Environmental Mutagens & Carcinogens 15-2 : 88-93 (1995)

Sensitization Effects of Hyperthermia on Bleomycin-induced DNA Strand Breaks and Replication Inhibition in CHO-K_1 Cells *in Vitro*

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(Received August 10, 1995)

ABSTRACT: **Effects of hyperthermia on the induction of DNA single strand breaks and replication inhibition were studied in bleomycin-treated CHO-K_1 cells by alkaline elution and alkaline sucrose gradient sedimentation. Bleomycin-induced DNA single strand breaks of DNA were dose-and time-dependently increased, and these strand breaks of DNA were gradually rejoined as post-incubation time passed. Treatment with hyperthermia alone did not affect the induction of DNA single strand breaks. However, pre-exposure of cells to hyperthermia followed by bleomycin treatment greatly increased the single strand breaks, and also reduced the rejoining processes of bleomycin-induced DNA single strand breaks. Bleomycin selectively inhibited the replicon initiation. The combined treatment with hyperthermia and bleomycin markedly potentiated the nonspecific inhibition of replication.**

INTRODUCTION

Hyperthermia have been demonstrated to have a therapeutic potential in the treatment of human malignancy, especially when used in combination with radiotherapy or chemotherapeutic agents (Hahn *et al.*, 1975; Iliakis and Seanter, 1990; Auger *et al.*, 1993). The most promising agents for thermochemotherapy appear to be nitrosoureas, cis-platinum, adriamycin and bleomycin (Marmor *et al.*, 1979; Naito *et al.*, 1989). Bleomycin is an antineoplastic glycoprotein that causes cell death by producing single-and double-stranded DNA breaks (Mirabelli *et al.*, 1982; Auger *et al.*, 1993). Braun and Hahn (1975) reported that simultaneous administration of bleomycin and hyperthermia both enhanced cell killing and inhibited recovery from potentially lethal damage *in vitro*. This enhanced cell killing was subsequently observed *in vivo* with primary EMT6 tumors in BALB/C mice (Marmor *et al.*, 1979).

The mechanism for thermal potentiation of bleomycin is not well understood. However, it may involve inhibition of repair of DNA lesions (Auger *et al.*, 1993). Elevated temperature also appears to inhibit DNA synthesis (Wong and Dewey, 1982). Direct molecular studies in the hyperthermic inhibition of DNA synthesis have shown both a reduction of the initiation of replicons and reduction in the chain elongation of DNA (Waters and Stone, 1984) possibly due to inhibition of phosphorylation (Naito *et al.*, 1989). The inhibition of DNA replication is regarded as a result of DNA damage, and causes changes in both molecular weight of newly synthesized DNA and the rate of DNA synthesis (Park and Cleaver, 1979). Replication inhibition and its recovery processes now appear to be controlled by a variety of regulatory factors such as the excision repair ability, the replicon size, the chain growth rate and the cluster size at both DNA and chromatin levels (Park *et al.*, 1985; Lee *et al.*, 1991).

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The present studies, therefore, have undertaken to investigate the potentiation effects of hyperthermia on bleomycin-induced DNA strand breaks, inhibition of DNA synthesis and their recovery processes in CHO cells.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO-K_1) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator.

Treatment of hyperthermia and bleomycin

Exponentially growing cells were exposed to bleomycin (BLM; Umezawa *et at.*, 1966) in a serum-free medium immediately before or after exposure to hyperthermia in a temperature-controlled water bath at 43.5°C. The cells were washed three times with a serum-free medium, and processed for alkaline elution and alkaline sucrose sedimentation analyses either directly or after postincubation in the medium containing 10 μM cytosine arabinoside (araC, Sigma) and 3 mM hydroxyurea (HU, Sigma).

Alkaline elution experiment

DNA single strand breaks were measured by alkaline elution technique as described by Kohn *et al.* (1974), with minor modifications. Cells were labeled with 0.2 μCi/ml of [^{3}H]-thymidine (specific activity: 5-6 mCi/mmol) for three days. The cells were then harvested in PBS-Merchant solution (150 mM NaCl, 4.28 mM K_2HPO_4, 0.71 mM KH_2PO_4), filtered through polycarbonate filter with 2 μm pore size (Nucleopore Corp.), lysed in lysis solution (2% sodium dodecylsulfate, 25 mM Na_2EDTA, pH 9.7), followed by digestion with proteinase K (0.5 mg per ml of lysis solution). The lysates were then eluted (flow rate: 0.05 ml/min) with a solution consisting of 20 mM Na_2EDTA plus tetrapropylammonium hydroxide (pH 12.1-12.2). Fractions of radioactivity remaining on the filter were plotted semilogarithmically against elution time.

Alkaline sucrose gradient sedimentation analysis

Inhibiting DNA replication was measured by alkaline sucrose gradient sedimentation analysis as described by Cleaver (1974). Exponentially growing CHO-K_1 cells were prelabeled with 0.01 μCi/ml [^{14}C]-thymidine (spec. act., 64 mCi/mmol) for 48 hr, treated with bleomycin before or after treatment with hyperthermia, washed three times with the serum-free medium, and subsequently pulse-labeled with 10 μCi/ml [^{3}H]-thymidine (spec. act., 60 Ci/mmol) for 10 min. The cells were then harvested and applied directly to a layer of lysing solution (0.5 N NaOH, 0.02 M Na_2EDTA) placed on top of a preformed 5~20% alkaline sucrose gradients (Park and Cleaver, 1979; Cleaver *et al.*, 1983). Cells were lysed for 4 hr to facilitate DNA strand unwinding, after which the DNA was sedimentated in SW28 rotor (Beckman) at 25,000 rpm for 4 hr. The molecular weights of DNA contained in each fraction were calculated according to Studier's equation : $Si=0.0523\ Mi^{0.4}$, where Mi represents the molecular weight in the i-th fraction (Studier, 1965), with [^{14}C]-labeled phage DNA ($Mw=1.65\times10^7$ dalton) used as a standard. The [^{3}H]/[^{14}C] ratio calculated from the sums of total [^{3}H] and [^{14}C] in the gradients were used to estimate the total inhibition of DNA synthesis.

RESULTS

1. DNA Single Strand Breaks

These experiments were concerned with the induction of single strand breaks of DNA and their rejoining in conjunction with hyperthermia and bleomycin. The quantitation of strand breaks was based on the relative percentage of [^{3}H]-thymidine incorporated DNA remaining on filters. Since strand breaks cause the reduction of molecular weight of DNA, they increase the rate at which DNA passes through the filters (Kohn *et al.*, 1974).

Fig. 1A represents DNA strand breaks induced by various concentrations of bleomycin. In control, the percentage of DNA remaining on filters decreased to 70% for 10 hours. In experimental groups that received 50~200 g/ml of bleomycin for 30 minutes, the elution rates were increased in dose-dependent manner and the percentages of DNA remaining on the filters appeared about 35~55%. These results indicated that the dose response of DNA strand breaks induced by bleomycin did oc-

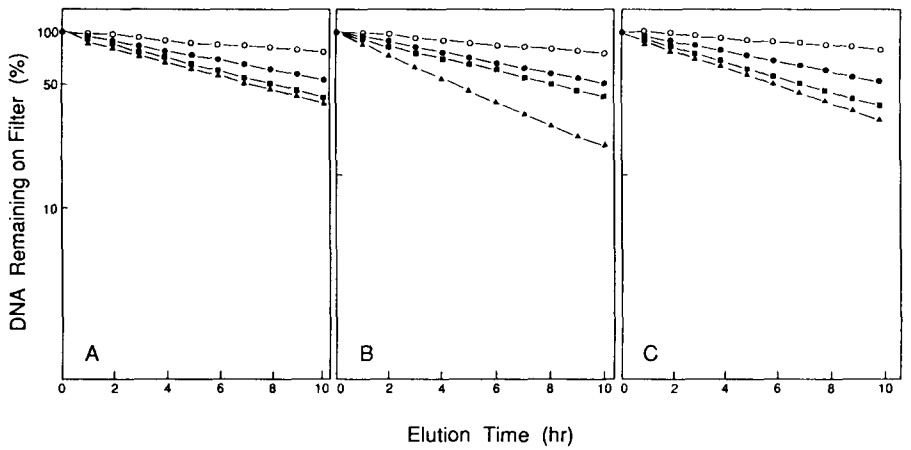

Fig. 1. Bleomycin-induced DNA single strand breaks as determined by alkaline elution. A. Dose-resonse of bleomycin (○, control; ●, 50 μg/ml; ■, 100 μg/ml; ▲, 200 μg/ml). B. Time-dependence of bleomycin (○, control; ●, 200 μg/ml for 0.5hr; ■, 200 μg/ml for 1hr; ▲, 200 μg/ml for 2hr). C. Effects of post-incubation times after bleomycin (○, control; ▲, 200 μg/ml, 0.5hr chased for 0.5hr; ■, 200 μg/ml, 0.5hr chased for 1hr; ●, 200 μg/ml, 0.5hr chased for 2hr)

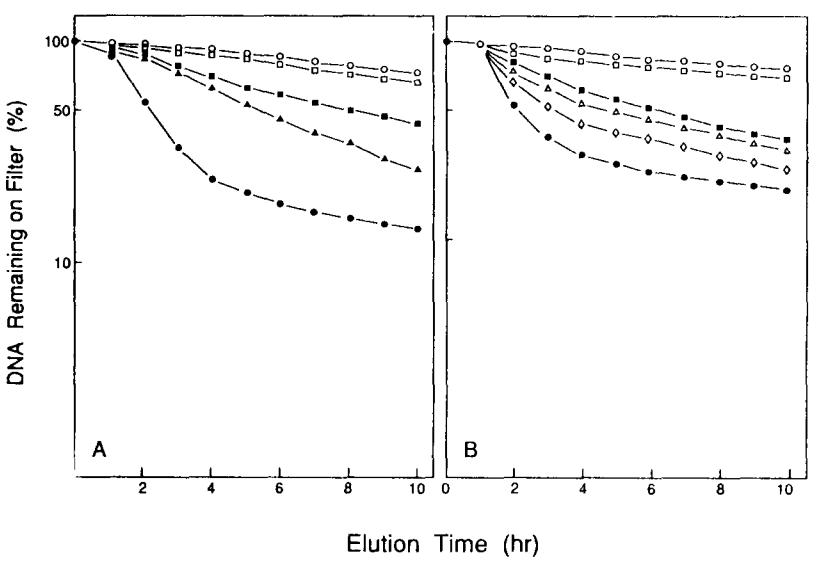

Fig. 2. Effects of hyperthermia on bleomycin-induced DNA single strand breaks. A. Effects of single or combined treatment (○, control; □, hyperthermia, 43.5°C for 10min; ■, bleomycin 200 μg/ml for 1hr; ▲, bleomycin 200 μg/ml for 1hr followed by hyperthermia, 43.5°C for 30 min; ●, hyperthermia 43.5°C for 30 min followed by bleomycin 200 μg/ml for 1hr). B. Effects of post-incubation times after single or combined treatment (○, control; ■, bleomycin 100 μg/ml for 1hr; □, bleomycin 100 μg/ml for 1 hr chased for 2hr; ●, hyperthermia, 43.5°C for 30 min followed by bleomycin, 100 μg/ml for 1hr; ◇, hyperthermia, 43.5°C for 30 min followed by bleomycin, 100 μg/ml for 1hr, chased for 2 hr; △, hyperthermia, 43.5°C for 30 min followed by bleomycin, 100 μg/ml for 1 hr, chased for 4 hr)

cur as dose increased. The amount of DNA remaining on filters in bleomycin treated groups also decreased in direct proportion to the duration of treatment increased (Fig. 1B).

Effects of post-incubating time on the rejoining process of strand breaks induced by 200 μg/ml of bleomycin for 30 minutes showed that single strand breaks induced by bleomycin were gradually rejoined as time passed and reached to about 50% of control at 2 hours of incubation (Fig. 1C). The

Table 1. Effects of hyperthermic treatment combined with bleomycin on DNA strand breaks.

Treatment	Differential Elution Rates[b] (x10²hr)		Enhancement Rates (%)[c]
DNA-damaging agents[a]	Heat		
	none	43.5°C	
none			
Heat		0.303	1.99
BLM	2.334		
Heat+BLM		7.452	182.6
BLM+Heat		4.442	68.46

[a]Heat was treated for 10 min at 43.5°C and other agents were described in Materials and Methods.

[b]Differential elution rates (Δk) show the pure effect of agents: Δk=log[Mv(control)/Mv(agent)]/T, where Mv is the value mean value of fraction of DNA remaining on filter at the end of elution time, T.

[c]The effect of hyperthermia on the DNA strand breaks was expressed as enhancement ratio (E): E=Δk(agent+heat)/[Δk (agent)+Δk(heat)]-1.

amounts of DNA remaining on filters were increased in proportion to incubation time due to rejoining of DNA strand breaks induced by bleomycin.

Fig. 2A shows the effects of the combined treatment with hyperthermia and bleomycin on DNA strand breaks. Hyperthermia treatment at 43.5°C for 10 minutes showed essentially no significant difference in the elution rate as compared to control. However, there was a marked increase in the elution rate of ^{3}H-labeled DNA when cells were exposed to bleomycin followed by hyperthermia. This potentiation effects of hyperthermia on bleomycin-induced DNA strand breaks were the greatest in the pretreatment of hyperthermia. The enhancement ratio, defined as the potentiation effect of hyperthermia on the formation of DNA strand breaks was up to 182% for the pretreatment of hyperthermia and 18.5% for the post-treatment (Table 1). These results indicated that the sequence in which hyperthermia and bleomycin are exposed is important.

Effects of post-incubation time on DNA strand breaks induced by the combined treatment with hyperthermia and bleomycin showed that the fraction of DNA remaining on filters was increased with incubation times (Fig. 2B), that DNA strand breaks induced by hyperthermia and bleomycin were slowly recovered with time due to the rejoining of single strand breaks.

2. Replication Inhibition

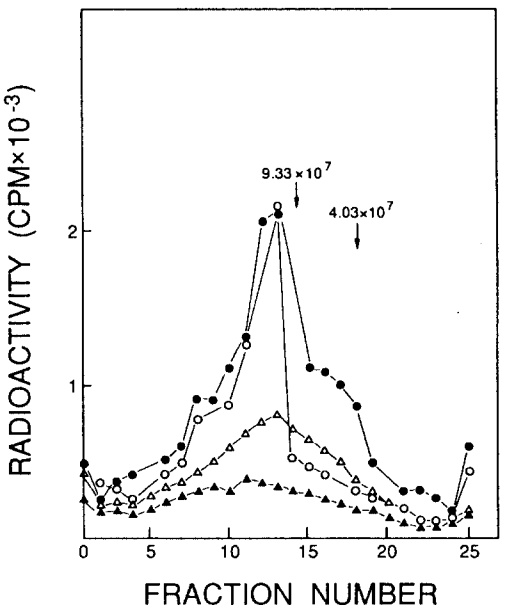

Fig. 3. Alkaline sucrose gradient profiles showing the effect of hyperthermia on the accumulation of DNA single strand breaks induced by bleomycin (●, control; ○, bleomycin, 200 μg/ml for 0.5hr; △, hyperthermia 43.5°C for 5min; ▲, hyperthermia, 43.5°C for 5min followed by bleomycin 200 μg/ml for 0.5hr)

The replicon size of CHO-K$_1$ cells is determined to be 42 μm (Kapp *et al.*, 1979) and the molecular weight of the half replicon size is 4.03×10^7 dalton, which corresponds to the 15th fraction from alkaline sucrose gradient employed in the present study (Cleaver, 1974). Painter (1977) differentiated the two aspects of DNA replication inhibition induced by DNA damaging agents as follows; inhibition of replicon initiation is defined as the reduction of the nascent DNA molecules below half replicon size whereas inhibition of chain elongation is defined as the interruption of synthesis of DNA molecules having the size above half replicon.

Fig. 3 shows the distribution patterns of nascent DNA molecules of CHO-K$_1$ cells which were prelabeled with [^{14}C]-thymidine, exposed to hyperthermia followed by bleomycin, chased, and subsequently pulse-labeled with [^{3}H]-thymidine for 10 min. The replicon initiation was selectively inhibited by the treatment with bleomycin, whereas the replication was markedly interrupted by the combined treatment with hyperthermia and bleomycin. These results suggest that hyperthermia do not seem to potentiate the bleomycin-induced inhibition of replicon initiation. Rather they sensitize the nonspecific inhibition of replication.

DISCUSSION

The present investigation demonstrated that pre-exposure of cells to hyperthermia enhanced the bleomycin-induced DNA single strand breaks and reduced the rejoining processes of strand breaks caused by bleomycin in cultured mammalian cells. This study also showed that the combined treatment with hyperthermia and bleomycin potentiated the non-specific inhibition of replication.

Hyperthermia-induced radio-or chemosensitization has been correlated with repair inhibition of induced DNA damage (Urlino and Kohn, 1989, Auger *et al.*, 1993). Furthermore, it has been suggested that combined applicaiton of hyperthermia with radiation may enhance conversion of repairable into irreparable lesions, as well as enhance the probability of misrepair of a subset of radiation-induced lesions (Iliakis and Seaner, 1990). These processes may, at least partly, be related to the inactivation induced by heat in DNA-dependent DNA polymerases (Auger *et al.*, 1993).

We have also demonstrated that the sequence in which hyperthermia and bleomycin are administered is important; treatment with hyperthermia followed by bleomycin resulted in the greatest enhancement in the induced DNA strand breaks and replication inhibition, suggesting that the cytotoxic effects of the two agents act synergistically. These results suggest that bleomycin is an outstanding candidate to be combined with hyperthermia in future clinical trials.

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CHO-K$_1$ 세포에 있어 Bleomycin에 의한 DNA 단사절단과 복제 억제에 미치는 열처리의 영향

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적 요

Bleomycin 처리한 **CHO-K$_1$** 세포에서 **DNA** 단사절단과 복제억제에 미치는 고온 처리의 영향을 알카리유출법 및 알카리농도구배 초원심분리법으로 연구하였다. **Bleomycin**에 의한 **DNA** 단사절단은 처리농도와 처리시간에 따라 증가하며, 이 **DNA** 단사절단은 처리후 배양시간에 따라 점차 재결합한다. 고온 단독처리는 **DNA** 단사 유발에 아무런 영향을 미치지 않는다. 그러나, **Bleomycin** 처리에 앞서 고온을 처리 할 경우 **DNA** 단사절단은 대단히 증가하며 이 절단의 재결합을 현저히 지연시킨다. 또한 **Bleomycin**은 **DNA** 복제단위의 복제개시를 선택적으로 억제하나, 고온과 **Bleomycin**을 복합 처리할 경우 불특정 복제억제를 증가시킨다.