Brief Definitive Report

Heat-Shock Proteins Protect Cells from Monocyte Cytotoxicity: Possible Mechanism of Self-Protection

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Summary

We have previously shown that major heat-shock protein (hsp 70) protects WEHI-S tumor cells from cytotoxicity mediated by tumor necrosis factor α (TNF-α) and TNF-β. In the present study, the effect of altered expression of hsp70 and low molecular weight heat-shock protein, hsp27, on tumor cell sensitivity to monocytes and lymphokine-activated killer (LAK) cells was studied. Constitutive and stable expression of transfected human hsp70 rendered cells almost completely resistant to monocytes. Conversely, inhibition of endogenous hsp70 by expression of antisense hsp70 RNA enhanced the sensitivity of cells to monocyte-mediated killing. Surprisingly, overexpression of human hsp27, which does not protect WEHI-S cells from TNF killing, conferred partial resistance to monocytes. Only approximately 60% of monocyte-mediated killing of WEHI-S cells could be blocked by neutralizing TNF-α antibody or immunoglobulin G–TNF receptor chimeric protein, suggesting the presence of both TNF-dependent and TNF-independent lytic mechanisms. As free radicals have been suggested to be mediators of monocyte cytotoxicity, we tested the sensitivity of transfected cells to oxidative stress. Overexpression of either hsp70 or hsp27 rendered cells partially resistant to hydrogen peroxide. No significant changes in the susceptibility of cell lines overexpressing hsp70 or hsp27 to cytotoxicity mediated by LAK cells were observed. Interestingly, monocytes but not LAK cells contained detectable levels of hsp27 and hsp70 in nonstressed conditions. Taken together, these data indicate that hsp70 protects tumor cells from TNF-mediated monocyte cytotoxicity and that both hsp27 and hsp70 confer resistance to TNF-independent, probably free radical–mediated lysis by monocytes. Moreover, hsp27 and hsp70 may provide monocytes with a protective mechanism against their own toxicity.

All organisms and cells respond to supraoptimal temperatures and various other environmental stresses by a rapid synthesis of a small group of evolutionarily highly conserved proteins, heat-shock proteins (hsp's) (1, 2). As the amino acid sequences of hsp's have been highly conserved throughout evolution, it has been assumed that they serve some universally important functions. Recent results from several laboratories suggest that hsp's participate in such crucial phenomena as protein import and assembly, protection from environmental stress, immunity, autoimmunity, and cancer (1, 2).

Cells capable of killing a broad spectrum of tumor cells without apparent specificity may be important in the first line of defence against malignancy. Effector cells with a capacity for such spontaneous killing include NK cells, natural cytotoxic cells, and monocytes (3–6). The spectrum of target cells killed by NK cells can be further enlarged by culture with IL-2 resulting in a cell population called LAK cells (7). Based on the target cell spectrum and on the studies with neutralizing antibodies, TNF-α has been claimed to be a major mediator of lysis by monocytes (3, 4, 8). Even though lymphocytes also express TNF-α mRNA, other mediators of cytotoxicity are likely to contribute to the cytotoxicity mediated by them (9).

The induction of heat-shock response in tumor cells renders them partially resistant to cytotoxicity mediated by TNF-α and -β, activated monocytes, and cytotoxic lymphocytes (10–13). To determine if individual hsp's are responsible for stress-induced tumor cell resistance to TNF killing, we transfected genes encoding human hsp70 and human hsp27 into the highly TNF-sensitive murine fibrosarcoma cell line WEHI-S (14). The results clearly showed that hsp70 but not hsp27 protects tumor cells from cytotoxicity mediated by TNFs. In the present study we have investigated the sensitivity of transfected cell lines with altered levels of hsp27 and hsp70 to oxidative stress and cytotoxicity mediated by monocytes and LAK cells.

Materials and Methods

Cell Lines and Culture Conditions. The WEHI-S cell line is a highly TNF-sensitive subclone of WEHI 164 murine fibrosarcoma cells (15). WEHI-S cells have been transfected with human hsp70

and hsp27 genes along with plasmid pSV2neo conferring G418 resistance by electroporation (14). Clones Wn-101, Wn-102, and Wn-104 are randomly chosen G418-selected control cell lines transfected with pSV2neo. G418-selected clones Wn-113, Wn-117, and Wn-1112 express exogenous human hsp70, and clones Wn-136, Wn-137, and Wn-139 express exogenous human hsp27 constitutively as analyzed by Western blot analysis (14). Clone Wn-172 expresses antisense hsp70 RNA and reduced levels of endogenous hsp70 as analyzed by Northern blot and immunoprecipitation analyses, respectively (14). The transfected clones used in this study include all the positive clones obtained in the transfection experiment.

Cells were cultured in complete medium, DME (Gibco Ltd., Paisley, UK) supplemented with 10% heat-inactivated FCS (Gibco Ltd.), glutamine, and antibiotics as described previously (14). Medium used for transfected cell lines was further supplemented with 0.2 μg/ml G418 (Gibco Ltd.).

Effector Cells. Fresh heparinized blood was from healthy laboratory personnel. Mononuclear cells were obtained by Lymphoprep gradient centrifugation (Nycomed Pharma AS, Oslo, Norway). To enrich LGLs, mononuclear cells were incubated on petri dishes for 1 h at 37°C at the concentration of 1–3 $\times$ 10^5 cells/cm^2. Subsequently, the nonadherent cells were washed and centrifuged in discontinuous Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradients ranging from 52.5% (vol/vol) to 42.5% with 2.5% concentration steps. Fractions, layering the 45–47.5% areas, were collected and washed twice. They consisted of 70–85% LGLs as judged morphologically from Giemsa-stained cytocentrifuge cell smears. To obtain LAK cells, LGLs were resuspended at the concentration of 1–2 $\times$ 10^6 cells/ml in complete medium supplemented with IL-2 (300 U/ml; EuroCetus Co., Amsterdam, Holland). After 3–5 d in culture, cells were washed twice before experiments.

To obtain monocytes, mononuclear cells were centrifuged twice through a Percoll layer (43%). The cell layer in the interphase was collected, washed, and incubated on petri dishes for 1 h at 37°C at the concentration of 1–3 $\times$ 10^5 cells/cm^2. After washing of the nonadherent cells, adherent cells were scraped using a rubber policeman, washed, resuspended in complete medium, incubated overnight at 4°C, and washed once before experiments. As judged morphologically the obtained fraction consisted of 75–90% monocytes.

Cytotoxicity Assays. Chromium release cytotoxicity assays were performed as described previously (10, 12). TNF inhibitors, mAb specific for TNF-α (kindly provided by Dr. G. R. Adolph, Ernst-Boehringer Institute, Vienna, Austria) and IgG heavy chain–TNF receptor chimeric protein (16; kindly provided by Dr. B. Beutler, Southwestern University, Dallas, TX) were mixed with effector cells 30 min before the experiment. Inhibitors were used at the concentrations capable of neutralizing 50 ng/ml of TNF-α (which is significantly more TNF-α than the amount of monocytes used in the assay can produce).

E/T Cell Binding Assay. The assay was set up in triplicate in 96-well microtiter plates (Greiner Gmbh, Frickenhausen, Germany). Target cells, (1,000 cells in 50 μl of complete medium per well) were added to wells, and after 1 h at 37°C indicated amounts of monocytes were added in 50 μl of complete medium. Monocytes bound to target cells (minimum of 500 target cells) were counted after incubation for 90 min at 37°C in a light microscope.

Western Blot Analysis. Cells were washed twice with cold PBS, lysed in Laemmli sample buffer (17), homogenized by passing through a 25-gauge needle, and boiled for 3 min. Cells were heat-shocked by incubating (1–3 $\times$ 10^6 cells/ml complete medium) for 1 h at 43°C and for 2 h at 37°C followed by washing and lysis as above. Lysates from 10^6 cells/lane were subjected to electrophoresis through 10% SDS-PAGE, and blotted to a nitrocellulose filter (Schleicher & Schuell, Inc., Dassel, Germany) as described previously (14). Filters were incubated at 37°C for 1 h in blocking buffer (3% BSA in PBS), and 2 h in diluting buffer (1% BSA in PBS) containing a 1:500 dilution of mAb specific for heat-inducible hsp70 (Amersham Corp., Amersham, UK) or a 1:1,000 dilution of mAb specific for human hsp27 (clone G3.1, StressGen, Victoria, British Columbia, Canada). After three 15-min washes in Tween buffer (0.2% Tween in PBS), filters were incubated for 1 h at 37°C in diluting buffer containing a 1:1,000 dilution of peroxidase-conjugated rabbit IgG against mouse IgG (Dako-Immunoglobulins A/S, Glostrup, Denmark). After three 15-min washes in Tween buffer, they were exposed for 2 min to citric acid–phosphate buffer (0.1 M citric acid, 0.1 M disodium hydrogen phosphate, pH 5) supplemented with 0.67 μl/ml of 30% hydrogen peroxide and 1:3 (vol/vol) of DONS-TMB (0.02 M dioctyl sulfosuccinate, 0.01 M 3,3',5,5'-tetramethyl benzidine in ethanol) and rinsed in distilled water.

Statistical Analysis. A two-tailed t test was used to test the statistical significance of the results.

Results and Discussion

Effects of hsp27 and hsp70 on Cellular Sensitivity to Monocytes. We have previously shown that a short heat treatment of tumor cells protects them from subsequent exposure to monocytes (12). To study the role of individual hsp's in this phenomenon, we tested the sensitivity of transfected WEHI-S cells with altered levels of hsp70 and hsp27 to monocyte-mediated cytotoxicity. Parental WEHI-S cells were effectively killed by unstimulated monocytes in a 7-h cytotoxicity assay in the absence of metabolic inhibitors (Fig. 1 *a*). Three control cell lines transfected with pSV2neo alone (Wn-101, Wn-102, and Wn-104) were as sensitive to monocytes as parental cells, indicating that transfection procedure and selection of clones by G418 had no effect on cellular sensitivity to monocytes (Fig. 1 *a*). As shown for one representative clone (Wn-102), 1-h pretreatment of control cells at 43°C 1 h before the experiment rendered them almost completely resistant to monocyte cytotoxicity.

All three cell lines constitutively expressing human hsp70 (Wn-113, Wn-117, and Wn-1112) were as resistant to monocytes as heat-treated control cells (Fig. 1, *a* and *b*). Moreover, cells expressing antisense hsp70 RNA and thereby reduced levels of endogenous hsp70 protein (Wn-172) were rendered significantly more sensitive to monocytes than control cells (Fig. 1 *b*).

The overexpression of low molecular weight stress protein hsp27 also rendered cells slightly but significantly more resistant to monocyte killing (Fig. 1 *c*). All three clones (Wn-136, Wn-137, and Wn-139) constitutively expressing human hsp27 were from 15 to 35% more resistant to monocytes than control cells.

WEHI-S cells used in this study have been obtained from WEHI 164 cells by two subclonings and selections for sensitivity to TNF-α (15). Original WEHI 164 cells have been reported to be resistant to unactivated monocytes in a 7-h assay in the absence of metabolic inhibitors (18). TNF-sensitive subclone WEHI-S used in this study was, however, effec-

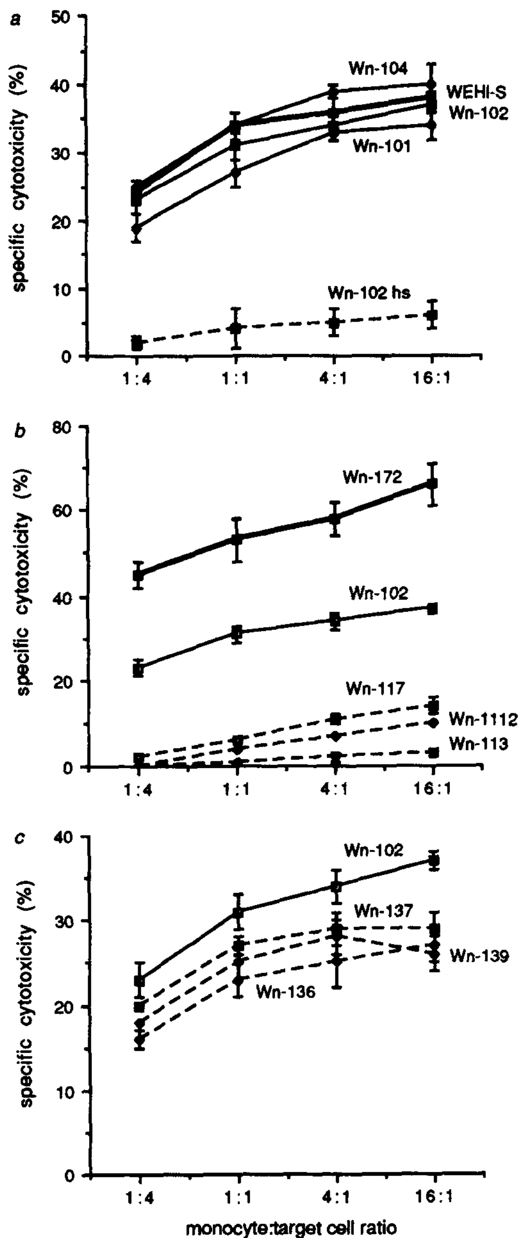

Figure 1. Effect of modified levels of hsp70 and hsp27 on WEHI-S cell sensitivity to monocytes. Sensitivity of transfected clones to freshly isolated monocytes at indicated E/T cell ratios was measured in a 7-h chromium release assay. Clones used: (*a*) parental WEHI-S cells and control clones transfected with pSV2neo (Wn-101, Wn-102, and Wn-104). When indicated "hs," cells were heat-shocked for 1 h at 43°C 1 h before the experiment. (*b*) Clones overexpressing hsp70 (Wn-113, Wn-117, and Wn-1112) and a clone expressing antisense hsp70 (Wn-172). One of the control clones (Wn-102) is shown for comparison. (*c*) Clones overexpressing hsp27 (Wn-136, Wn-137, and Wn-139) and one of the control clones (Wn-102). The values represent means of a triplicate experiment ± SD. Experiments were performed from four to six times with essentially the same results.

tively killed by unstimulated monocytes in a similar assay, suggesting that selection for TNF sensitivity also selects for sensitivity to monocytes. WEHI-S cells and especially Wn-172 cells, which are killed by monocytes at E/T cell ratios as low as 1:100 (data not shown), can prove useful as tools for measuring the activity of freshly isolated monocytes in a short-term assay.

TNF-α in Monocyte-mediated Cytotoxicity. TNF-α is one of the major mediators of monocyte cytotoxicity (3, 4, 8). Our earlier data indicate that overexpression of hsp70 but not that of hsp27 renders cells resistant to TNF-α-mediated cytotoxicity (14). Thus, hsp70-mediated resistance to TNF-α could partly explain the resistance of Wn-113, Wn-117, and Wn-1112 cells to monocytes. However, as cells overexpressing hsp27 have unchanged sensitivity to TNFs but are partially protected from monocytes, other mediators of cytotoxicity have to be involved in killing of WEHI-S cells by monocytes. This conclusion is further supported by data showing that mAb against TNF-α and a strong TNF inhibitor, IgG-TNF receptor chimeric protein (16), at concentrations capable of neutralizing 50 ng/ml of TNF-α could block only 60% of monocyte-mediated lysis of control cells (Fig. 2). TNF inhibitors had no significant effect on the sensitivity of Wn-1112 to monocytes, as could be expected by the fact that these cells are almost resistant to TNFs. Interestingly, in the presence of TNF inhibitors, Wn-137 cells were as resistant as Wn-1112 cells and significantly more resistant than control cells to monocyte-mediated cytotoxicity. This supports the idea that overexpression of hsp27 protects WEHI-S cells from non-TNF-mediated toxicity of monocytes.

Effect of hsp27 and hsp70 on Cellular Sensitivity to Hydrogen Peroxide. Production and release of free oxygen radicals is a part of the lytic program used by monocytes to kill tumor cells (4, 19). Therefore, we tested the sensitivity of our cell lines with altered sensitivities to monocytes to hydrogen peroxide. Control-transfected cells had a similar sensitivity to hydrogen peroxide as WEHI-S cells (data not shown), being

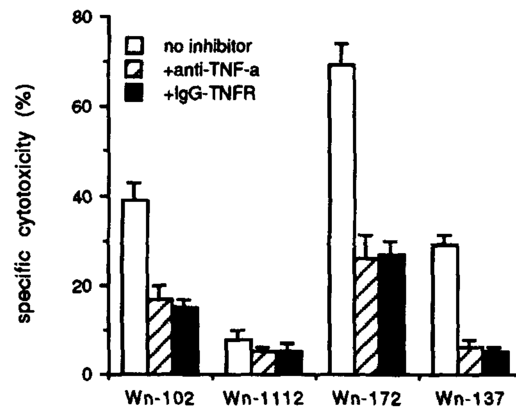

Figure 2. Inhibition of monocyte-mediated lysis of tumor cells by neutralizing TNF-α antibody or IgG-TNF receptor chimeric protein. Sensitivity of tumor cells to monocytes at an E/T cell ratio of 16:1 in the absence (*open bars*) or presence of TNF inhibitors (mAb to TNF-α; *hatched bars*; IgG-TNF receptor chimeric protein; *filled bars*) was measured in a 7-h chromium release assay. Clones used: control cells (Wn-102), cells overexpressing hsp70 (Wn-1112), cells expressing antisense hsp70 (Wn-172), and cells overexpressing hsp27 (Wn-137). The values represent means of a triplicate experiment ± SD. Experiments were repeated twice with essentially the same results.

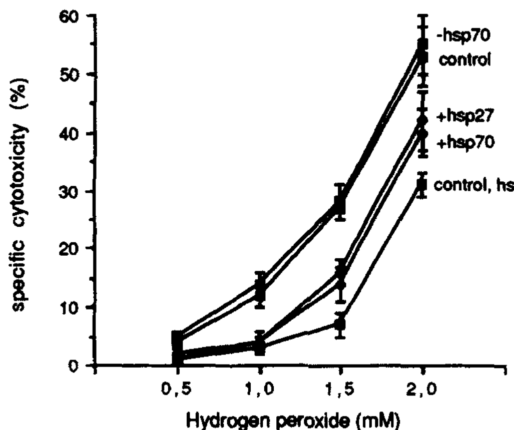

Figure 3. Protection of tumor cells from toxicity of hydrogen peroxide by overexpression of hsp's. Sensitivity of various clones with altered levels of hsp's to indicated concentrations of hydrogen peroxide was measured in a 5-h chromium release assay. The values represent means of results from triplicate experiments with three different clones; control (Wn-101, Wn-102, and Wn-104); +hsp70 (Wn-113, Wn-117, and Wn-1112); +hsp27 (Wn-136, Wn-137, and Wn-139) ± SD except for *–hsp70*, which represents means of three different triplicate experiments using Wn-172 cells ± SD. When indicated, *hs* cells were heat-shocked for 1 h at 43°C 1 h before the experiment. Experiment was repeated twice with essentially the same results.

sensitive to cytotoxicity of hydrogen peroxide at concentrations >0.5 mM in a 5-h chromium release assay (Fig. 3). Induction of heat-shock response by pretreatment of control cells for 1 h at 43°C 1 h before the experiment protected them significantly against hydrogen peroxide (Fig. 3). The results showing that overexpression of either hsp70 or hsp27 protects cells partially from toxic effects of hydrogen peroxide suggest that these hsp's are partly responsible for the heat-induced protection. Surprisingly, inhibition of endogenous hsp70 synthesis by antisense hsp70 RNA failed to affect the sensitivity of WEHI-S cells to hydrogen peroxide under the assay conditions used (Fig. 3).

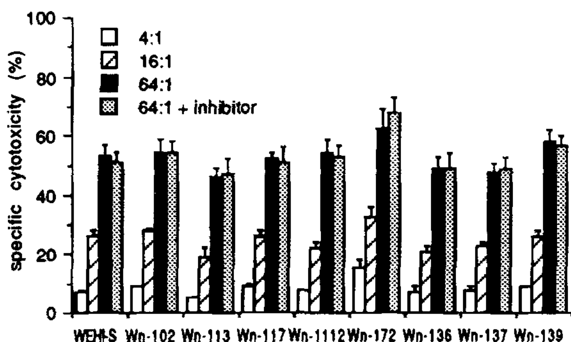

Figure 4. Effect of modified levels of hsp70 and hsp27 on WEHI-S cell sensitivity to LAK cells. Sensitivity of transfected clones of WEHI-S cells to LAK cells at indicated E/T cell ratios was measured in a 4-h chromium release assay. Clones used were as in Fig. 1. IgG-TNF receptor chimeric protein (16) was used to inhibit TNF-mediated cytotoxicity (*dotted bars*). The values represent means of a triplicate experiment ± SD. Experiments were performed four times with essentially the same results.

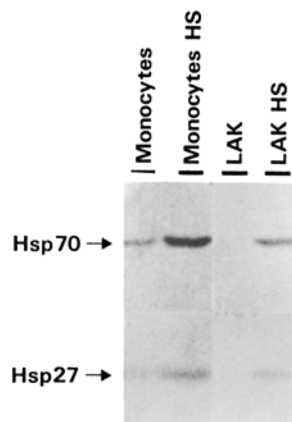

Figure 5. Expression of hsp27 and hsp70 in monocytes and LAK cells. Western blot showing expression of major heat-inducible hsp70 and low molecular weight hsp27 in freshly isolated monocytes and LAK cells. When indicated *HS* cells were heat-shocked for 1 h at 43°C 2 h before the lysis.

Hsp27-associated and part of the hsp70-associated tumor cell resistance to monocyte cytotoxicity may be due to the improved tolerance to oxidative stress. Furthermore, it is interesting to note that intracellular free radical production has been suggested to be part of the lytic pathway induced by TNFs (20). Thus, hsp70-associated resistance to oxidative stress may also partly explain hsp70-mediated TNF resistance.

Binding of Monocytes to Target Cells. No significant differences were found in the ability of monocytes to bind to different clones used in this study at E/T cell ratios ranging from 1:5 to 20:1 (data not shown). Thus, differences in the sensitivity of various clones to monocyte cytotoxicity are likely to be due to resistance mechanisms required after effector cell binding to target cells.

Effects of hsp27 and hsp70 on Cellular Sensitivity to LAK Cells. Parental WEHI-S cells were effectively killed by LAK cells in a 4-h cytotoxicity assay (Fig. 4). Nonstimulated LGLs were ineffective (specific lysis, <10%) in a similar assay at E/T cell ratios up to 100:1 (data not shown). Interestingly, the sensitivities of all clones with altered levels of hsp70 and hsp27 to cytotoxicity mediated by LAK cells were unchanged (Fig. 4). These results suggest that the mechanism of cytotoxicity of LAK cells differs essentially from that of monocytes and is independent of TNF and free radicals. This is further supported by the results showing that addition of TNF inhibitor to the assay had no effect on the lysis of target cells by LAK cells (Fig. 4).

Expression of hsp's in Monocytes and LAK Cells. The data presented above clearly show that both hsp27 and hsp70 protect tumor cells from cytotoxicity mediated by monocytes but not by LAK cells. Therefore, it was interesting to study whether effector cells themselves express hsp's and thereby protect themselves against toxic mediators. Interestingly, the result of a Western blot analysis showed that freshly isolated monocytes, indeed, have detectable levels of the major heat-inducible hsp70 and hsp27 (Fig. 5). Expression of hsp's was also seen in partly purified monocytes after Lymphoprep and Percoll gradient centrifugations performed at 4°C, indicating that the expression was not due to the induction associated with purification (data not shown). In LAK cells hsp27 and hsp70 could only be detected after heat-shock treatment (Fig.

5). Freshly isolated LGLs were negative and monocytes cultured for 3 d were positive for both hsp's tested, suggesting that differences in expression of hsp's between monocytes and LAK cells is not due to different times in culture (data not shown).

Heat-shock response is characterized by a rapid and specific synthesis of hsp's when cells or whole organisms are abruptly exposed to supraoptimal temperatures or various other environmental stresses (1). Synthesis of hsp's has for a long time been associated with the acquired thermotolerance seen in cells after induction of heat-shock response. Recent results from transfection studies have given direct evidence in support of this hypothesis. Overexpression of human hsp27 and human hsp70 in Chinese hamster ovary cells and rat fibroblasts, respectively, renders these cells resistant to subsequent exposure to heat (21, 22). Our results show that the protective role of hsp's is not limited to acquired thermotolerance, but that they also protect cells from immunological effector cells and oxidative stress. Whether the mechanisms involved in protection from such diverse stresses are similar remains to be studied and is expected to shed light on the modes of action of hsp's. Interestingly, expression of both hsp27 and hsp70 is more commonly seen in tumors than in benign tissues of same origin (23–26). Therefore, it is interesting to speculate that these proteins may play a crucial role in the early stages of tumorigenesis by allowing tumor cells to escape from immunological surveillance. Moreover, expression of hsp27 and hsp70 in freshly isolated monocytes but not in lymphocytes suggests that hsp's may also protect monocytes from the highly toxic metabolites that they themselves produce upon activation.

We thank B. Beutler for IgG-TNF receptor chimeric protein and constructive criticism on the manuscript; G. R. Adolf for recombinant TNF-α and its mAb; P. A. Bauer for technical assistance; and J. Zeuthen for his support.

This work was supported by grants from the Danish Cancer Society and the Novo Foundation.

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Received for publication 7 August 1992 and in revised form 6 October 1992.

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