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Induction of Synthesis of Heat Shock Protein 72 in Tumor Necrosis Factor Gene-transduced Cells

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Heat shock protein (HSP) and endogenous tumor necrosis factor (enTNF) both act as resistance factors against the cytotoxicity of various cellular stresses. To clarify the relationship between these two stress response systems, we investigated whether or not enTNF is capable of inducing HSP72. Without heating, no difference was found in HSP72 synthesis between enTNF-nonexpressing L-M cells and cells expressing L-R or L-M (pcDV-TNF). After initiation of heat treatment, however, a remarkable increase in HSP72 synthesis was noted in enTNF-expressing cells compared to enTNF-nonexpressing L-M cells. These findings indicated that enTNF augments heat-inducible HSP72 synthesis.

Key words: Tumor necrosis factor — Heat shock protein — TNF expression vector

The heat shock proteins (HSPs) are a group of highly conserved proteins that are rapidly induced in cells exposed to a variety of environmental stresses, including temperatures a few degrees above normal growth temperature, and that act as an intracellular resistance factor.[1-3] We have previously reported that endogenous tumor necrosis factor (enTNF) also acts as a cross-resistance factor against the cytotoxicity of heat,[4] adriamycin[5] and exogenous TNF[6-9] by induction of manganous superoxide dismutase (MnSOD), thereby scavenging reactive oxygen free radicals. Therefore, cancer cells show resistance with two different stress response systems, i.e. HSPs and enTNF, against heat, various anticancer drugs and cytokines. However, the relationship between these two stress response systems is not clear. In the present study, we therefore investigated whether or not enTNF is capable of inducing HSPs.

Heat-sensitive L-M (mouse tumorigenic fibroblast) cells, originally expressing no enTNF, were transfected with a human TNF expression vector, pcDV-TNF, and the transfectants were designated as L-M (pcDV-TNF).[4,7] L-R cells were TNF-resistant cells obtained by incubating L-M cells in the presence of gradually increasing concentrations of recombinant human TNF (1 to 5×10^5 units/ml) to express enTNF.[4,6] L-M (pcDV-TNF) and L-R cells showed apparent resistance to heat treatment compared to parental L-M cells.[4]

Cells (1×10^6 cells/35 mm dish) were heated at 42°C for 1 h and incubated at 37°C for 1, 2, 3, 4, 8 and 16 h, then washed two times with methionine-free medium (ICN Biomedicals Inc., CA) and labeled with 3.7 MBq/ml of ^{35}S-methionine (DuPont/NEN Research Products, MA) for 1 h. They were then washed with phosphate-buffered saline (PBS) and solubilized in lysis buffer (1% NP-40, 0.15 *M* NaCl, 50 m*M* Tris-HCl pH 8.0, 5 m*M* EDTA, 2 m*M* N-ethylmaleimide, 2 m*M* PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin) for 30 min at 4°C. After centrifugation at 10,000*g* for 30 min, the supernatant was incubated with anti-HSP72 monoclonal antibody (Amersham, Bucks, UK) at 4°C for 18 h and subsequently mixed with CL-4B Sepharose beads (Protein A Sepharose CL-4B, Pharmacia Biotech Norden AB, Sollentuna, Sweden) and shaken for 1 h at 4°C. The beads were washed three times with lysis buffer and boiled for 3 min in Laemmli's sodium dodecyl sulfate (SDS) sample buffer to elute the immunoprecipitate. Samples were then analyzed by SDS-10% polyacrylamide gel electrophoresis(PAGE) and autoradiography. Finally, HSP72 synthesis was quantitated by densitometry (dual-wavelength TLC scanner CS-910, Shimadzu Co., Kyoto) at 750 nm. Time courses of HSP72 levels of enTNF-expressing L-R cells and nonexpressing L-M cells were assessed by exposing these cells to 42°C for 1 h.

As shown in Fig. 1 (without heating), the expressions of HSP72 of L-M and L-R cells were weak and no difference was observed. After initiation of heat treatment, expression of HSP72 increased rapidly, reached a peak at 2–3 h and rapidly returned to basal levels during the recovery period (4–8 h) in both cells. However, a remarkable increase of HSP72 expression, to 4.2 times higher than that of L-M cells at 3 h, was noted in L-R

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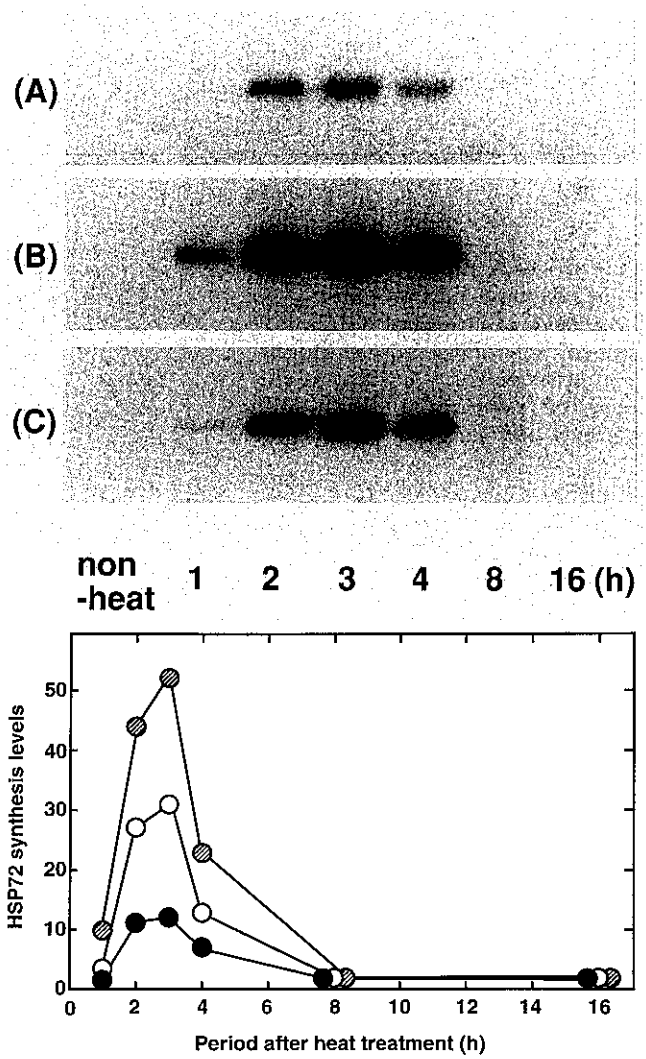

Fig. 1. Time course of HSP induction after treatment at 42°C for 1 h. Cells were heated at 42°C for 1 h and incubated at 37°C for 1, 2, 3, 4, 8 and 16 h. Then the cells were labeled with 3.7 MBq/ml ^{35}S-methionine at 37°C for 1 h. The cell lysate was incubated with anti-HSP72 monoclonal antibody at 4°C for 18 h and subsequently mixed with CL-4B Sepharose beads. The immunoprecipitates eluted from the beads were analyzed by SDS-10% PAGE and autoradiography (upper panel). Lane (A), L-M cells; lane (B), L-R cells; lane (C), L-M (pcDV-TNF) cells. Using densitometry, the relative density of each band was estimated by arbitrarily normalizing the value of that of L-M cells 1 h after heat-treatment to 1 (lower panel). L-M cells (●), L-R cells (▨) and L-M (pcDV-TNF) cells (○).

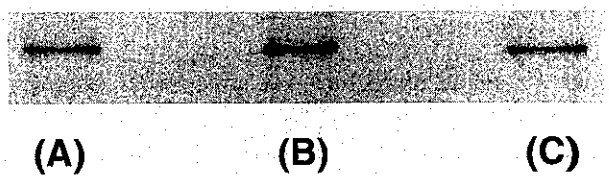

Fig. 2. Effect of anti-TNF antibody on the synthesis of HSP 72 in heat-treated L-R cells. Cells were heated at 42°C for 1 h and incubated at 37°C for 3 h in control medium (A) and medium containing anti-mouse TNF rabbit serum (B) or normal rabbit serum (C). Then the cells were labeled with 3.7 MBq/ml ^{35}S-methionine at 37°C for 1 h. The cell lysate was incubated with anti-HSP72 monoclonal antibody at 4°C for 18 h and subsequently mixed with CL-4B Sepharose beads. The immunoprecipitates eluted from the beads were analyzed by SDS-10% PAGE and autoradiography.

cells. This result suggests that enTNF may be related to the expression of HSP72.

In order to clarify the relationship between enTNF and HSP72 expression, TNF-nonexpressing L-M cells were transfected with pcDV-TNF and expression of HSP72 was examined. L-M (pcDV-TNF) cells, which are stable transfectants, secreted TNF into the culture medium as reported previously.[4-7] A substantial increase of HSP72 expression was also noted in L-M (pcDV-TNF) cells as compared to parental L-M cells (Fig. 1).

These findings indicated that HSP72 synthesis after heat treatment is enhanced in TNF-producing cells as compared to TNF-nonproducing cells. However, it is still unknown whether the induction of synthesis of HSP72 by enTNF is exerted in an intracellular or extracellular (autocrine) manner. In the present study, therefore, we examined the effect of the addition of anti-mouse TNF rabbit serum (Asahi Chemical Industry Co., Ltd., Tokyo), which neutralized the mouse TNF activity, to the culture medium on the synthesis of HSP72 in heat-treated L-R cells. As shown in Fig. 2, no difference was observed in HSP72 synthesis between anti-TNF antibody-treated and non-treated L-R cells. Furthermore, we observed a similar increase of HSP72 expression in L-M cells transfected with a nonsecretory-type TNF expression vector (pTNFΔpro), after heat treatment (data not shown). Therefore, it is conceivable that enTNF exerts HSP72-inducing activity intracellularly.

These findings suggest that enTNF participates not only in intrinsic resistance against heat, adriamycin and exogenous TNF via induction of MnSOD but also in acquisition of heat resistance via augmenting HSP expression induced by heat treatment. The mechanism whereby HSP synthesis in induced by enTNF remains to be investigated.

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