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Reduction of TNF α-induced Oxidative DNA Damage Product, 8-Hydroxy-2′-Deoxyguanosine, in L929 Cells Stably Transfected with Small Heat Shock Protein

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Previous studies have demonstrated that oxidative stress involving generation of reactive oxygen species (ROS) is responsible for the cytotoxic action of TNF α. Protective effect of small heat shock proteins (small HSP) against diverse oxidative stress conditions has been suggeted. Although overexpression of small hsp was shown to provide an enhanced survival of TNF α-sensitive cells when challenged with TNF α, neither the nature of TNF α-induced cytotoxicity nor the protective mechanism of small HSP has not been completely understood. In this study, we have attempted to determine whether TNF α induces oxidative DNA damage in TNF α-sensitive L929 cells. We chose to measure the level of 8-hydroxy-2′-deoxyguanosine (8 ohdG), which has been increasingly recognized as one of the most sensitive markers of oxidative DNA damage. Our results clearly demonstrated that the level of 8 ohdG increased in L929 cells in a TNF α dose-dependent manner. Subsequently, we asked whether small HSP has a protective effect on TNF α-induced oxidative DNA damage. To accomplish this goal, we have stably transfected L929 cells with mouse small hsp cDNA (hsp25) since these cells are devoid of endogenous small hsps. We found that TNF α-induced 8 ohdG was decreased in cells overexpressing exogenous small hsp. We also found that the cell killing activity of TNF α was decreased in these cells as measured by clonogenic survival. Taken together, results from the current study show that cytotoxic mechanism of TNF α involves oxidative damage of DNA and that overexpression of the small hsp reduces this oxidative damage. We suggest that the reduction of oxidative DNA damage is one of the most important protective mechanisms of small HSP against TNF α.

Key Words: TNF α, HSP, Oxidative DNA damage, 8 ohdG

INTRODUCTION

Tumor necrosis factor α (TNF α) is a cytokine derived from activated monocyte-macrophages. Among many properties, its cytotoxic and cytostatic potential against some transformed cell lines has been of interest as a candidate for anti-cancer therapy (Carswell et al, 1975; Creasey et al, 1987; Flick & E. 1984; Sato et al, 1986; Shirai et al, 1985; Sugarman et al, 1985; Wang et al, 1985; Watanabe et al, 1987). Although the mechanism of its selective anti-tumor activity is not completely understood, reactive oxygen species (ROS) induced by TNF α have been implicated as potent mediators of cell killing activity of cytokine (Godfrey et al, 1987; Park et al, 1992; Zimmerman et al, 1989).

ROS are known to be involved in many pathophysiological processes (Halliwell & Gutteridge, 1990). Although cells have developed various enzymatic and nonenzymatic systems to control ROS, a certain fraction escapes the cellular defenses and causes permanent or transient damage to proteins, lipids and nucleic acids (Sandstrom et al, 1994; Stadman, 1992). Since unrepaired DNA damage can serve as a significant source of mutations that lead to a variety of human pathologies including cancer (Ames 1989), the identification and measurement of oxidative damage

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to DNA, in particular, has received great attention. Representative DNA lesions resulting from exposure to ROS are modified bases and strand breaks (Halliwell & Gutteridge, 1984). Among them, a modified base product, 8-hydroxy-2′-deoxyguanosine (8 ohdG) has been increasingly recognized as an excellent marker of oxidative DNA damage partly because 8 ohdG represents one of the major products generated by a wide array of treatments associated with oxidant damage (Cheng et al, 1992; Floyd et al, 1990). Moreover, 8 ohdG can be measured with very high sensitivity by oxidative electrochemistry using HPLC (Floyd, 1986; Frenkel, 1992). The ratio of 8 ohdG to deoxyguanosine (dG) has been used as a sensitive index of the degree of oxidative damage to DNA both in vivo and in vitro (Cao et al, 1988; Floyd 1990; Roy et al, 1991).

Different froms of DNA damage such as fragmentation and ring-saturated thymine derivative, thymine glycol, were shown to be induced by TNF α (Rubin et al, 1988; Zimmerman et al, 1989). However, it is not known whether TNF α induces 8 ohdG formation. Consequently, the relationship between the generation of 8 ohdG and the cell killing activity of TNF α has not been established to date. The immediate goal of current study was to determine the amounts of 8 ohdG both before and after TNF α treament in TNF α-sensitive L929 cells.

Recently, it was reported that small heat shock proteins (small HSPs) have ability to decrease ROS by raising the intracellular concentration of glutathione (Mehlen et al, 1996). The authors also reported that it is a common property of small HSPs from different species. We hypothesized thet if small HSPs reduce ROS induced by TNF α, they should also reduce the level of oxidative DNA damage product, 8 ohdG. To test this hypothesis in TNF α-sensitive L929 cells, it was necessary to introduce exogcnous small HSP into these cells as L929 cells are devoid of endogenous small hsps (Lee et al, 1992; Mehlan et al, 1995). Therefore, we constructed an expression vector by placing small hsp cDNA under the control of CMV promoter. Since L929 cells are originated from mouse, we chose to use mouse small hsp, hsp25 (Frohli et al, 1993) for this study. We transfected L929 cells with the constructed vector and selected clones overexpressing small hsp. Here we report that the level of 8 ohdG was reduced in cells overexpressing hsp25. Survival respones of these cells were also improved significantly. Taken together, we suggest that the modified base product, 8 ohdG is one of the major DNA lesions generated by TNF α and that the reduction of the oxidative DNA damage products is one of the important protective mechanisms conferred by small HSP against cell killing activity of TNF α.

METHODS

Cells and culture conditions

Murine fibroblastoid L929 cells were grown in monolayer in Dulbecco's MEM containing 10% FCS. The cultures were kept in a humidified 37℃ incubator with a mixture of 95% air and 5% CO_2. Mycoplasma contamination was routinely monitored.

Measurement of cell survival

For the measurement of the cell survival, cells were trypsinized, diluted, and plated for colony formation. Colonies were allowed to grow 14 days, fixed and stained (Puck & Marcus 1956).

Tumor necrosis factor α

Recombinant murine TNF α (10^7 U/mg) was from Boehringer Mannheim Biochemicals (Meylan, France). The purity before lyophilization was 95% and the endotoxin level was 0.058 ng per vial.

DNA isolation and enzymatic hydrolysis

DNA was washed twice with 70% ethanol, dried, and dissolved in 200 μl of 10 mM Tris-HCl/0.1 mM EDTA and 100 mM NaCl (pH 7.0) for its enzymatic digestion (Wei & Frenkel 1992). One hundred μg of DNA was incubated with 50 units of DNaee I in 40 μl Tris HCl (10 mM) and 10 μl of 0.5 M $MgCl_2$ (the final concentration of 20 mM) at 37°C for 1 hr. The pH of the reation mixtrue was then lowered with 15 μl of sodium acetate 0.5 M (pH 5.1) and 10 μl of nuclease P1 (5 units) and 30 μl of 10 mM $ZnSO_4$ (to give a final concentration of 1 mM) were added. The resulting mixture was incubated for 1 hr and pH was readjusted with 100 μl of 0.4 M Tris-HCl (pH 8.0) followed by the addition of 20 μl of alkaline

phosphatase (3 units). Enzymes were precipitated and the supernatant was evaporated to dryness.

Measurement of 8-hydroxy-2′ -deoxyguanosine (8 ohdG)

The DNA hydrolysate prepared as above were dissolved in HPLC-grade water and filtered through a 0.2 μm syringe filter before applied to a Waters ODS HPLC column (250×4.6 mm, 5 μm paricle size). The amount of 8 ohdG in the DNA digest was measured by electrochemical detection as previously described (Floyd et al, 1988). Elution conditions were as described by Richter et al (1988) using filtered and vacuum-degassed 50 mM phospohate buffer solution, pH 5.5, containing 8% methanol. The amounts of dG was quantified by UV absorbance detector placed in line between the HPLC column and the electrochemical detector (Floyd et al, 1990). Standard samples of dG and 8 ohdG were analyzed to assure their good separation and to allow indentification and quantitation of those derived from sample DNA. 8 ohdG was synthesized by the method of Kasai and Nishimura (Kasai & Nishimura, 1984).

Plasmids

Murine small hsp cDNA (hsp25) was excised from a vector, phsp6 (Frohli et al, 1993). Expression vector, pBC25 was constructed by placing hsp25 cDNA under the control of CMV promoter. CMV promoter was from Clonetech (Clonetech, Palo Alto, CA).

Transfection and selection

Exponentially growing L929 cells were plated into 60 mm petri dishes at 4×10^5 cells per plate two days prior to transfection. Cells were transfected with 20 μg of purified plasmid DNA in the presence of LipotecACE reagent (Life Technologies, Gaithersburg, MD) for 24 hr exposure at 37°C. Stable transfectants were selected with 400 g/ml geneticin (Life Technologies, Gaithersburg, MD) for approximately one week, followed by continued growth in the presence of 200 μg/ml genetichin to obtain colonies suitable for isolation. The clones were frozen at low passage number. Transfected cell lines with passage numbers between 4 and 10 were used for the experiments.

Polyacrylamide gel electrophoresis and western blot

For one dimensional polyacrylamide gel electrophoresis (PAGE) and western blot, cells were solubilized with lysis buffer (Laemmli, 1970). Equal amounts of protein (50 μg) were analyzed on SDS-PAGE. Five percent acrylamide was used for the stacking gel and 10~18% linear gradient for the separating gel. Protein content was measured by Lowry method (Lowry et al, 1951). After electrophoresis, the proteins were transferred onto nitrocellulose membrane and processed for immunoblotting with HSP25 (1:5000 dilution) monoclonal antibody (StressGen, Canada). Goat anti rabbit IgG conjugated with alkaline phosphatase (Promega, Madison, WI) was diluted to 1:3000 for HSP25 detection. Specific bands were visualized using nitro blue terazolium (NBT) and 5-bromo-4- chloro-3-indolphosphate(BCIP).

Metabolic labeling and two dimensional polyacrylamide gel electrophoresis

L929 cells and transfectants derived from these cells were examined for HSP25 protein expression by metabolic labeling with 50 μCi/ml of [^{3}H]-leucine (Amersham, UK) for 8 hr at 37°C. Heat inducibility was also examined after heating at 45°C for 10 min. The cells were washed twice in Hank's balanced salt solution and lysed in sample buffer containing 8 M urea, 1.7% Nonidet p-40, and 4.3% 2-mercaptoethanol. Protein content of the cell lysates were determined by the method of Lowry(Lowry et al, 1951). Samples were first resolved by isoelectric focusing gels (pH 3.5-10). The focusing gels were then placed into the wells of 10 to 18% linear gradient SDS-PAGE. After electrophoresis, the gels were fixed in 30% trichloroacetic acid (TCA) for 20 min and processed for fluorography by dehydration using consecutive 15 min washes in 25%, 50%, and glacial acetic acid. Gels were then placed in 125 ml of PPO solution (20%(w/v) 2,5-diphenyloxazole in glacial acetic acid) for 2.5 hr, then in distilled water with gentle agitation for 16 hr prior to drying at 60°C for 2.5 hr. The autoradiogram was prepared by exposing dry gels to a kodak XAR fim at −70°C (Eastman Kodak co. Rochester, NY).

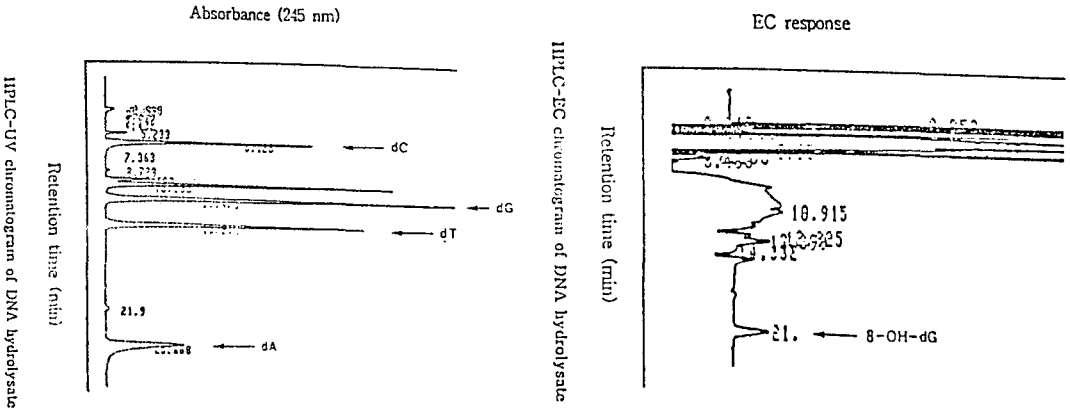

Fig. 1. HPLC chromatograms of DNA hydrolysate obtained from L929 cells.
DNA was isolated from L929 cells and subsequently processed for the HPLC analysis according to the procedure described in "Materials and Methods." The chromatogram shown above represent a typical EC response (right panel) and a UV absorbance response at 245 nm (left panel) of the digested samlple DNA. UV absorbance detector were placed in line between the HPLC column and the electrochemical detector so that both EC and UV signals from the same sample could be detected simultaneously.

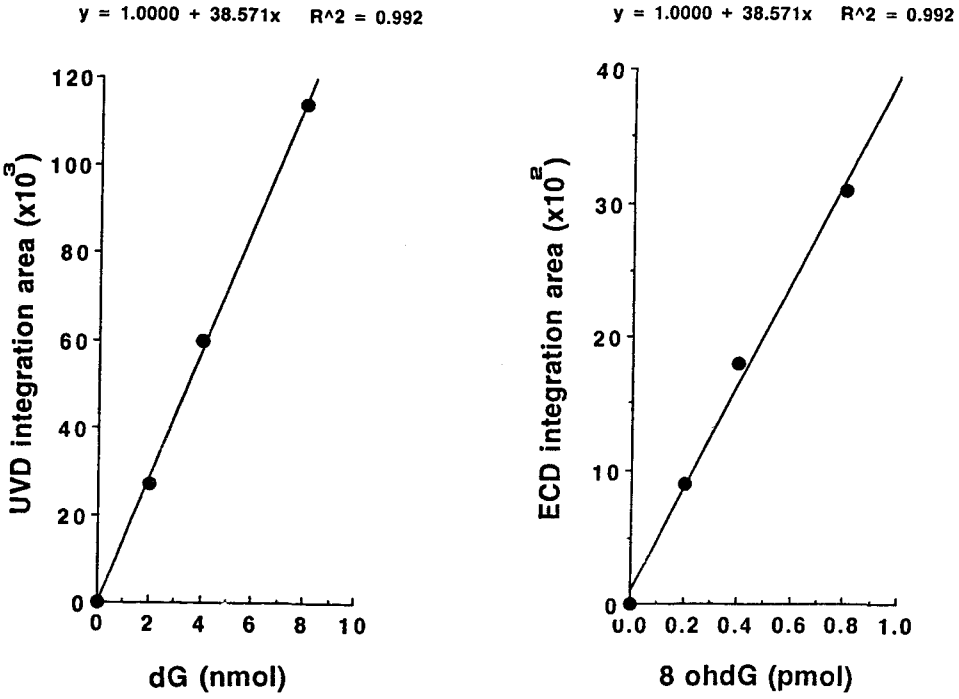

Fig. 2. Standard curves of 8 ohdG and dG.
Standard samples of 8 ohdG and dG were analyzed by HPLC. Standard curves generated from analyzing known amounts of 8 ohdG (right panel) and dG (left) are shown.

RESULTS

HPLC analysis of 8-hydroxy-2′-deoxyguanosine (8 ohdG).

The immediate goal of this study was to determine whether TNF α induces oxidative DNA damage in TNF α-sensitive L929 cells. We examined the possibility by determining the level of 8 ohdG per 10^6 dG present in DNA. UV absorbance detector was placed in line between the HPLC column and the EC detector so that the dG signal and 8 ohdG can be analyzed simultaneously. A typical HPLC-EC (right panel) and an HPLC-UV chromatogram (left panel) obtained from L929 DNA hydrolysate are shown in Fig. 1.

We treated L929 cells with 1000 units/ml of TNF α for increasing time intervals at 37°C. At the end of the incubation period, DNA was isolated and processed for the HPLC analysis as described in Materials and Methods. Standard curves were generated by analyzing known amounts of 8 ohdG (Fig. 2, right panel) and dG (Fig. 2, left panel). As shown in Fig. 3, the amounts of 8 ohdG was increased progressively as the incubation time increased, peaking at 5 hr of TNF α treatment. Our data clearly demonstrate that TNF α increases oxidative DNA damage products, 8 ohdG, in TNF α-sensitive L929 cells. It is very likely that the generation of 8 ohdG was mediated by reactive oxygen species (ROS) derived by TNF α.

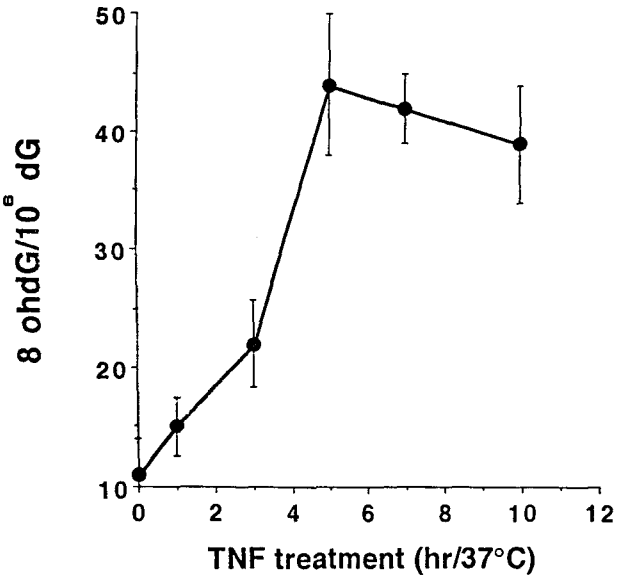

Fig. 3. Increase of 8 ohdG in DNA isolated from L929 cells treated with TNF α.
L929 cells were treated with 1000 units/ml of TNF α for increasing time intervals. The cells were harvested at the time points indicated and the amounts of 8 ohdG in their DNA hdrolysates were determined. The amounts of 8 ohdG were expressed as the number of 8 ohdG per 10^6dG. Each point represents the mean ± standard deviation. Experiments were repeated at least three times.

Construction of murine small heat shock protein (small hsp) expression vector.

Since small HSP was shown to decrease the level

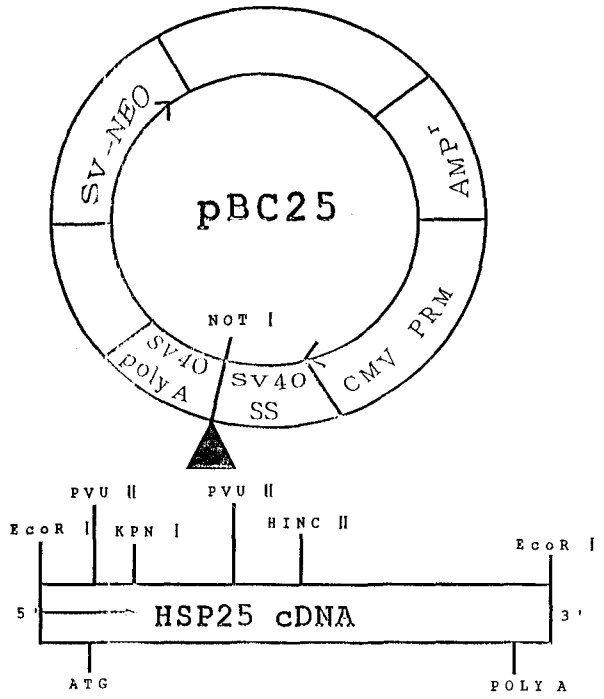

Fig. 4. Schematic representation of the small heat shock protein expression vector.
Plasmid pBC25 containing the small heat shock protein gene, hsp25, was flanked by a CMV promoter (CMV PRM, cytomegalovirus promoter) and a SV40 polyadenylation signal (SV40 polyA). The hsp25 cDNA shown as a box was placed for correct orientation for the expression. The starting codon (ATG) and polyadenylation signal (poly A) are shown under box. Neomycin resistance gene (SV-NEO) and ampicillin resistance gene (AMP) are present to allow for eukayotic and prokaryotic selection, respectively.

of TNF α-derived ROS (Mehlen et al, 1996), next question we asked was whether small hsp can reduce the amounts of 8 ohdG in L929 cells treated with TNF α. To answer this question, it was necessary to introduce exogenous small hsp into L929 cells, since these cells are devoid of small hsp expression. We therefore constructed an expression vector, pBC25, by placing mouse small hsp cDNA fragment (hsp25) under the control of CMV promoter. Schematic representation of the expression vector is shown in Fig. 4.

Expression of exogenous small heat shock protein in L929 cells.

We then stably transfected L929 cells with small hsp expression (hsp25) vector, pBC25. Partent vector that does not contain hsp25 gene was also transfected into L929 Cells to use as a control. We selected several clones (L25#2, 3, 5, 6, 7 and 8) transfected with pBC25. Western blot results probed with anti-HSP25 antibody are shown in Fig. 5. Those cells transfected with pBC25 were found to express hsp25 constitutively. Variable level of basal expression was detected; highest level of hsp25 was detected in clone #8, which was used for the subsequent studies. Control clone (pBC) transfected with the parent plasmid did not show any detectable hsp25 expression. We subsequently asked the heat inducibility of the introduced hsp 25. As shown in Fig. 5, we did find the heat inducible expression of hsp25 in those clones transfected with pBC25. Neither basal nor inducible expression of hsp25 was detected in control clone pBC.

Another possibility we examined was whether the expression of exogenous small hsp could influence the induction of other hsps. We looked at the HSP70 antibody (C92) recognizing the inducible form of hsp70. As shown in Fig. 6, the inducible expression of HSP70 was normal in all clones, suggesting that the possibility is unlikely.

Phosphorylation of transfected small heat shock protein.

It is known that the phosphorylation status of small HSP changes after heat shock, resulting in the appearance of several isoforms of small hsp slightly differing in their pI points. We examined pI profile change of the introduced HSP25 after heat shock. We heated L929 cells at 45°C for 10 min and labeled with [^{3}H] leucine during incubation at 37°C for 12 hr. Autoradiograms obtained from a representative hsp25- transfected clone (L25#8, right panel) or parent vector-transfected control clone (pBC, left panel) are shown in Fig. 7. Isoelectric focusing was per-

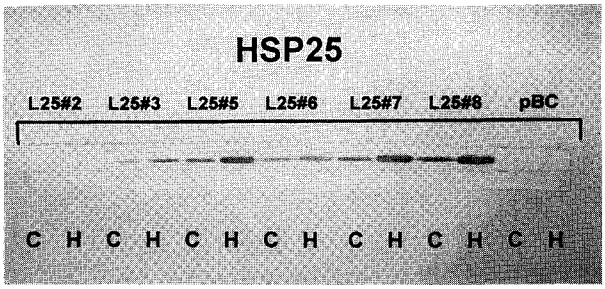

Fig. 5. Expression of transfected hsp25 in L929 cells. Immunoblot obtained from proteins extracted from pBC25-transfected clones (L25#2, 3, 5, 6, 7, and 8) were probed with HSP25 antibody. pBC represents a control clone transfected with the parent plasmid which does not contain hsp25 gene. The hsp25 transfected clones expressed some basal level of HSP25 and varying levels of heat inducible HSP25 (C, control; H, heated). Neither the basal nor the inducible expression of HSP25 was not detected in the control clone tranfected with the vector alone (pBC).

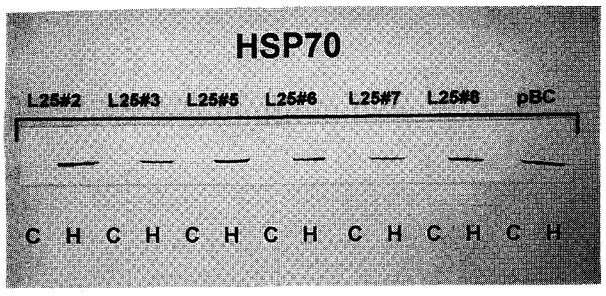

Fig. 6. Expression of hsp70 in L929 cells. Immunoblot obtained from proteins extracted from pBC25-transfected clones (L25#2, 3, 5, 6, 7, and 8) were probed with HSP70 antibody that specifically recognizes inducible from of HSP70. pBC represents a control clone transfected with the parent plasmid which does not contain hsp25 gene. No differences in hsp70 induction was detected between the pBC transfected clones and control pBC-clone.

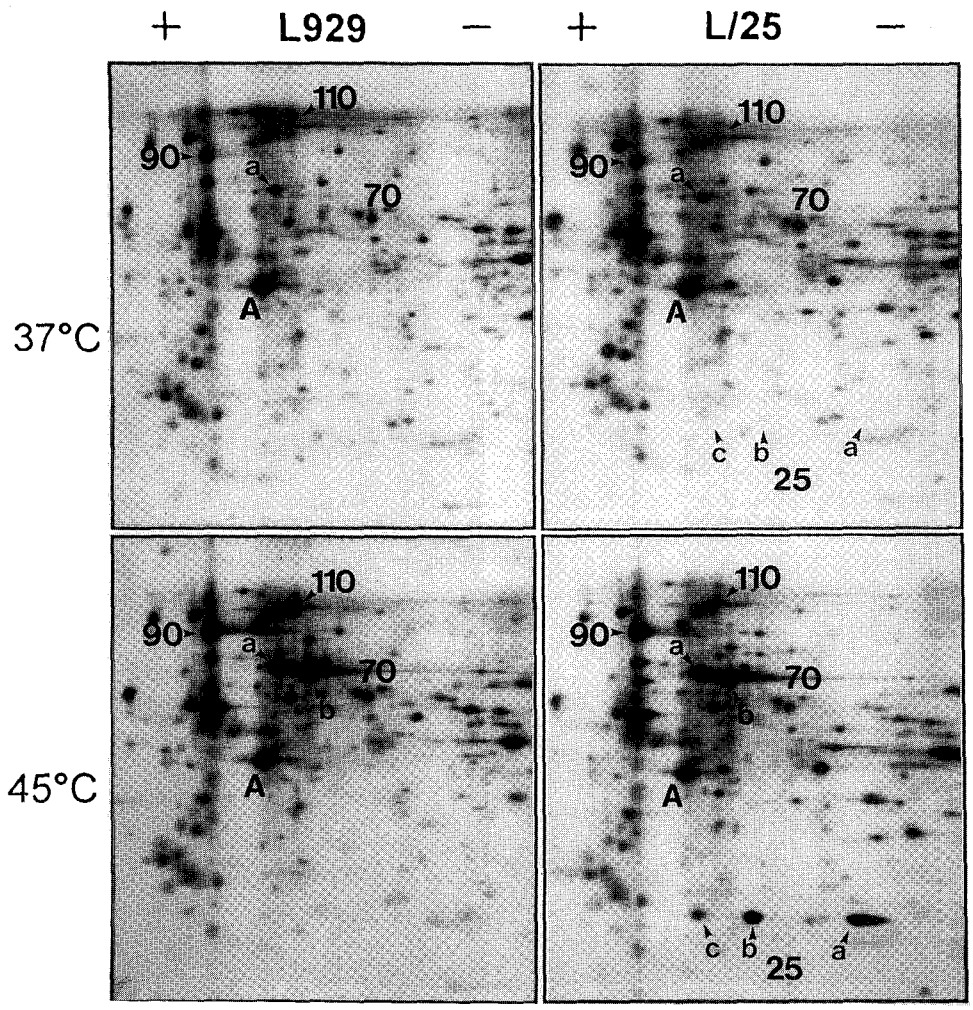

Fig. 7. Two-dimensional PAGE analysis of heat shock protein expression in L929 cells.

Autoradiograms of 2D-PAGE gels obtained from a representative-pBC25 transfected clone (L25#8) are shown in the right panel. Both constitutive (upper) and heat-inducible expression (lower) patterns are shown. Nonphosphorylated HSP25 (a) and two phosphorylated forms (b and c) of HSP25 were detected after heat shock as illustrated in the right lower panel. Both constitutive (upper) and heat-inducible expression (lower) patterns of identically treated control clone (pBC) are shown for comparison (left panel).

formed with the cathode(−) on the right and the anode(+) on the left, therefore basic proteins are located to the right and acidic proteins to the left. The locations of HSP70, 90, and 110 kD are indicated by arrows and actin (A) is identified. Locations of the isoforms of small hsps are indicated by arrows (a, b, and c).

As shown in Fig. 7 (right panel), L25#8 clone displayed heat inducible expression and phosphorylation of HSP25 in a pattern consistent with documented expression of this protein in other rodent cells (Landry et al, 1989). Nonphosporylated (a) and two phosphorylated forms (b and c) of hsp25 were detected. Labeled proteins from control cells confirmed the absence of endogenous HSP25 expression both before (upper panel) and after heat shock (lower panel). However, both basal and heat-inducible expression of other hsps such as hsp110 and hsp90 as well as hsp70 were evident in these cells. In fact, other than hsp25, there were no significant differences in the expression

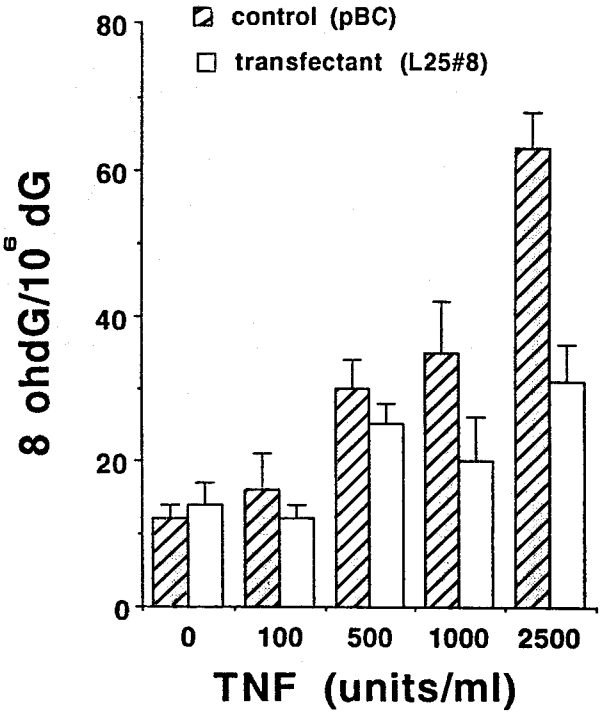

Fig. 8. Reduced 8 ohdG content in L929 cells overexpressing HSP25.
A control clone containing vector control (pBC) or the representative transfectants, L25#8 were treated with increasing concentrations of TNF α for 5 hr at 37°C hummidified CO_2 incubator. At the end of the incubation period, their DNA was isoated, hydrolyzed and subjected to 8 ohdG analysis as described in "Materials and Methods." Note the steady-state levels of endogenous 8 ohdG (8 ohdG/10^6dG) present in cells without TNF α treatment. Each bar represents the mean ± standard deviation. Experiments were repeated at least three times.

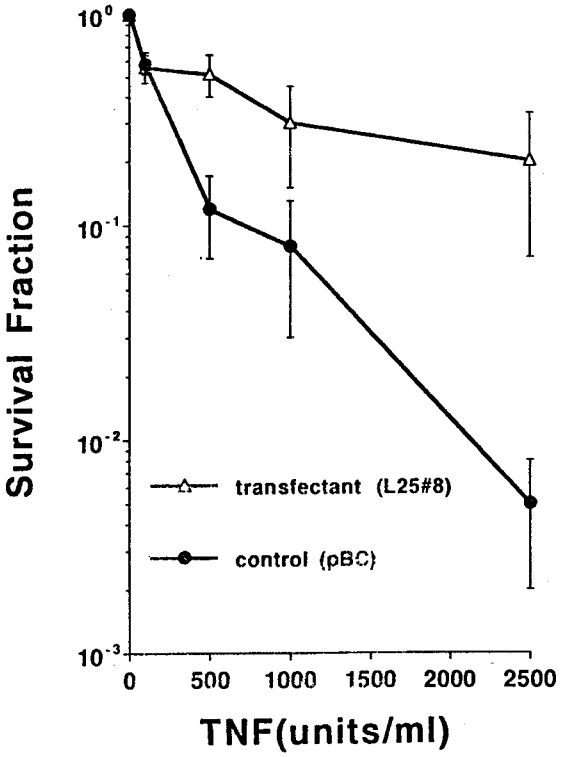

Fig. 9. Increased survival upon TNF α treatment in L929 cells overexpressing HSP25.
A control clone containing vector control (pBC) or the representative transfectants, L25#8 were treated with increasing concentrations of TNF α for 5 hr at 37°C humidified CO_2 incubator. Following treatment, cells were trypsinized, diluted, and plated for colony formation as described in "Materials and Methods."

of endogenous hsps between clones pBC and L25#8.

Reduction of TNF α-induced oxidative DNA damage and cell killing by small heat shock protein.

After confirming the normal expression profile of the exogenously introduced hsp25 in L25#8, we asked if the level of TNF α-induced 8 ohdG decreased in these cells. We treated these cells for 5 hr with increasing concentrations of TNF α. As shown in Fig. 8, the amounts of 8 ohdG was increased in a TNF α dose-dependent manner. The level of 8 ohdG in L25#8 was lower than that found in control cells (pBC) at all concentrations tested. The basal level of 8 ohdG was comparable between L25#8 and pBC: 12±2 8 ohdG/10^6dG in control and 14±3 8 ohdG/10^6dG in L25#8.

We also found that the survival response of L25#8 was enhanced significantly when compared with parent vector-transfected pBC (Fig. 9). These results suggest that the 8 ohdG is one of the major oxidative DNA lesions generated by TNF α and that the reduction of the oxidative DNA damage is an important protective mechanism conferred by small hsp against cell killing activity of TNF α.

DISCUSSION

Results from the current study show that cytotoxic mechanism of TNF α involves generation of oxidative DNA damage product, 8 ohdG, and that over-

expression of the small hsp reduces the level of 8 ohdG. Our data suggest that the reduction of oxidative damage products, which we measured with 8 ohdG, is an important protective mechanism of small hsp against TNF α. It is evidenced by the enhanced survival response together with the reduced 8 ohdG formation in cells overexpressing small hsp.

Several types of oxidative DNA damage products have been reported, which include modified bases and strand breaks (Halliwell & Gutterodge, 1984). Oxidative stress induced by TNF α is very likely to give rise to DNA damage products. One of the modified bases, 8 ohdG has been used as an indicator of oxidative DNA damage in vivo and in vitro (Cao et al, 1988; Floyd 1990; Roy et al, 1991) in part because 8 ohdG represents one of the major products generated by a wide array of treatments associated with oxidant damage (Cheng et al, 1992; Floyd et al, 1990). Moreover, the well established measurement procedure based on the oxidative electrochemistry using HPLC system makes 8 ohdG a sensitive marker of oxidative DNA damage (Floyd 1986; Frenkel 1992).

Since cytotoxic mechanism of TNF α has been recognized to involve the generation of reactive oxygen species (ROS), we probed whether TNF α induces oxidative DNA damage product, 8 ohdG. Our data shown here clearly demonstrate that 8 ohdG is induced by TNF α and it increases in a TNF α dose-dependent manner. Our finding is in accordance with the results of previous investigators, who reported that TNF α induces oxidative derivatization of thymine (thymine glycol) (Zimmerman et al, 1989). Our results presented here provide additional new data toward the elucidation of TNF α-specific oxidative damage using sensitive HPLC-EC measurement system. We noted a substantial level of endogenous 8 ohdG in control cells. It appears that normal metabolic pathway generates de novo a certain level of oxidative damage, although we can not completely exclude the possible artifactual oxidation during sample preparation.

Small heat shock protein (small HSP) have been implicated in the protection angainst a variety of cellular stresses including oxidative stress. The mechanism of its protection, however, is not fully understood. Elegant study by Mehlen et al (Mehlen et al, 1996) recently demonstrated that small HSP has ability to decrease reactive oxygen species (ROS) by raising the intracellular concentration of glutathione (Mehlen et al, 1996). We hypothesized that if small HSP reduces ROS induced by TNF α, it should reduce the level of oxidative damage product, 8 ohdG. Our finding that 8 ohdG was reduced in L929 cells transfected with small hsp (hsp25) proves that this hypothesis is valid. Further, our finding that the survival responses of these cells were enhanced indicates that elevated level of small HSP can protect TNF α-induced cell killing presumably by reducing oxidative damage to DNA.

It is possible that the decision as to whether TNF α treated cells will be resistant is dependent on the extent of intracellular ROS formation and the ability of the cell to buffer the oxidative burst. One of these buffers could be SOD. Indeed, induction of superoxide dismutase proved to be protective against cytotoxic action of TNF α (Park et al, 1992; Wong et al, 1989; Wong & Goeddel, 1988). Another is probably tripeptide intracellular thiol glutathione which is known as a powerful detoxifier of ROS. The ability of small HSP to decrease 8 ohdG may be related to the ability of small HSP to modulate the intracellular level of glutathione. Mehlen et al (Mehlen et al, 1996) recently demonstrated that decreasing the intracellular glutathione level abolished small HSP-mediated protection against TNF α. Although we were not able to find a direct correlation between the level of small HSP and the amounts of intracellular glutathione present in transfectants, we did find increase in basal level of glutathione in small HSP transfectants, in general (data not shown). Further, glutathione oxidation index (oxidized glutathione/total pool of glutathione) as measured by HPLC appeared to be clearly different between a representative small hsp transfectants, L25#8, and parent vector-transfected, pBC (data not shown).

It is also possible that the protective activity of small HSP is associated with changes in the phosphorylation, oligomerization and localization of this protein (Mehlen et al, 1995). The ability of mammalian cells to phosphorylate HSP25 has been suggested to be an important factor in regulating some of the activities of these proteins in cells (Lavoie et al, 1995; Marin et al, 1996). It would be interesting to see whether phosphorylation of small HSP is essential for its ability to modulate cellular glutathione and/or reduce 8 ohdG upon TNF α treatment. This issue can be tested relatively easily by con-

structing an expression vector with phosphorylation defective- hsp25 gene. L929 cell system appeared to be one of the best system to study the function of small HSP since these cells are intrinsically devoid of endogenous small hsps. Moreover, as suggested by previous investigators and confimed in the current study, the overexpression of small hsp can be achieved without eliciting a compensatory expression of other endogenous heat shock protein (Lee et al, 1992; Mehlan et al, 1995).

Taken together, our data show that cytotoxic mechanism of TNF α involves the generation of oxidative DNA damage as evidenced by the dose-dependent increase of oxidative damage product, 8 ohdG. Our data also show that small HSP provides protection against TNF α -induced cytotoxicity as evidenced by the results that the overexpression of the exogenous small HSP reduces the level of 8 ohdG as well as cell killing activity of TNF α. These results indicate that small HSP confers resistance to the cytotoxic action of TNF α, presumably by reducing the oxidative DNA damage in L929 cells.

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