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Hyperthermia-induced Apoptosis is Independent upon DNA Strand Breaks in Human Lymphoid Cells

Hwa Jin Jung, Won Hye Ka, Jee Na Hwang, and Young Rok Seo

Department of Pharmacology, Medical Research Center (MRC), Kyung Hee University School of Medicine, Seoul 130-701, South Korea

Heat shock (43°C for 60 minutes) is sufficient to induce apoptosis in a wide number of cell lines. In this study, we asked whether DNA strand breaks are responsible for this phenomenon. Using the highly sensitive comet assay for DNA damage detection, we were unable to demonstrate DNA breaks immediately after heat shock in Raji human lymphoid cells. It showed that DNA breaks were not necessary for hyperthermic apoptosis, since its activity is indicative of DNA lesions. Here, we present a suggestion that a protein(s) is the major target for heat shock apoptosis. We firstly found glycerol, which reportedly stabilizes protein structure, showed a protective effect in Raji cells against hyperthermic apoptosis. In addition, quercetin, which modulates transcription of the heat shock protein family members, enhanced apoptotic death induced by hyperthermia. Furthermore, Raji cells are protected by a pre-mild heat treatment prior to the killing dose of heat shock.

Key Words: Apoptosis, Hyperthermia, DNA damage, Heat shock protein, Comet assay

INTRODUCTION

Apoptosis, a physiological type of cell death, plays an important role of selective deletion of cells in divergent situation of various tissues (Levine et al, 1991; White, 1995). Especially, there has been an increasing interest in the roles of apoptosis in tumor cell for therapy (Olive et al, 1993). A more complete understanding of mechanisms of apoptosis would be useful, since new strategy based on the possibility of reducing tumor growth rate by apoptosis could provide alternative ways of dealing with human malignant disease.

Apoptosis can be initiated by a wide of environmental stimuli and involve an active cellular process which requires active RNA and protein synthesis (Wyllie, 1985). The events that are able to induce apoptosis are incredibly diverse but are generally classified into one of three categories: (1) induction by direct DNA damage e.g. strand breaks, chromosomal aberrations, etc., (2) induction by transduced signals e.g. FAS/APO-1 transmembrane signals, and (3) stress mediated apoptosis. It is the third class of inducers that has been most difficult to understand and delineate.

Hyperthermia, a typical environmental stress, has long been known as toxic to cells. It has been recognized the mode of cell killing to be influenced by severity of the heat treatment (Harmon et al, 1990). A number of reports have been published to demonstrate the induction of apoptosis by mild hyperthermia (Deng & Podack, 1993; Mosser & Martin, 1992; Sellins & Cohen, 1991). The onset of programmed cell death is a major response to hyperthermia treatment in many tumors, though the extent and kinetics of apoptosis induced by hyperthermia in tumor cells is highly variable (Armour et al, 1993; Takano et al, 1991). However, the mechanism of heat-induced apoptosis has not been fully understood. Some of the possibilities are that thermal injury may initiate a death signal, target certain heat labile proteins, or cause direct or indirect DNA damage leading to apoptosis.

In the present study, we undertook to determine the possibility that hyperthermia induces DNA strand breaks in apoptosis-sensitive cells from human using highly sensitive comet assay, and sought to ask another suggestion that proteins (heat shock proteins) are the major target for hyperthermic apoptosis.

Corresponding to: Young Rok Seo, Department of Pharmacology, Medical Research Center (MRC), Kyung Hee University School of Medicine, Seoul 130-701, Korea. (Tel) 82-2-961-0674, (Fax) 82-2-963-0674, (E-mail) dream21@khu.ac.kr

METHODS

Tissue culture

Raji cell lines (ATCC, USA) were maintained at exponential phase growth in RPMI 1640 plus 10% FBS (Sigma, USA). Cell viability was routinely monitored using trypan blue exclusion. Cells were exposed to various treatment regimens at a density of 10^5 cells/mL.

Cell treatment

Heat shock apoptosis was induced using a Fisher Immersion Circulator at 43°C as described elsewhere (Fairbairn & O'Neill, 1996). Glycerol was prepared at 0.67 M in complete RPMI 1640. Quercetin (Sigma, USA) was

ABBREVIATIONS: HPS, heat shock protein.

prepared at a stock concentration of 10 mM. Control experiments with carrier alone were conducted to confirm background toxicity reduction; ethanol concentrations were kept below 0.5%.

Comet assay

The alkaline comet assay was performed essentially as described by Olive and coworkers (Olive et al, 1993). Cells in PBS were suspended in a low melt agarose gel on a frosted microscope slide. The cells were lysed (0.03 M NaOH, 1.0 M NaCl, 0.1% SLS) and the DNA was electrophoresed in alkaline solutions (0.03 M NaOH plus 2 mM EDTA) at 0.7 V/cm for 20 minutes, neutralized, and stained with propidium iodide for DNA visualization. Comet images were captured and detected by an attached CCD camera with a fluorescence microscope (Leica, Switzerland). Tail moment, a relative measure of the mean fragment length, was determined by NIH image analysis software (NIH, USA).

Measurement of apoptosis induction

Morphological features of apoptotic phase were assessed with acridine orange/ethidium bromide staining. Condensed chromatin or fragmented (dots) nucleus shows clearly in green as an indicator in apoptotic cells. According to these criteria, the evaluation of apoptotic phase was performed with a fluorescence microscope (Leica, Switzerland). After washing once with PBS, the treated cells were stained with 4 g/ml acridine orange and 4 g/ml ethidium bromide dissolved in PBS. Subsequently, apoptotic bodies were visually scored, and the fraction was determined as the percentage of cells.

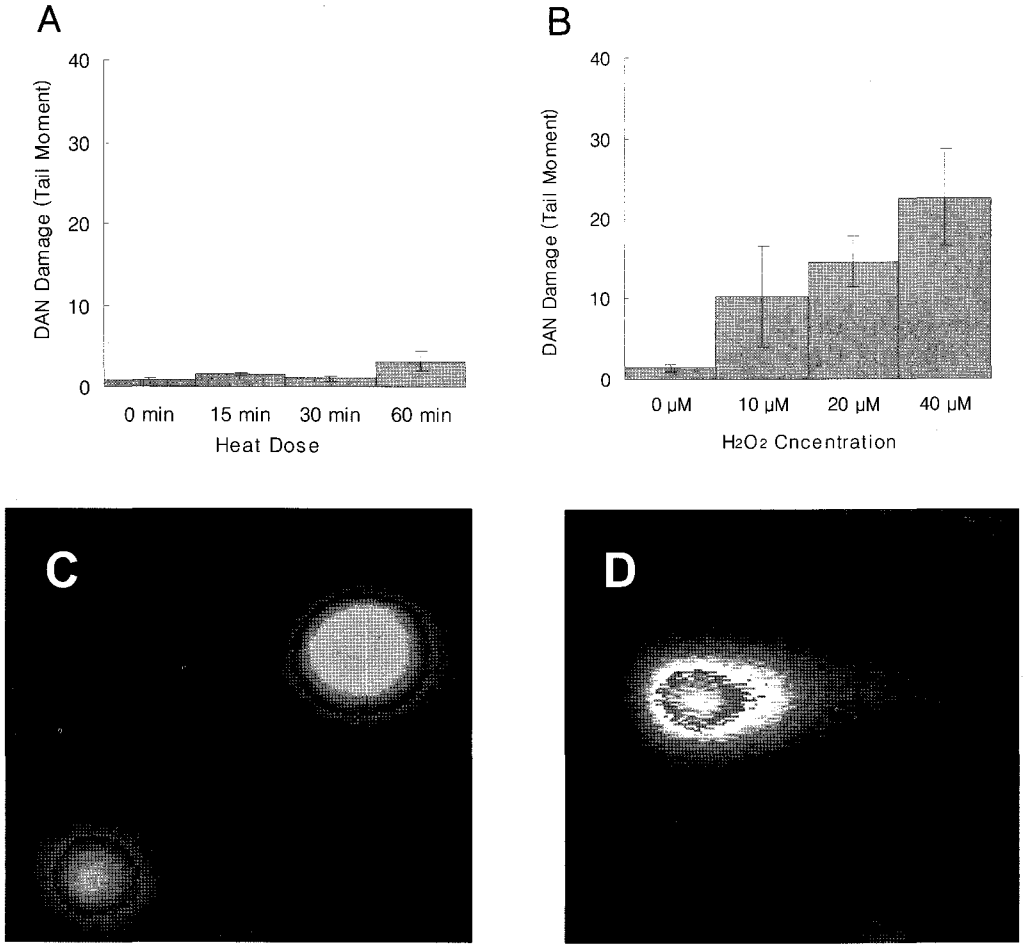

Fig. 1. Measurement of DNA damage (measured in tail moment) induced by heat shock and hydrogen peroxide in Raji human lymphoid cells. For heat treatment, Raji cells were incubated at 43°C for 60 minutes 24 hours before the analysis of apoptotic induction. For treatment with hydrogen peroxide, Raji cells were incubated with 50 μM hydrogen peroxide in PBS for 20 minutes on the ice immediately before execution of apoptotic analysis. (A) DNA strand breakage was not detected in heat-treated cells. (B) Hydrogen peroxide contributed to DNA strand breaks in dose dependent fashion. (C) Representative comet picture of heat-treated cells, indicating hyperthermic stress-induced response is independent of DNA strand breaks. (D) Representative comet picture of hydrogen peroxide-treated cells, showing significant amount of strand breaks.

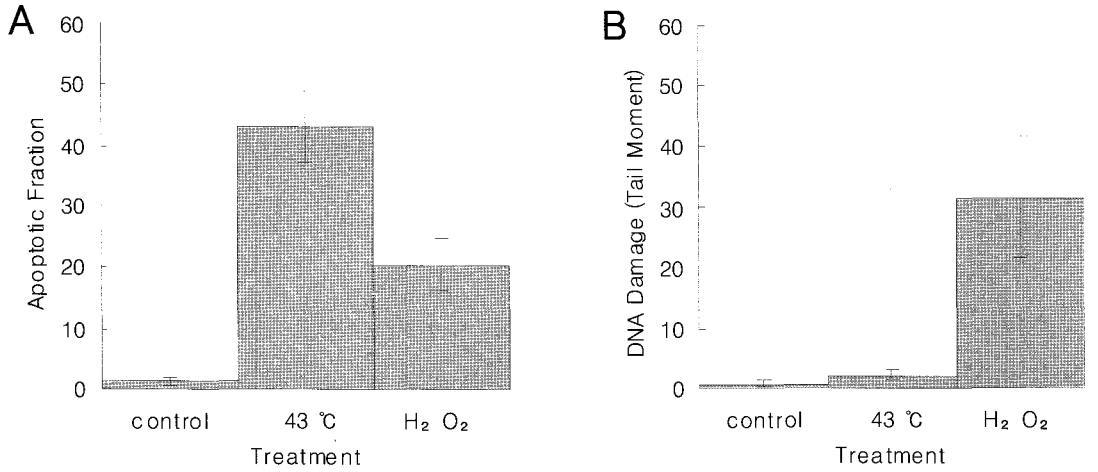

Fig. 2. (A) Direct comparison of the apoptotic induction induced by heat shock (at 43°C for 60 minutes) and hydrogen peroxide (50 μM hydrogen peroxide for 20 minutes). (B) Direct comparison of DNA damage levels (tail moment) induced by heat shock and hydrogen peroxide. Significant apoptotic induction was observed in both heat-treated and hydrogen peroxide-treated cells, whereas DNA damage was detected only in cells exposed to hydrogen peroxide.

Statistical analysis

Data points from all experiments of this paper reflect the mean of three independent experiments; bars, SD. Statistical analysis (t-test) was conducted with SigmaPlot Software (SPSS Inc., USA).

RESULTS AND DISCUSSION

Our experiments were conducted to address the problem of whether DNA is the critical target of heat shock, and whether DNA damage is a necessary event leading to hyperthermic apoptosis. We have approached this question in several ways.

First, we tested whether heat shock induces DNA strand breaks of human lymphoid cells using the sensitive comet assay. Raji cells were exposed to hydrogen peroxide on ice or hyperthermia for the indicated time periods. As seen in Fig. 1A and C, we were unable to observe any evidence of DNA strand breaks in heat treated cells, whereas hydrogen peroxide-treated cells showed a dose-dependent increase in DNA strand breaks (Fig. 1B and D). In addition, the direct comparison of cellular responses to heat shock and hydrogen peroxide has been performed. As seen in Fig. 2, significant apoptotic induction was observed in both heat-treated and hydrogen peroxide-treated cells, whereas DNA damage was detected only in cells exposed to hydrogen peroxide. These data strongly suggest that hyperthermic apoptosis is independent upon DNA strand breaks in human lymphoid cells. This finding would not be surprising if the putative DNA lesions were not strand breaks or alkali-labile sites. However, if unknown non-strand break DNA lesions, insensitive to alkali-induced cleavage, were responsible for hyperthermic apoptosis, then cellular attempts to repair the lesion would be detectable by the comet assay after given incubation periods. This would not be unlike the detection of UV-induced strand breaks during the excision repair process as reported by others (Arlett et al, 1993; Gedik et al, 1992). Indeed, the detectable DNA damage is found only at later time periods. However, this extensive damage is consistent with apoptotic DNA damage, and variable levels of damage are not observed in this or our previous studies. In addition, according to this kind of interpretation, cells refractory to heat shock apoptosis would be predicted to maintain resiliency on the basis of more efficient repair processes. However, the possibility that irreparable non-strand break inducing lesions may be responsible for the cellular response can not be directly discounted by these experiments.

To examine alternative mechanisms for apoptotic induction, we tested for possible protection against apoptosis using glycerol. Raji cells were pre-incubated with 0.67 M glycerol for 30 minutes. They were subjected to heat shock for 60 minutes, and subsequently washed 3 times in PBS before 24 hour incubation with complete RPMI 1640 media and analyzed apoptotic morphology. Glycerol was found to protect Raji cells from hyperthermic apoptosis, resulting in the significant decline of the apoptotic fraction (Fig. 3A). However, we were not able to abrogate the extent of DNA damage in cells exposed to hydrogen peroxide with glycerol treatment as determined by image analysis of DNA comets (Fig. 3B).

Glycerol has been widely shown to prevent hyperthermic toxicity in a large number of varied systems (Dubois et al, 1991; Ho & Lin, 1991; Lin et al, 1984; Pinto et al, 1991). The proposed mechanism of action is the prevention of protein denaturation. We were interested to test the potential protective effect of glycerol against apoptosis, and found that the concurrent treatment with hyperthermia is sufficient to reduce cellular toxicity significantly (Fig. 3). The fact that we observed the reduced apoptosis without demonstrating evidence that glycerol is able to reduce strand break frequencies provides additional evidence against a role for strand breaks in hyperthermia-induced apoptosis.

The flavonoid quercetin has long been recognized to act as a sensitizer toward hyperthermic toxicity (Kim et al,

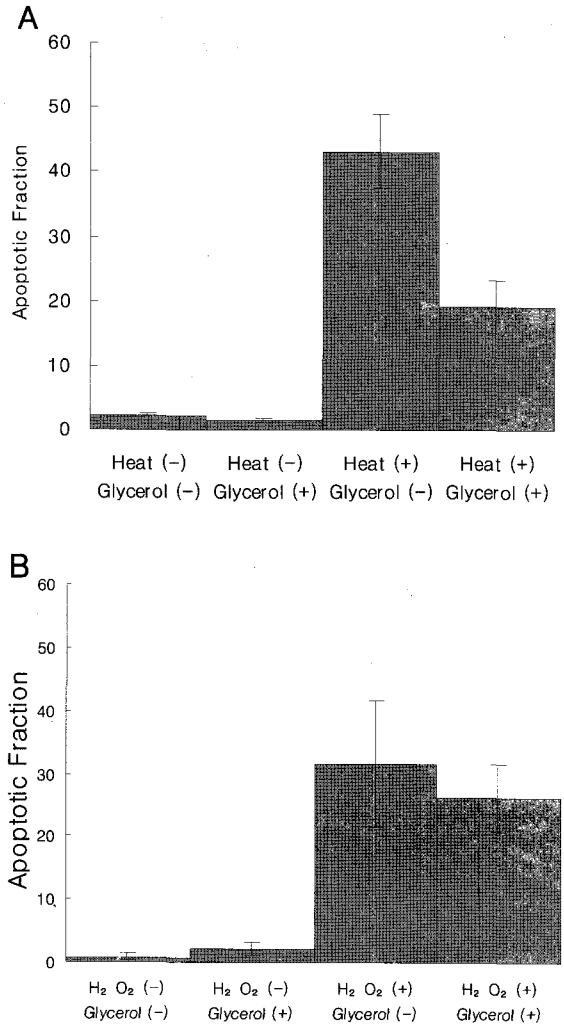

Fig. 3. Glycerol protects from heat shock-induced apoptosis (at 43°C for 60 minutes) (A) ($p<0.05$), but not from hydrogen peroxide-induced apoptosis (50 μM hydrogen peroxide for 20 minutes) (B). Cells were pre-incubated in the presence or absence of 0.67 M glycerol for 30 minutes.

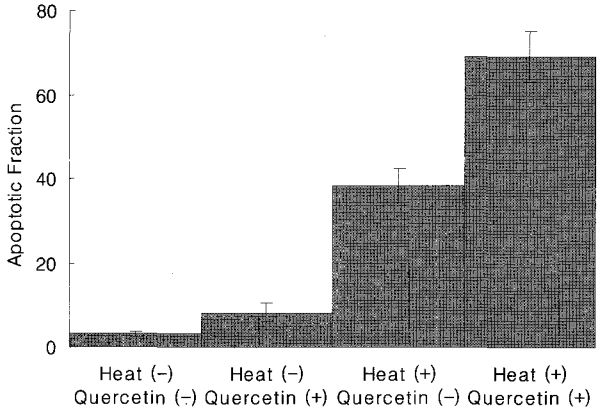

Fig. 4. Quercetin significantly sensitizes heat-treated Raji cells ($p<0.05$). Cells were pre-incubated in the presence or absence of 200 μM quercetin for 4 hours. Analysis of apoptotic induction were performed 24 hours after the heat treatment (at 43°C for 60 minutes).

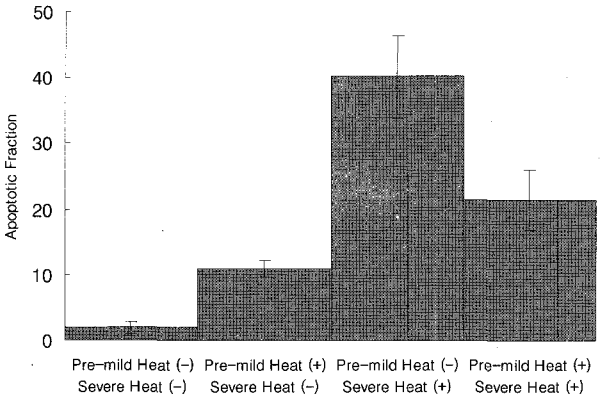

Fig. 5. Thermotolerance protects Raji cells from subsequent hyperthermic apoptosis. Heat treatment ($p<0.05$): Pre-mild heat, 30 minutes at 43°C, given 6 hours prior to the severe heat treatment; severe heat, 60 minutes at 43°C. Analysis of apoptotic induction was performed 24 hours after the severe heat treatment.

1984). A recent report by Wei et al. (Wei et al, 1994) indicated that quercetin-induced apoptosis proceeds by inhibiting synthesis of heat shock protein 70 (HSP70), and that loss of HSP70 itself is sufficient to lead to apoptotic death. Several other investigators have shown a demonstrable relationship between quercetin treatment and prevention of HSP transcription (Elia & Santoro, 1994; Hosokawa et al, 1992; Lee et al, 1994; Nagai et al, 1995). If quercetin acts by blocking heat-shock protein production, concurrent quercetin and 43°C exposure should result in increased death compared to heat-shock alone. As seen in Fig. 4, Raji cells were found to have significant excessive death with increasing concentrations of quercetin. In addition, mild hyperthermic pre-treatment six hours before the apoptosis-inducing treatment, which allows production of protective HSP members, is capable of modulating apoptosis (Fig. 5).

These results together point to the importance of a thermal-labile target, the protection of which is sufficient to cause a significant decline in the fraction of cells entering an apoptotic program. In addition, the prevention of protection, i.e., by quercetin exposure, is sufficient to enhance apoptotic death. Indeed, overexpression of the bcl-2 gene has previously shown to be protective against thermal apoptosis (Cuende et al, 1993; Strasser & Anderson, 1995). It is likely, therefore, that the important cellular target in thermal injury which leads to apoptosis is a protein target, such as Bcl-2, a Bcl-2 family member, or some as yet unknown target in conjunction with a number of other targets. The possibility of a single target necessary and sufficient for apoptosis seems unlikely in this model, and we expect the mechanism to be complex. A more likely scenario, consistent with other published data, is that apoptosis is the result of a combination of the thermal destruction (directly or indirectly) of apoptosis protecting molecules with a concurrent production of killing molecules

which then execute the death sentence.

In summary, we argues against the possibility that DNA strand breaks are the physiologically important consequence of heat shock. As an alternative pathway, we suggest that a protein(s) is the major target for heat shock-induced apoptosis. Raji cells are protected by glycerol or a protective mild heat treatment prior to the killing dose. In addition, quercetin, a transcriptional modulator of the heat shock protein family members, enhances apoptotic death induced by heat shock. Our study might give an insight into understanding a possible mechanism of hyperthermia-induced apoptosis in human lymphoid system, although further studies are needed.

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