sankyo Co., Ltd. (Tokyo).

Cytotoxicity assays were carried out as described previously.[2] V79 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum at 37 °C in a humidified incubator under an atmosphere of 5% CO_2 in air. In the early log-phase, the cells were treated with EDTA–trypsin solution and collected by centrifugation. The prepared cells were suspended in fresh medium at a concentration of 5×10^6 cells/ml. The cell suspension (0.2 ml) was added to 0.8 ml of Dulbecco's phosphate-buffered saline (PBS) (for the control experiment and thermal treatment without drugs) or to the same medium containing an appropriate concentration of drugs (for the drug treatment). They were then incubated at 37 or 43 °C for 1 h. After this procedure was completed, the cells were collected by centrifugation and subjected to the second treatment. When the initial treatment was chemical exposure, the centrifuged cells were washed once with 3 ml of PBS before the thermal treatment. The second treatment, chemical or hyperthermal, was performed in the same manner as the first treatment. The cells were then resuspended in 1 ml of fresh medium and, after appropriate diluting, were seeded in a 60-mm culture dish. They were then incubated at 37 °C for 7 d and the colonies were counted. In the simultaneous treatment, the cells were incubated at 37 °C for 1 h in order to expose them to the same conditions as cells in the sequential treatment.

The surviving fraction of the chemical-treated cells was normalized with respect to that of the untreated cells at the corresponding temperature.

Results

Genotoxic Drugs Each genotoxic drug examined proved to exert a synergistic cytotoxicity when simultaneously combined with hyperthermia, as already documented for some chemotherapeutic agents.[1-5] In a sequential combination of a drug with hyperthermia, regardless of whether drug exposure preceded or followed hyperthermia, a synergistic effect was also observed, but to a lesser extent than that observed in the simultaneous exposure. No significant difference was found when drug exposures preceded and followed hyperthermia with any of the genotoxic drugs examined in this study.

Other Chemicals As shown in Fig. 2A, ethanol was severely cytotoxic at 43 °C even at a dose range of less than 1%, although it did not exert any cytotoxic effect at 37 °C at doses as high as 5%. Sequential exposure induced some synergistic cytotoxicity which, to some extent, reflected the order of treatment.

DMSO was also severely cytotoxic at 43 °C at doses of more than 2%, although it produced almost no effect at

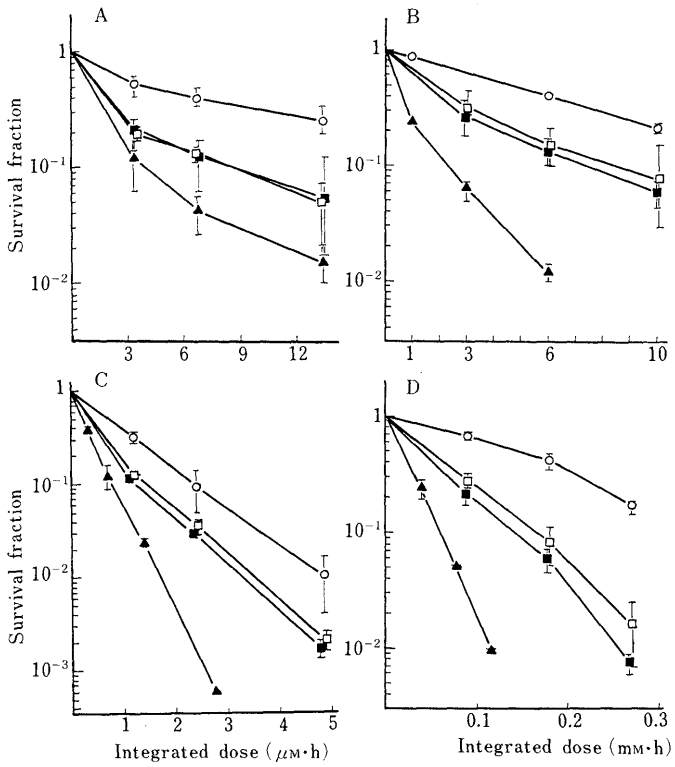

Fig. 1. Survival Fractions *versus* the Integrated Doses after Treatment of Cultured Chinese Hamster V79 Cells with Some Genotoxic Substances

A: bleomycin. B: paraquat. C: ACNU. D: MNU. Surviving fraction of the chemical-treated cells was normalized with respect to that of the untreated cells at the corresponding temperatures. ○, 1 h at 37 °C, followed by 1 h with chemical at 37 °C; ▲, 1 h at 37 °C, followed by 1 h with chemical at 43 °C; □, 1 h with chemical at 37 °C, followed by 1 h at 43 °C; ■, 1 h at 43 °C, followed by 1 h with chemical at 37 °C.

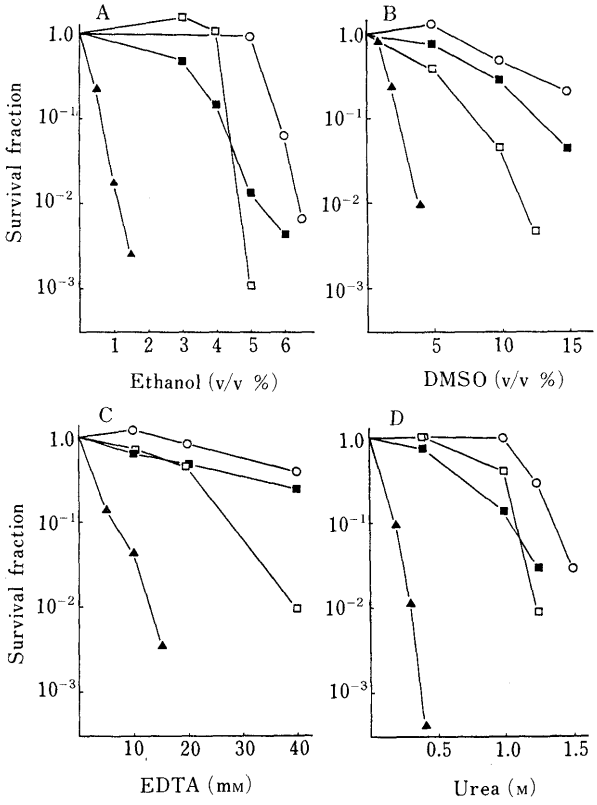

Fig. 2. Survival Fractions *versus* Chemical Concentrations

See the legend to Fig. 1 for details.

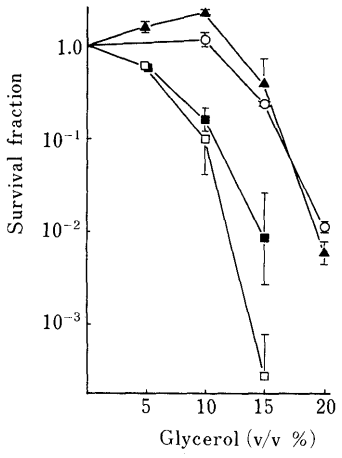

Fig. 3. Survival Fractions *versus* Glycerol Concentrations

See the legend to Fig. 1 for details.

37 °C even at doses of more than 10%,[13] as shown in Fig. 2B. Sequential exposure to DMSO preceding hyperthermia induced a synergistic response almost half that observed with the simultaneous treatment, whereas drug exposure following hyperthermia resulted in even less synergy.

EDTA, which is sometimes added to cultured cell systems, did not affect cell viability at 37 °C even at a 40 mM dose, but at 43 °C, severe cytotoxicity was observed at doses as low as 5 mM, as shown in Fig. 2C. No synergistic effect was induced when chemical exposure followed hyperthermia. But when exposure preceded hyperthermia, there was a synergistic response at a rather high dose of 40 mM.

Urea was also cytotoxic at 43 °C, as shown in Fig. 2D, although, at 37 °C, it did not affect cell viability at less than 1 M dosage. Sequential exposure to urea, regardless of whether it preceded or followed hyperthermia, induced a synergistic cytotoxicity to a slight degree.

Glycerol is known to protect cells against thermal death.[9,10] In fact, as we previously reported,[14] when cells were heated at 43 °C for 1 h in the absence of glycerol, the survival fraction was reduced to 0.084, whereas in the presence of 10% glycerol, this fraction was as high as 0.43.[14] The present study reproduced this heat-protective behavior of glycerol when used simultaneously with heat. However, sequential exposures to glycerol and hyperthermia gave rise to marked decreases in the number of cell survivors, regardless of the order of sequencing, as shown in Fig. 3. The destructive effect brought about by sequential treatment with glycerol and hyperthermia is noteworthy, although the mechanism involved is not yet clear.

Discussion

All the chemicals examined in this study exerted synergistic cytotoxicity when sequentially combined with hyperthermia. The degree of enhancement when drug exposure preceded or followed hyperthermia was somewhat less than that induced by simultaneous exposure. It is thought, therefore, that the synergistic effect of drug exposure and hyperthermia is attributable not only to the relationship of temperature and chemical injury as defined by the Arrhenius law, but also to a lethal interaction between the biological consequences of chemical injury and thermal damage. We previously reported[2] that the biphasic nature

of the Arrhenius relationship found for BLM and PQ might be related to an interaction between the biological consequences of the two types of cell injuries. However, the present study demonstrates that hyperthermal synergy found for NMU and ACNU, which gave monophasic, *i.e.* linear, Arrhenius plots, is also attributable, in part, to an interaction of the biological consequences.

It is worth emphasizing that a variety of chemicals which are expected to affect cell membranes and proteins exerted synergistic cytotoxicities similar to those shown by the genotoxic drugs.[14] With ethanol, the shoulder region of the survival curve at 37 °C disappeared under hyperthermal conditions. No increase was observed in the slope of the linear portion of the dose–response plot. Apart from mechanistic considerations, since ethanol, DMSO, and EDTA are often added to cultured cell assay systems, attention should be paid to the fact that an extraordinary increase in cytotoxicity might be induced with these common additives under hyperthermal conditions.

Lin *et al.* reported[3] that hyperthermia preceding BLM exposure induced a greater cytotoxicity than hyperthermia following BLM exposure. In the present study, we were unable to demonstrate any appreciable difference in cytotoxicity between BLM exposures preceding and following hyperthermia.

In conclusion, it appears that enhanced cytotoxicity induced by drugs, simultaneously or sequentially combined with hyperthermia, might be due to certain thermal effects which aggravate a wide variety of cell injuries including deoxyribonucleic acid (DNA) lesions and cell membrane disorders.

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