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Decreased heat- and tumor necrosis factor-mediated hsp28 phosphorylation in thermotolerant Hela cells

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Heat shock or tumor necrosis factor rapidly stimulated the phosphorylation of the mammalian low molecular weight stress protein hsp28. We have found that both phenomena are greatly decreased in cells which are made tolerant to heat. This observation correlated with a better survival of thermotolerant cells exposed to either heat or TNF treatment. The results suggest that the phosphorylation of hsp28 may be linked to the resistance of the cells to the deleterious effects induced by either heat or a mediator of inflammation such as TNF.

Thermotolerance; Heat shock protein phosphorylation; Tumor necrosis factor

1. INTRODUCTION

Cells or organisms exposed to supra-optimal temperatures stimulate the synthesis of the heat shock proteins (hsps) and develop a tolerance to different types of stress. Transiently, thermotolerant cells are able to survive an otherwise lethal heat shock challenge [1,2]. The hsps are supposed to protect against cellular damages induced by non-physiological conditions and may play a crucial role in the acquisition of thermotolerance [3–6]. One of these proteins, the mammalian hsp28, is a phophoprotein of unknown function sharing homology with a region of bovine lens α-crystallin [7,8]. Following heat shock, the phosphorylation of this protein is strongly stimulated [9–14] and affects at least 50% of hsp28 polypeptides [13]. This phenomenon is transient and disappears several hours after the heat shock treatment [11,13]. Hsp28 is present under normal cellular growth conditions [10–14] and the corresponding Drosophila protein (hsp27) is developmentally regulated [15,16] with a pattern of tissue-specific expression resembling that of the ras oncogen [16].

Tumor necrosis factor-α/cachectin (TNF) is a monocyte/macrophage-derived pro-inflammatory cytokine. It causes the necrosis of solid tumors in vivo, fever, endothelial cell resorption and the acute-phase response [17,18]. An early cellular event of TNF action is the phosphorylation of 28 kDa proteins [14,19–23] identified as hsp28 isoforms [14,23]. Interleukin-1 (IL-1) which shares several biological properties with TNF induced a similar phenomenon [23,24].

Since thermotolerance protects cells against environmental stress, we investigated whether the C-kinase independent [14] heat- or TNF-mediated phosphorylation of hsp28 was altered in thermotolerant cells.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

HeLa cells growing on 35-mm Falcon dishes were incubated at 37°C. Heat or TNF treatment was performed in the presence of 5% CO_2. Cell death was monitored by using the vital dye Trypan blue (Sigma).

2.2. *Reagents*

Human recombinant TNF-α (10^7 U/mg) was from Dr. J.-M. Dayer (Dept. of Immunology, University of Geneva, Switzerland). [^{32}P]orthophosphoric acid (carrier free) was from Amersham, UK. The specificity of anti hsp28 serum has already been described [11,13,14].

2.3. *Radiolabelling*

HeLa cells were incubated for 20 min in Dulbecco modified Eagle medium lacking phosphate before being exposed at 37°C to 0.5 mCi/ml [^{32}P]orthophosphate (carrier free; Amersham, UK) in the same medium. Labelling was (i) for 2 h immediately after the heat stress, or (ii) for 1 h before adding TNF. After further incubation with TNF for the required time, the labelling medium was removed and the cells were quickly washed in ice-cold phosphate-buffered saline (PBS) and lysed in boiling SDS sample buffer.

2.4. *Two-dimensional gel electrophoresis and immunoblotting*

Two-dimensional gel electrophoresis and immunoblotting, using hsp28 antiserum were performed as described previously [13,14].

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3. RESULTS

3.1. *Thermotolerance reduces the stress-mediated changes in protein phosphorylation*

Fig. 1a-c shows that in Hela cells labelled with $^{32}P_i$ (see section 2) the phosphorylation of several 28 kDa polypeptides is more intense after a heat stress at 44°C for 45 min than after an incubation for the same time at 42°C. These 28 kDa polypeptides were shown to be hsp28 isoforms [10,11,13,14]. It is shown here that several other proteins also increased their level of phosphorylation after a heat stress, while some exhibited a marked dephosphorylation. Therefore we investigated the pattern of protein phosphorylation in cells which were made thermotolerant before the heat stress. To this end, Hela cells were heat-treated at 43°C for 90 min and allowed to recover for 10 h at 37°C in order to maximally develop thermotolerance [2,13]. In these cells, hsp28 phosphorylation was only weakly increased by the 44°C heat stress (Fig. 1d). In addition, the phosphorylation pattern of the other polypeptides described above was unaffected by the heat stress. Immunoblot experiments, using an antiserum recognizing hsp28, also showed a decreased accumulation of the stress-induced phospho-isoforms of this protein in thermotolerant stressed cells (Fig. 2A). Most of hsp28 was then recovered at the level of the non-phosphorylated 'a' isoform.

Analysis of unstressed cells showed a higher level of $^{32}P_i$ labelled hsp28 isoforms in thermotolerant cells (Fig. 2B). However, as previously described [13] and confirmed in the immunoblot presented in Fig. 2B, the quantitative level of hsp28 is higher in these cells. This suggests that the specific level of hsp28 phosphorylation is roughly the same in normal and thermotolerant unstressed cells. Consequently, the reduction of the stress-induced hsp28 phosphorylation in stressed thermotolerant cells shown in Fig. 1d may be underestimated.

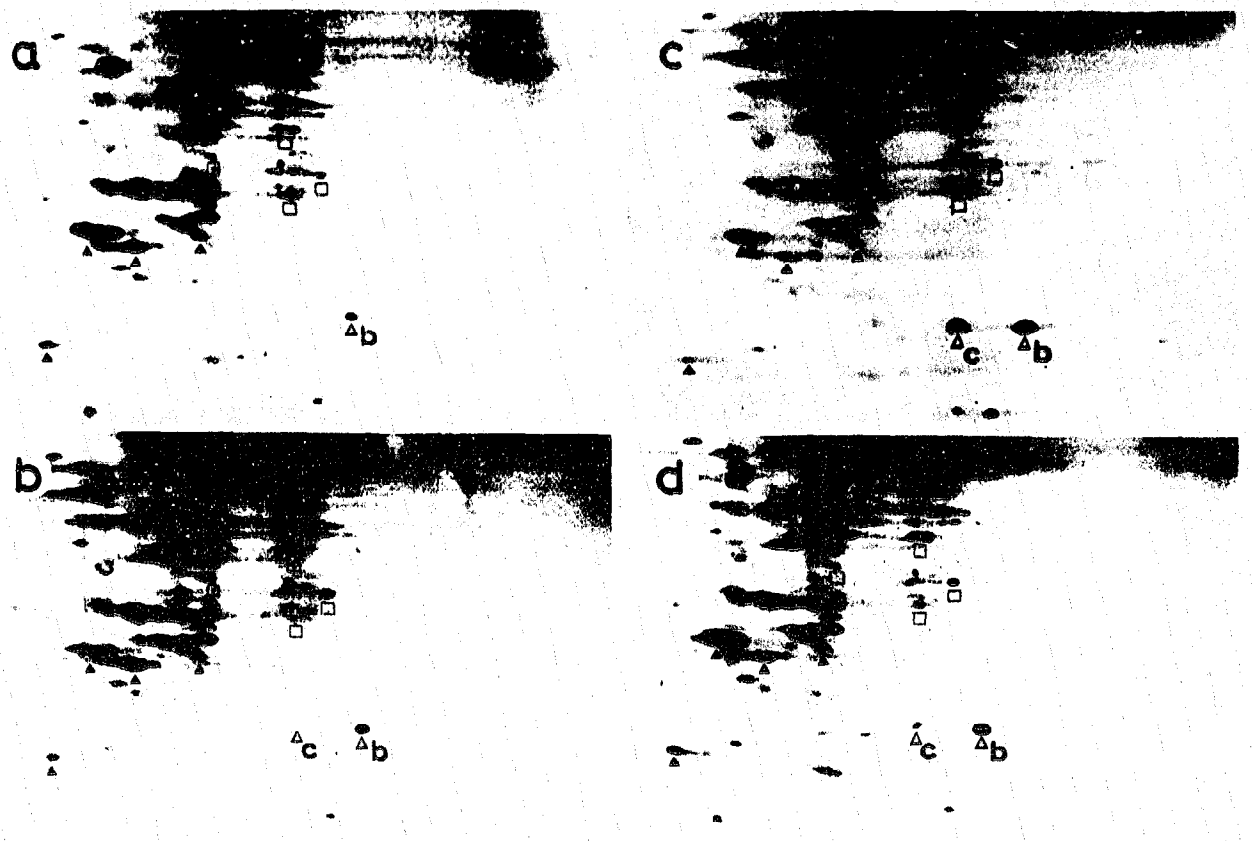

Fig. 1. Decreased heat-induced phosphorylation of hsp28 in heat-treated thermotolerant Hela cells. Normal and thermotolerant HeLa cells either kept at 37°C, or heat-treated at 42°C for 45 min were labelled with [^{32}P]orthophosphate and the phosphoproteins analyzed in two-dimensional gel electrophoresis as described in section 2. The acidic end is to the left. Autoradiographs of the two-dimensional gels are presented. (a) Control cells kept at normal temperature; (b) cells heat-treated at 42°C; (c) cells heat-treated at 44°C; (d) as (c), but in this case the cells were thermotolerant. The open arrow-heads noted 'b' and 'c' indicate the position of the 28 kDa phospho-polypeptides. The open squares indicate the position of other proteins with an increased phosphorylation status after heat shock but which are not observed in heat-treated thermotolerant cells. The black arrow-heads indicate the position of proteins exhibiting a decreased phosphorylation after heat shock which are not observed anymore in heat-treated thermotolerant cells.

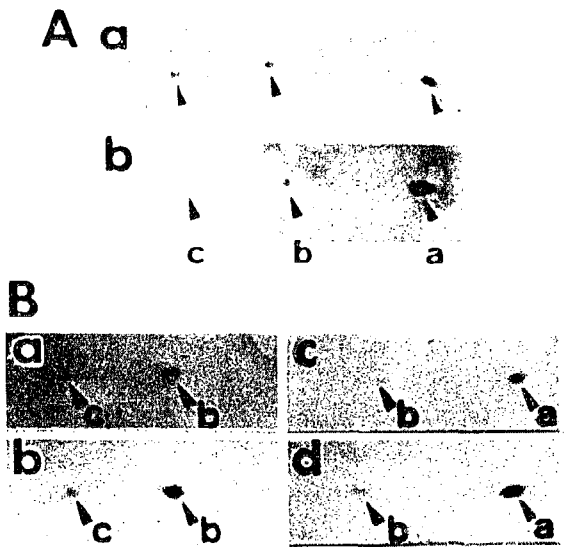

Fig. 2. (A) Decreased accumulation of hsp28 'b' and 'c' isoforms in heat-treated thermotolerant Hela cells. Anti hsp28 antiserum was used to probe protein blots of two-dimensional gels from either heat-treated cells (1) or heat-treated thermotolerant cells. The fraction of the blot showing hsp28 isoforms is presented. The acidic end is to the left. The arrow heads noted 'a' to 'c' indicate the position of hsp28 isoforms. The more basic isoform of hsp28 ('a') does not contain phosphate. (B) Similar specific levels of hsp28 phosphorylation in normal and thermotolerant unstressed Hela cells. (a,b) Labelling experiment: control (a) and thermotolerant (b) Hela cells labelled with $^{32}P_i$ and analyzed in two-dimensional gels as described in section 2. Autoradiographs of the fraction of the gels showing hsp28 isoforms are presented. (c,d) Immunoblot experiment: anti hsp28 antiserum was used to probe protein blots of two-dimensional gels from either normal (c) or thermotolerant (d) unstressed cells. As above, the fraction of the blot showing hsp28 isoforms is presented. The arrow-heads noted 'a','b','c' indicate the position of hsp28 isoforms. The 'c' isoform is not detectable in the immunoblots.

3.2. *Thermotolerance reduces the TNF-mediated hsp28 phosphorylation.*

In Hela cells stimulated by high doses of TNF-α a rapid phosphorylation of hsp28 has been observed [14,23]. Both the heat- and TNF-mediated hsp28 phosphorylation are independent of C-kinase [14]. We therefore investigated whether this TNF-induced phosphorylation was also altered in thermotolerant Hela cells. As seen in Fig. 3, the induction of hsp28 phosphorylation by this cytokine (2000 U/ml) was less effective in thermotolerant cells. This result was confirmed by immunoblots of two-dimensional gels using anti-hsp28 antibody (Fig. 4).

3.3. *Thermotolerance protects against TNF-mediated cytolysis in Hela cells*

Heat shock performed before a TNF-treatment was found to protect several cell types against the cytokine-induced cytolysis [25,26]. Table I shows that this is also valid for Hela cells. Although Hela cells are rather resistant to TNF-mediated cytolysis, we reproducibly

Table I

Decreased cytotoxicity induced by heat shock and TNF in thermotolerant Hela cells

Cells	Treatment	% of Trypan blue positive cells
Normal	-	4.5 ± 1.5
Thermotolerant	-	6.0 ± 1.0
Normal	Heat shock	18.0 ± 2.0
Thermotolerant	Heat shock	10.0 ± 2.0
Normal	TNF	27.0 ± 3.5
Thermotolerant	TNF	14.0 ± 3.5

Normal and thermotolerant cells were either kept at 37°C, exposed to a drastic heat stress at 45°C for 30 min or incubated with 2000 U/ml TNF-α for 1 h at 37°C. Cytotoxicity was monitored as the percentage of the cells stained by the vital dye Trypan blue. Each value represents the percentage of cells stained by the dye and is averaged. Standard deviations are indicated (n = 57).

observed that thermotolerance induced a higher cell survival to this cytokine as monitored by the staining with the vital dye Trypan blue. The degree of protection induced by thermotolerance was similar in heat- and TNF-treated cells. In contrast, no protection was observed when TNF was added before or immediately after the heat stress when no thermotolerance had developed (not shown). Thus, the decreased TNF-mediated hsp28 phosphorylation in tolerant cells is correlated with a decreased cytotoxicity of this cytokine.

4. DISCUSSION

We have found that thermotolerance reduced the level of the stress-induced alterations of protein phosphorylation. This suggests a protection of the protein kinase/phosphatase machinery and/or that the stress-induced changes in protein phosphorylation do not present any advantages for the stressed thermotolerant cells. Similarly, it is interesting to note the reduction of the stress-induced changes in cellular localization and oligomerization of hsp28 in thermotolerant cells [13,28]. The protection of several vital cellular functions have also been described in these cells [2,27].

The cytokine tumor necrosis factor α (TNF α) is a modulator of inflammation which rapidly induces the phosphorylation of hsp28 [14,23]. Both heat- and TNF-mediated phosphorylations of hsp28 are C-kinase independent phenomena [14] and occur at serine/threonine residues [9,23]. In this report we show a decreased TNF-mediated hsp28 phosphorylation in thermotolerant Hela cells. In addition, these cells were more resistant to the cytotoxic effects induced by TNF, a result which is in agreement with the findings of Jäättelä et al. [25,26]. Our results favor the hypothesis that hsp28 phosphorylation is linked to the resistance of the cells to the deleterious effects induced by either heat or TNF. It appears that TNF surface receptors are not lost

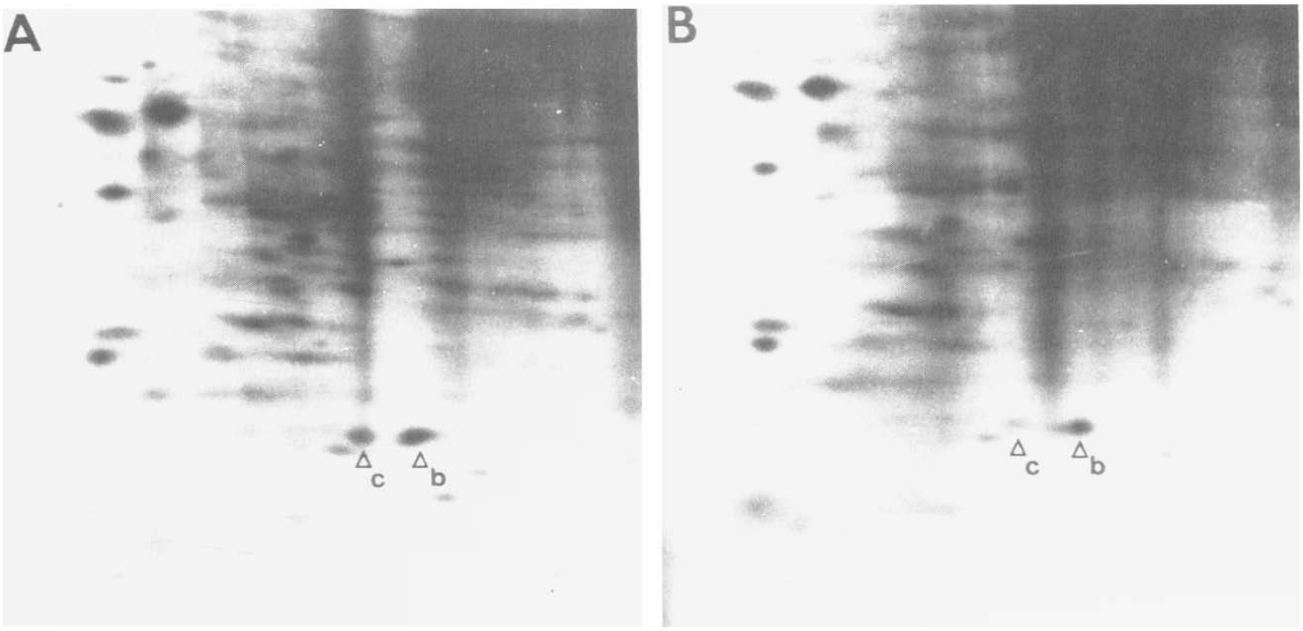

Fig. 3. Decreased heat-induced phosphorylation of hsp28 in TNF-treated thermotolerant Hela cells. Normal or thermotolerant HeLa cells were labelled with $^{32}P_i$ and further incubated with TNF (2000 U/ml) for 30 min at 37°C as described in section 2. The labelled proteins were analyzed as above and autoradiographs of the two-dimensional polyacrylamide gels are presented. The acidic end is to the left. (A) control cells, (B) thermotolerant cells. The open arrow-heads noted 'b','c' indicate the position of hsp28 phospho-isoforms.

following heat treatment [25]. Thus, the inhibition of TNF-mediated cytotoxicity in tolerant cells may be due to post-binding events such as altered signal transduction mechanisms. The accumulation of the hsps and/or detoxificant enzymes, such as manganous superoxide dismutase (SOD) [25,29], may also explain this decreased cytotoxicity of TNF. Perhaps thermotolerance is part of a mechanism that prevents the destruction of stressed cells by inflammatory mediators during their recovery from damage.

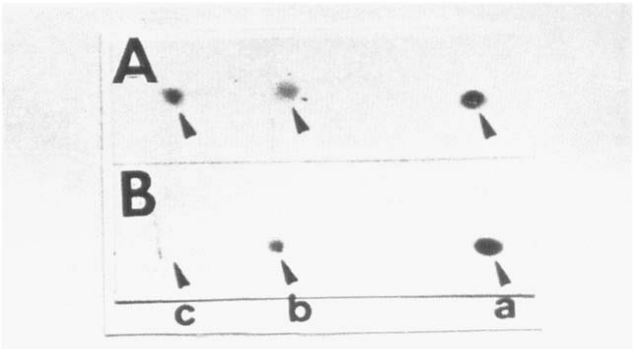

Fig. 4. Decreased accumulation of hsp28 acid isoforms in TNF-treated thermotolerant Hela cells. Immunoblot experiment using anti hsp28 serum of two-dimensional gels from either normal (A) or thermotolerant (B) TNF-treated cells. TNF-treatment was performed as described in Fig. 3. The fraction of the immunoblot showing hsp28 isoforms is presented. The arrow-heads noted 'a','b','c' indicate the position of hsp28 isoforms.

Contrasting with these observations, synergistic killing effects of TNF and heat shock have been described [30]. However, in these experiments the heat shock treatment was performed after the beginning of the incubation with this cytokine. We have made similar observations (unpublished). An intriguing possibility may be that TNF present before the heat stress sensibilizes cells to heat shock and abolishes the development of thermotolerance.

Thus, in the case of fever induced by the injection of TNF to rabbits [31], the killing ability of this cytokine may be enhanced. However, febrile conditions may also induce thermotolerance suggesting that the in vitro induction of TNF resistance and the decreased TNF-mediated hsp28 phosphorylation are probably functionally significant also in vivo. Therefore, it can be concluded that heat shock and the acquisition of thermotolerance may modify the immune response by modulating the activity of TNF.

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