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M M Simon, … , M Jäättelä, T Schwarz

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Research Article

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Heat Shock Protein 70 Overexpression Affects the Response to Ultraviolet Light in Murine Fibroblasts

Evidence for Increased Cell Viability and Suppression of Cytokine Release

Manuel M. Simon,*‡ Arnold Reikerstorfer,§ Agatha Schwarz,* Christine Krone,* Thomas A. Luger,* Marja Jäättelä,‖ and Thomas Schwarz*

**Ludwig Boltzmann Institute of Cell Biology and Immunobiology, Department of Dermatology, University Münster, D-48149 Münster, Germany; ‡Institute of Biochemistry and Molecular Cellbiology, Biocenter, University of Vienna, A-1030 Vienna, Austria; §Research Institute of Molecular Pathology, A-1030 Vienna, Austria; and ‖Department of Tumor Cell Biology, Danish Cancer Society Research Center, Division of Cancer Biology, DK-2100 Copenhagen, Denmark*

Abstract

To elucidate cellular concepts for protection against ultraviolet (UV) light we investigated the effect of heat shock protein 70 (hsp70) overexpression on cell viability and on the secretion of UV-inducible immunological cytokines. Transfected murine fibrosarcoma cells (WEHI-S), overexpressing hsp70 or a sham transfected control were used. Overexpression of hsp70 was sufficient to markedly increase cell viability upon treatment with UVB (290–320 nm). Since long wave UV (UVA, 320–400 nm) as well as UVB turned out to stimulate the release of O_2^- radicals we studied the cell viability upon oxidative stress. Hsp70 overexpression increased viability upon treatment with hydrogen peroxide or menadione, but had no influence on UV-induced O_2^- release. UV-light is known to upregulate immunologic and proinflammatory cytokines such as IL-1 and IL-6. Oxidative stress appeared to exert a similar effect. Hsp70 overexpression markedly decreased the release of IL-6 induced by UVA, UVB and oxidative stress. To test whether the hsp70 mediated suppression is confined to events caused by UV-light we determined IL-1–mediated effects. IL-1–induced IL-6 release was reduced by hsp70 overexpression, whereas the IL-1 mediated activation of nuclear factor κB was not affected. Our data suggests that hsp70 plays a central role not only in cell protection against UV-light, but also in the regulation of proinflammatory cytokine release induced by UV-exposure. (*J. Clin. Invest.* 1995. 95:926–933.) Key words: interleukin 1 • interleukin 6 • oxidative stress • NFκB • protection

Introduction

Stress events induce the expression of a set of highly conserved proteins entitled heat shock proteins (hsps)[1] in bacteria, fungi as well as eukaryotes. The expression of these proteins is induced by elevated temperature and a variety of other conditions like heavy metals, viral infections, alcohol, particular cytokines, and growth factors or decreased temperature (1, 2) and has been shown to protect against cytotoxic environmental conditions (3, 4). A cytoprotective function of hsps induced by elevated temperatures against cytolysis by TNF α and β has been previously reported (5, 6). This function has been attributed to one of the major heat shock proteins, hsp70 (7, 8), a member of the 70-kD family of hsps which are proposed to serve as molecular chaperonines (reviewed in 9). However, how hsp70 as well as most of the other heat inducible gene products provide protection remains unclear to date. Recently, a linkage emerged between hsps and immunological functions. Bacterial hsps which share high homology with the mammalian proteins (10) have the capacity to serve as potent antigens (11). In addition, hsps are involved in autoimmune reactions (12). On the other hand, heat-treated antigen presenting cells exhibit an impaired ability to stimulate the specific T cell response (13). The cooperation of hsps and the cytokine network has been illustrated by the finding that these proteins were shown to be under control of certain immunological cytokines (14) and that a member of the hsp70 family was suggested to be involved in antigen processing (15).

Ultraviolet (UV) light represents one of the most important environmental impacts for humans. This subject continues to gain even more prominance due to the increased UV-influx caused by ozone depletion (16) and due to the fact that the majority of the population continues to engage in extensive UV-exposure. UV-light, however, can represent a hazard to human health by causing induction and promotion of cancer, cell death, inflammation, and immunosuppression (17). Normal cells are equipped with programs and instruments to lower their sensitivity against toxic environmental conditions, and hsps appear to be integral to these processes. Since hsps can exhibit cytoprotective functions we were interested to study the effect of hsps on the cellular response to UV-light. A murine fibrosarcoma cell line (WEHI-164) derived clone (WEHI-S) transfected with the entire human coding region (10) under control of the SV40 early promoter and a sham transfected control were used as described (7). Here, we demonstrate hsp70 overexpression to be sufficient to provide protection against treatment with up to lethal doses of UVB-light (290–320 nm) as well as against exposure to oxidative stress. However, the generation of reactive oxygen intermediates (ROI), early cellular responses to UV-irradiation, is not influenced by high hsp70 concentrations. Moreover, the present data indicate that the release of IL-1 and IL-6, proinflammatory cytokines secreted in response to these types of

Address correspondence to Thomas Schwarz, M.D., Department of Dermatology, University Münster, Von-Esmarchstrasse 56, D-48149 Münster, Germany. Phone: 251-836565; FAX.: 251-836522.

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1. *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; hsps, heat shock proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; ROI, reactive oxygen intermediate; UV, ultraviolet light.

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stress, is reduced when hsp70 is overexpressed. Taken together, our data provide evidence that hsp70 represents an important regulatory factor controlling the release of stress response peptides and that hsp70 is able to modify the cellular response to UV-light.

Methods

Cell culture. An isogenic set of transfected WEHI-S clones was used throughout the study. The WN-113 clone was transfected with the human hsp70 gene driven by the SV40 promoter as described (7) along with plasmid pSV2neo. Control cells were transfected with SV2neo alone, a combined culture of 12 single clones with stably transfected neomycin resistance was denominated as WN10×. These cells express transfected neomycin resistance gene coding for amino glycoside phospho transferase and thus were used as a control group of cells transfected with a cDNA encoding a protein considered irrelevant to the UV-response. WN113 cells were repeatedly tested for the expression of transfected hsp70 by Western blot analysis and found to express constitutively high hsp70 levels as already reported (7). In contrast, WN10× cells did not express human hsp70. Cells were propagated in DME supplemented with 10% FCS, 1% antibiotic/antimycotic solution, 2 mM L-glutamine and 200 μg/ml G418 (all from GIBCO BRL, Gaithersburg, MD). The cell density was kept subconfluent, cells were passaged three times per week. Culture medium or PBS used was prewarmed at 37°C to avoid an induction of hsps by cold shock. In all experiments, cells were seeded into dishes at a density of $7 \times 10^5/cm^2$ in supplemented DME but without G418 16 h before the experiment. Heat shock of WN10× cells was performed by placing the culture dishes at 43°C for 1 h and subsequent incubation at 37°C for 1 h. Hydrogen peroxide was purchased from Boehringer Mannheim (Mannheim, FRG). Menadione (2-methyl-1,4-naphtochinone; Sigma Chemical Co., St. Louis, MO) was used from a freshly prepared, sterile filtered 100 mM stock solution. Recombinant murine IL-1α was purchased from Cistron Biotechnology (Pine Brook, NJ).

UV irradiation and light sources. UV-irradiation was performed as described (18). Briefly, cells were seeded in DME containing 10% FCS into dishes at a density of $7 \times 10^5/cm^2$ and grown for 16 h. Immediately before irradiation, cells were washed twice with prewarmed PBS and exposed to the respective UV spectrum through PBS. Warming of the PBS during the irradiation procedure was negligible. UVA irradiation was performed using a Sellas Sunlight 2001 lamp (Sellas Corp., D-58285 Gevelsberg, FRG) emitting wavelengths > 320 nm with an emission peak of 365 nm. The UV energy measured at 365 nm using an UVA-meter (Waldmann, D-89443 Schwenningen, FRG) was 540 W/m^2 at a tube to target distance of 55 cm. For UVB irradiation, a bank of 4 FS20 bulbs (Westinghouse Electric Corp., Pittsburgh, PA) was used which emit most of their energy within the UVB range (290–320 nm) with an emission peak at 313 nm. The UV output measured at 310 nm using an IL1700 research radiometer (International Light, Newport, MA) was 8.0 W/m^2 at a tube to target distance of 28 cm. The highest dose of UVB applied was 280 J/m^2 which requested an exposure time of 35 s. The UVA amount concomitantly emitted measured with the Waldmann UVA-meter at 365 nm was 0.7 J/m^2. Since UVA in such a dose range is biologically not active, the UVA contamination is negligible for the purposes of this study. Immediately after UV-treatment, PBS was replaced by DME containing 10% FCS without G418.

Measurement of superoxide anion (O_2^-). Superoxide anion release was determined on the basis of cytochrome C reduction as described (19). Briefly, cells were seeded in 3.5-cm dishes, washed twice with warm PBS and irradiated through 1 ml PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose. Immediately after irradiation, horse liver cytochrome C (Sigma Chemical Co.) was added to a final concentration of 50 μM. The dishes were placed at 37°C for 30 min. After clearing the supernatant from detached cells by centrifugation, the absorbance of the supernatant was monitored at 550 nm in reference to the isosbestic wavelengths at 540 and 556 nm. The molar extinction coefficient of 0.021 $\mu M^{-1} cm^{-1}$ was used for calculation of the superoxide anion concentration released in 1 h. Hydrogen peroxide release was measured by a horseradish peroxidase coupled color reaction of phenol red as described (20).

Determination of survival rates and measurement of cytokines. Determination of survival rates was performed with MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide) as described (21). The spontaneous proliferation rate of WN113 and WN10× cells was not significantly different. For treatment with hydrogen peroxide appropriate concentrations were added to the culture medium, subsequently cells were incubated for 16 h at 37°C. The same incubation time was used after UV-light treatment. Cells were incubated with menadione at concentrations as indicated for 4 h at 37°C, washed out twice with PBS and then incubated for additional 12 h at 37°C in complete DME. A "zero survival" sample was prepared by complete lysis of cells by two freeze/thaw cycles and treated identically to the other samples. 1 ml of the conditioned medium was then removed for determination of cytokine content. MTT (5 mg/ml solved in PBS) was added to the remaining medium (final concentration of 500 μg/ml) and incubated at 37°C. After color development 1 ml 10% SDS, 50% formamide pH 4.7 was added and incubated 5 h at 37°C to lyse the cells and solve the formazane precipitate. The clear solution was measured at 595 nm against the "zero survival" control. Survival rates were calculated as percent OD595 of the untreated cells normalized to the "zero survival" value, the resulting survival rates represent the metabolic activity remaining after 16 h of of culturing. When WN113 or WN10× cells after irradiation or oxidative treatment were cultured for a longer period of time the metabolic activity increased again and cells could reach confluency indicating that they were not blocked to enter mitosis. Survival rates were determined in triplicates.

IL-6 was measured using the murine hybridoma cell line B9 as described (22) except that the proliferation was monitored with MTT added to the B9 cells to a final concentration of 1 mg/ml. After color development cells were lysed for 3 h at 37°C by the addition of 1 volume of 10% SDS, 50% formamide pH 4.7 for 4 to 16 h. Plates were read in an ELISA reader (Biorad, Munich, FRG) at 595 nm, IL-6 content of the supernatants was calculated by comparison with an IL-6 standard curve.

IL-1 was measured by a bioassay using D10 [N4]M cells kindly provided by Dr. S. J. Hopkins (University of Manchester, U.K.) as described (23). Proliferation rate was determined on base of a XTT ([sodium 3(1-phenylaminocarbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro]-benzenesulfonate) color reaction (EZ4U; Biomedica Corp., Vienna, Austria). All measurements of cytokines were done in triplicates. Experiments were performed at least three times and representative experiments are presented throughout the study.

Electrophoretic mobility shift assay (EMSA). 1×10^6 cells were washed with PBS and detached from the dishes with a rubber policeman. Preparation of extracts was essentially performed as described (24). Briefly, cells were allowed to swell in hypotonic buffer (10 mM Hepes, pH 7.8, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF) for 15 min on ice. After addition of NP-40 (Sigma Chemical Co.) to a final concentration of 0.5%, nuclei were collected by centrifugation (500 *g*, 5 min). Nuclear proteins were extracted from the remaining pellet with hypertonic buffer (20 mM Hepes, pH 7.9, 350 mM NaCl, 1 mM DTT, 1 mM PMSF). After clearing the extracts at 8,000 *g* for 5 min, the protein content of all samples was adjusted to 2 μg/μl protein (determined by Biorad protein assay; Biorad, Munich, FRG). A synthetic double stranded oligonucleotide with 5′ protruding XhoI compatible ends was provided by H. Zeller (Department of Experimental Dermatology, University of Muenster). It is derived from the human IL-6 promoter (−121 to −138): 5′TCGAGTGTGGGATTTTCCCATGAC3′ (NFκB consensus binding motif underlined). For nonspecific competition the following double stranded oligonucleotide was used: 5′TCG-ATGGAGTCTCCA3′. Binding reactions were performed with 6 μg of protein, ^{32}P-labeled (T4-polynucleotide kinase; Boehringer Mannheim) double-stranded oligonucleotide, 50 mM KCl and 1 μg poly dIdC

(Boehringer Mannheim), for 30 min at room temperature and electrophoretically separated on a 5% acrylamide gel.

RNAse protection assay. For determination of c-fos mRNA steady state concentrations a RNAse protection assay was performed as described (25). The murine c-*fos* antisense RNA probe was transcribed from a XmaI–EcoRI fragment from the vector pGc-fos (26) inserted into pSP64 (Promega, Madison, WI) using the SP6 promoter. The ^{32}P-labeled probe contained 340 nucleotides and gave rise to a c-*fos* mRNA protected fragment of 177 nucleotides. As an internal control we used a ^{32}P-labeled anti-sense probe of 345 nucleotides from an EcoRI–StyI fragment of the S16 cDNA which resulted in a 155 nucleotides S16 mRNA protected fragment as described (27).

Statistics. All values are expressed as means±standard deviation. Representative experiments were performed at least three times independently. Each of these was set up in triplicates. Amounts of IL-1 or IL-6 were calculated from a standard curve. For the determination of the statistical significance of groups a one way ANOVA and student's t test were used. Data obtained with WN10× cells were compared to those obtained with WN113 cells. *P* values of $P < 0.001$ were considered to be statistically significant.

Results

Hsp70 overexpression is sufficient for protection against UVB-light. For a variety of different types of lethal stress, overexpression of hsps by elevated temperatures has been shown to increase cell viability (3, 4). We addressed the question whether overexpression of hsp70 is sufficient to provide protection against UV-light. Cell damage as a result from exposure to short wavelength UVC (240–290 nm) as well as UVB (290–320 nm) light is known to include events such as pyrimidine dimer formation, lipid peroxidation and also protein denaturation. To analyse the effect of hsp70 overexpression on UVB-induced cell death, increasing doses of UVB-light were applied to WN113 cells which overexpress hsp70, the sham transfected WN10× cells which only express the amino glyocside phospho transferase and WN10× cells which were pretreated with heat shock. Fig. 1 demonstrates the result of a representative UVB-killing assay. Cells expressing high hsp70 levels showed markedly increased survival rates compared to the sham transfected cells. The half maximal lethal UVB-dose for WN113 cells was approximately twofold of that for WN10×. Induction of hsps by heat treatment of WN10× cells even slightly improved the viability after UVB-irradiation compared with the hsp70 overexpressing clone suggesting the presence of synergistically acting protective factors up-regulated by heat–shock treatment. Irradiated WN113 or WN10× cells with decreased metabolic activity were not blocked in entering mitosis since upon prolonged culturing cells were able to reach confluency. This was also reflected by a respective increase of the metabolic activity (data not shown). Thus we conclude, that hsp70 overexpression mediated protection is not due to a delayed cell death, but protects cells and enables long term survival. Killing experiments were also performed with UVA-irradiation (320–400 nm). To avoid a significant warming of PBS during the treatment UVA-doses only up to 800 kJ/m^2 were used, which, however, did not affect cell viability (data not shown).

UV-light induced ROI release is not abrogated by hsp70 overexpression. In the last decade, some evidence emerged for the involvement of reactive oxygen intermediates (ROI) in UV-light mediated cell damage (28); superoxide anion is released immediately from the cells upon UV-exposure apparently as a direct response to UV-irradiation. Therefore, we next studied

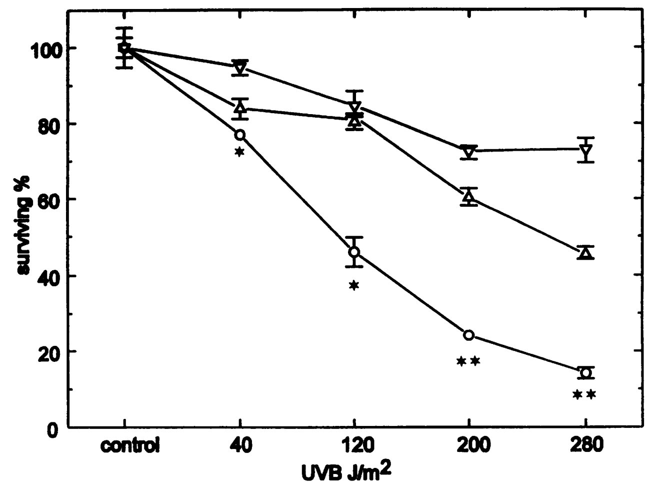

Figure 1. Survival rates after UVB irradiation. Control WN10× (○), hsp70 overexpressing WN113 (△), and WN10× exposed to 60 min of heat treatment (▽) were irradiated through prewarmed PBS with increasing doses of UVB-light. After treatment PBS was replaced by DMEM containing 1% FCS and subsequently incubated at 37°C for 16 h. After removal of half of the supernatant for determination of IL-6, total metabolic activity was measured by a MTT assay. Data shown are expressed as percent of metabolic activity of the untreated control. Statistical significance was calculated with values obtained from WN10× compared to WN113 cells treated with the same UV dose. (*$P < 0.001$, **$P < 0.0001$). Error bars represent the standard deviation of three samples.

whether overexpression of hsp70 affects the UVA- or UVB-mediated release of ROI. Fig. 2 demonstrates a representative determination of UVA- and UVB-mediated superoxide anion release by WN10× and WN113 cells. Both UV-spectra induced O_2^- release from the cells in a dose dependent manner. The O_2^- release from WN113 cells under these conditions did not significantly differ from that of sham transfected WN10× cells suggesting hsp70 not to be involved in downregulation of cellular ROI production and thereby providing a cytoprotection against UV-irradiation. We also tested for hydrogen peroxide release after UV-irradiation, but the concentrations were below the detection limit of our system.

Oxidative stress-induced cell damage is reduced by hsp70 overexpression. Since antioxidant vitamins such as α-tocopherol (29) or ascorbate (30) as well as the radical scavenging chemical *N*-acetylcysteine (M. Simon, unpublished observations) were shown to provide protection against UV-light induced cell death, we next investigated the effect of hsp70 overexpression on cell damage induced by oxidative stress. WN113, pre-heat shocked WN10× and untreated WN10× cells were exposed to increasing concentrations of oxidants. Here, we used hydrogen peroxide, known to form hydroxyl radicals by a Fenton type reaction and menadione, a redox cycling chinone. Reduced menadione in reaction with molecular oxygen leads to the production of superoxide anion from which via Fenton chemistry highly reactive hydroxyl radicals can be generated (31). Fig. 3 shows killing assays with increasing doses of both compounds. Hsp70 overexpression provides a profound cytoprotection upon both hydrogen peroxide and menadione treatment. These results are in accordance to previous experiments with hydrogen peroxide using a Cr release assay (8). Pre-heat shock of WN10× cells before oxidative treatment showed sur-

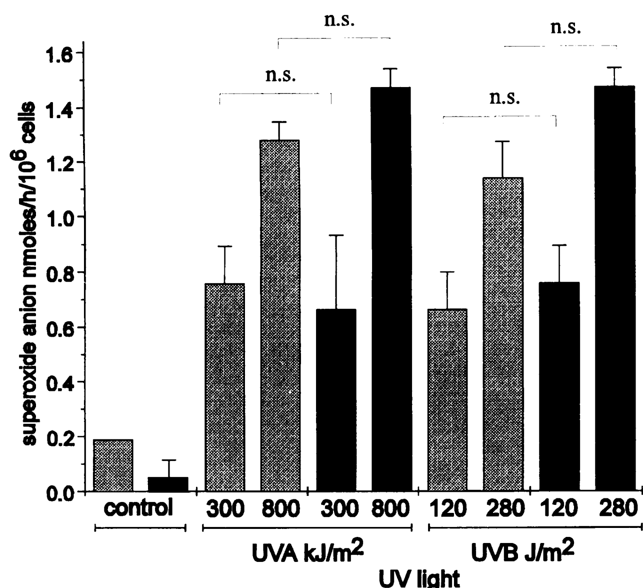

Figure 2. Determination of superoxide anion release after UVA and UVB treatment. Cells were irradiated through 1 ml PBS containing 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Immediately after UV exposure, cytochrom C was added followed by incubation at 37°C for 30 min. Superoxide anion concentration was determined by photometric absorbance of reduced cytochrom C as described. Dotted bar wild type WN10×, black bar hsp70 overexpressing WN113. Statistical significance was calculated with values obtained from WN10× compared with WN113 cells treated with the same UV dose. Error bars represent the standard deviation of three samples.

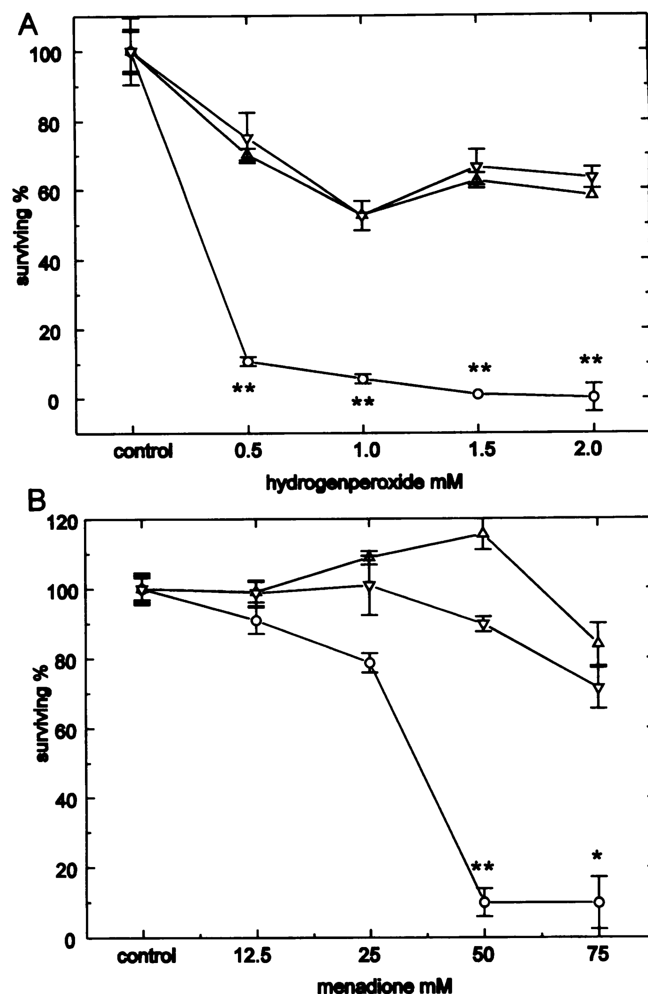

Figure 3. Survival rates after oxidative stress. Control WN10× (○), hsp70 overexpressing WN113 (△) and WN10× exposed to 60 min heat treatment (▽) were incubated with hydrogen peroxide (*A*) or menadione (*B*) by adding the concentrations as indicated to the culture medium. Cells treated with hydrogen peroxide were incubated for 16 h at 37°C, samples treated with menadione were incubated for 4 h at 37°C, then menadione was washed out twice with PBS, and incubated for additional 12 h at 37°C in complete DME. Thereafter, 1 ml supernatant culture medium was removed for determination of the IL-6 content. The total metabolic activity was measured by MTT assay. Data shown are expressed as percent of metabolic activity of the untreated control. *P* values were calculated with metabolic activity values of WN10× versus WN113 cells treated with the same dose of hydrogenperoxide or menadione. ($*P < 0.001$, $**P < 0.0001$). Error bars represent the standard deviation of three samples.

vival rates not significantly different from that of the hsp70 overexpressing strain WN113.

IL-6 release induced by UV-light or oxidative stress is reduced by overexpression of hsp70. UV-light previously was shown to trigger the induction of cytokines including IL-6 (22, 32) which appears to be involved in local and systemic inflammation following UV-exposure (33). Since ROI are immediate products of UV-irradiation we next investigated whether ROI could act as second messengers for the induction of IL-6 and whether UV or oxidative stress mediated IL-6 release is influenced by increased cellular hsp70 concentrations. In Fig. 4 the IL-6 release by WN10× and WN113 cells upon different types of stress (UVA, UVB and hydrogen peroxide) from representative experiments is shown. Similar to UV and oxidative stress in vitro, heat–shock treatment of WN10× cells was found to activate IL-6 release, too. Consequently, cells treated by this stress type failed to further respond with enhanced IL-6 release mediated by subsequent UV or hydrogen peroxide treatment (data not shown). The data presented in Fig. 4 and the cell viability data shown in Figs. 1 and 3 *A*, respectively, were obtained from the very same experiment. UVA (panel *A*), UVB (panel *B*) as well as the hydrogen peroxide (panel *C*) mediated IL-6 release was markedly decreased in WN113 cells which express enhanced amounts of hsp70 compared with the sham transfected WN10× cells. Interestingly, WN113 cells were not insensitive to UV-light or oxidative stress with respect to IL-6 secretion, but revealed an increased stimulation threshold for activation of the stress response. When the survival rates of WN10× and WN113 cells after UVB or hydrogen peroxide treatment depicted in Figs. 1 and 3 *A* and the amounts of IL-6 released by these stimuli (Fig. 4, *B* and *C*) were compared, a negative correlation can be observed. This may suggest that the fewer cells that survive a certain injurious dose, the more stress signal is released. On the other hand, we cannot exclude with absolute certainty that the increased release is a nonspecific manifestation of cell death with release of cytoplasmic contents. Consequently, we were interested to study the effect of IL-1, another stimulus for the IL-6 release.

Influence of hsp70 on stress induced IL-1 and IL-1 controlled signaling pathways. To further characterize the mechanism of the hsp70 mediated suppression of IL-6 response, we investigated the influence of hsp70 on IL-1, another UV-respon-

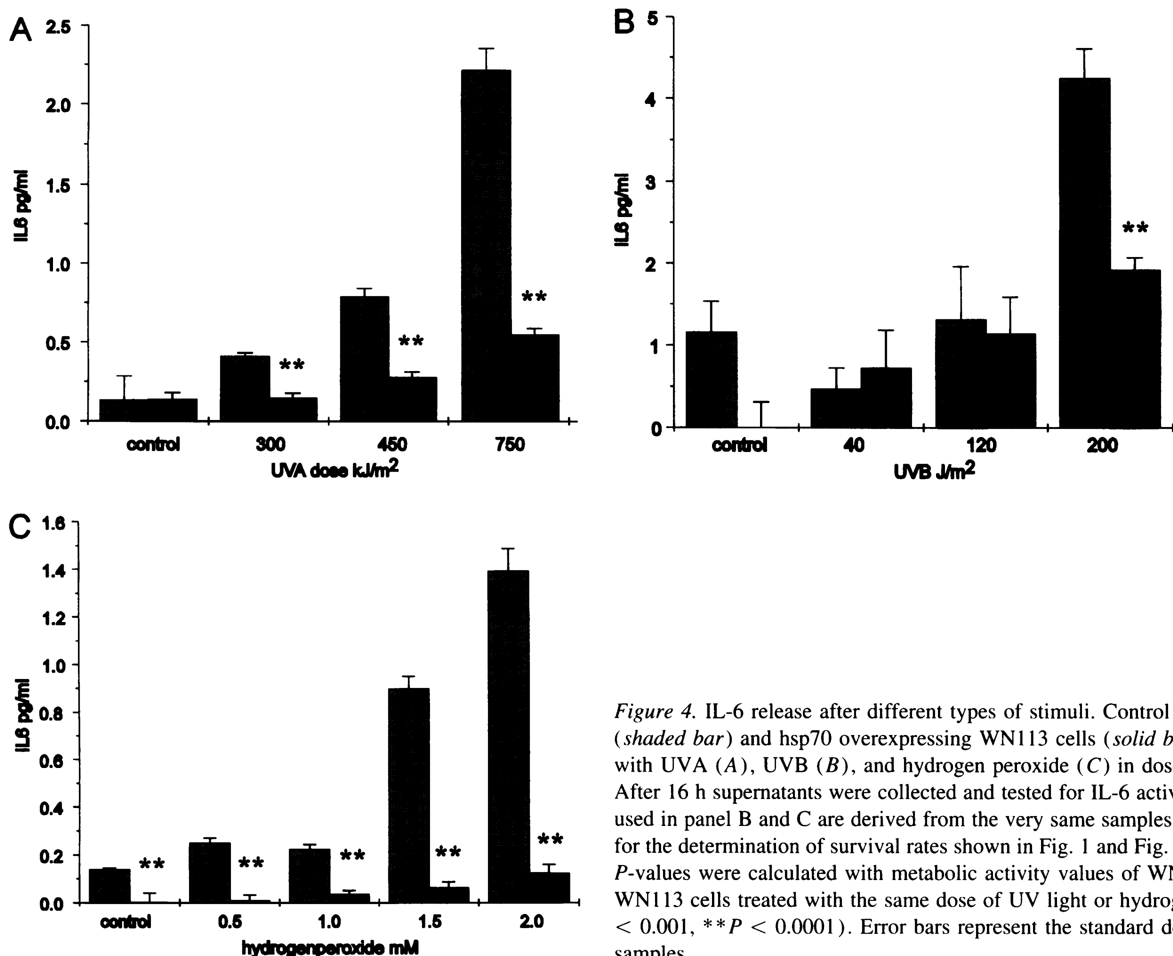

Figure 4. IL-6 release after different types of stimuli. Control WN10× cells (*shaded bar*) and hsp70 overexpressing WN113 cells (*solid bar*) were treated with UVA (*A*), UVB (*B*), and hydrogen peroxide (*C*) in doses as indicated. After 16 h supernatants were collected and tested for IL-6 activity. Supernatants used in panel B and C are derived from the very same samples which were used for the determination of survival rates shown in Fig. 1 and Fig. 2 *A*, respectively. *P*-values were calculated with metabolic activity values of WN10× versus WN113 cells treated with the same dose of UV light or hydrogenperoxide. ($*P < 0.001$, $**P < 0.0001$). Error bars represent the standard deviation of three samples.

sive cytokine which also is able to induce IL-6 (34): First, we investigated whether IL-1 secretion after UVA-irradiation is suppressed when hsp70 is overexpressed. As demonstrated in Fig. 5 *A* the amount of IL-1 released upon UVA-treatment is reduced by overexpression of hsp70. Next, we studied the effect of hsp70 on the IL-1 mediated IL-6 release. Fig. 5 *B* demonstrates that the IL-6 secretion induced by recombinant IL-1α was suppressed in WN113 compared with WN10× cells indicating that hsp70 protected cells also exhibit an increased threshold for the IL-1 stimulus as observed for UV- and oxidative stress signals (see Fig. 4).

One of the biological activities of IL-1 is its ability to activate the immediate early stress response transcription factor NFκB (reviewed in 35). NFκB in its inactive form bound to an inhibitory protein IκB is retained in the cytoplasm until the NFκB-IκB complex dissociates upon different types of stimuli including IL-1 (36), TNF (37), phorbol esters (38), LPS (39), or UV-irradiation (40, 41). Thereafter it migrates into the nucleus, binds to NFκB specific enhancer elements and activates gene transcription. An NFκB recognition sequence has been demonstrated to be an important regulatory element within the IL-6 promoter (42, 43). To elucidate whether the signaling pathway of the IL-1 stimulus leading to enhanced release of IL-6 is blocked when hsp70 is overexpressed, we performed an EMSA using a radioactive-labeled double-stranded DNA fragment of the human IL-6 promoter containing an NFκB binding motif (Fig. 6). Nuclear protein extracts were prepared from WN10× cells (lanes *1* and *2*) and WN113 cells (lanes *3* and *4*) which were left untreated (lanes *1* and *3*) or stimulated with 25 U/ml IL-1α (lanes *2* and *4*) for 90 min. The IL-1–mediated NFκB like binding is demonstrated to be specific by competition with a 50-fold excess of unlabeled NFκB oligonucleotide (lane *5*) and by the lack of competition when a 50-fold excess of a nonspecific oligonucleotide was present during the binding reaction (lane *6*). For the competition (lanes *5* and *6*), nuclear extracts from IL-1α treated WN10× cells (lane *2*) were used. In both cases, the control and the hsp70 overexpressing cells activation of NFκB like binding is induced upon IL-1 treatment, suggesting that at least this stress signaling pathway is not impaired by high hsp70 levels within the cell.

The inability to detect an effect of hsp70 overexpression on IL-1 induced NFκB activation suggests that hsp70 acts beyond the transcriptional control of IL-6. Since many other genes including c-*fos,* metallothionein and collagenase are known to be induced by UV-light (40) we were interested whether hsp70 overexpression interferes with the transcriptional induction of the oncogene c-*fos.* WN113 and WN10× cells were exposed to 100 J/m^2 and 200 J/m^2 UVB, respectively, 1 h later RNA was extracted and c-*fos* expression determined by a RNAse protection assay (Fig. 7). In accordance to previous data (40) c-*fos* was UV-inducible in both WN113 and WN10× cells. However, there was no obvious difference in the degree of c-*fos* induction by UVB-light between WN113 and WN10× cells suggesting that hsp70 overexpression does not influence c-*fos*

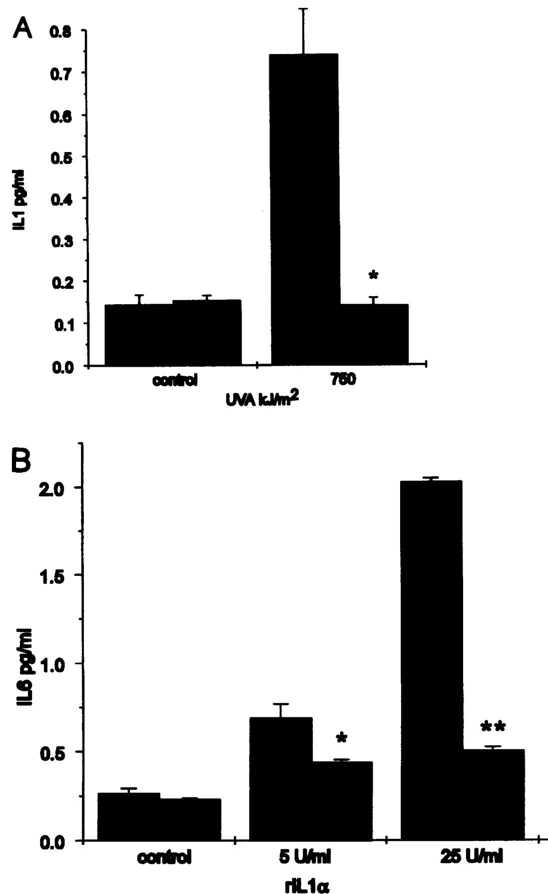

Figure 5. Determination of IL-1 release and effects of IL-1α. (*A*) Control WN10X cells (*shaded bar*) and hsp70 overexpressing WN113 cells (*solid bar*) were exposed to UVA irradiation, supernatants were collected after 16 h for measurement of IL-1 content. (*B*) Recombinant IL-1α was added to wild type WN10X cells (*shaded bar*) and hsp70 overexpressing WN113 cells (*solid bar*), supernatants were harvested after 16 h culture for determination of IL-6 content. Statistical significance was calculated with values of WN10X versus WN113 cells treated with the same dose of hydrogenperoxide or menadione. (*$P < 0.001$, **$P < 0.0001$). Error bars represent the standard deviation of three samples.

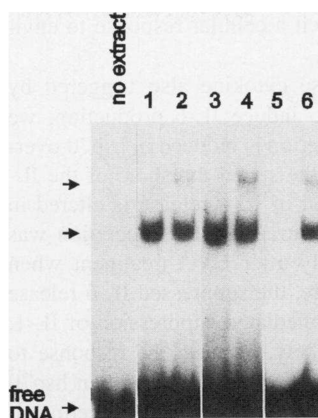

Figure 6. Activation of NFκB like binding by IL-1α. Nuclear extracts prepared from control WN10X (lanes *1* and *2*) cells and hsp70 overexpression WN113 (lanes *3* and *4*) cells were treated with IL-1α (25 U/ml) for 90 min (lanes *2* and *4*) or left untreated (lanes *1* and *3*) and used for EMSA. Arrows indicate the NFκB like binding complex, upper arrow IL-1α inducible, lower arrow constitutive binding. Specificity of the binding is demonstrated by competition with 50 fold molar excess of the NFκB motif bearing unlabeled oligonucleotide (lane *5*) or by addition of 50-fold excess of nonspecific unlabeled oligonucleotide (lane *6*).

mRNA expression. This further supports our suggestion that hsp70 might act at a posttranslational rather than at a transcriptional level.

Discussion

Expression of hsp70 is induced by a variety of stress events (1, 2) and mediates protection against adverse environmental conditions (3, 4). Since it has been shown that hsp70 overexpression alone is sufficient to establish cytoprotection against TNF (7, 8), we studied whether high cellular concentrations of hsp70 will also provide protection against UV-light. We demonstrate that hsp70 overexpression protects against treatment with otherwise lethal doses of UVB-light (290–320 nm) and enables long term survival. Several reports suggest reactive oxygen intermediates (ROI) to represent an immediate byproduct of UVB- as well as UVA- (320–400 nm) irradiation (28). We found that the reduced cell viability after exposure of cells to oxidative stress by menadione or hydrogen peroxide is dramatically improved when hsp70 is overexpressed. However, as shown in Fig. 2 both WN10X and WN113 cells release ROI upon UVA as well as UVB-exposure in a dose dependent manner. Therefore, the stabilizing effect of hsp70 is apparently not due to a down regulation of the ROI metabolism by its overexpression.

Hsp70 overexpression does not give rise to completely UV-resistant cells. In fact, in cells protected in such a way respond in a similar manner to the applied stress, however, the threshold intensity of a certain stress appears to be shifted to higher levels, when hsp70 is overexpressed. Cells exposed to deleterious environmental stress like UV-light activate an immediate early program determining the further fate of the cell. Intracellular protection factors and signaling peptides are produced, and decisions, e.g., to differentiate or to undergo apoptosis are made. Since IL-6 is regarded as one of these stress response signaling factors, we used this cytokine as a marker for a functional cellular stress response. Survival rates were employed as a measure for the degree of stress applied. Taking these two parameters together, our results demonstrate that overexpression of hsp70 not only augments cell viability, but also increases the

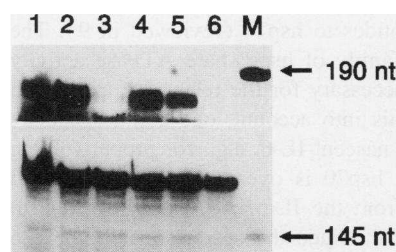

Figure 7. UVB-light mediated induction of c-fos mRNA is not affected by hsp70 overexpression. Subconfluent WN10X (lanes *1, 2,* and *3*) and WN113 cells (lanes *4, 5,* and *6*) were washed twice with prewarmed PBS and exposed to 200 J/m^2 (lanes *1* and *4*) and to 100 J/m^2 UVB, respectively (lanes *2* and *5*). Control samples were treated identically except UV-irradiation (lanes *3* and *6*). After UVB-treatment cells were incubated for 1 h in DME plus 10% FCS, subsequently total RNA was extracted and assayed for c-*fos* mRNA by a RNAse protection assay. Lane M contains a ^{32}P-labeled DNA length standard. The expected size of the protected fragment of the c-*fos* probe is 177 nucleotides (*upper panel*). To show that equal amounts of RNA were used S16 mRNA was probed as an internal control (expected size of the protected fragment 155 nucleotides; *lower panel*).

signal threshold necessary to elicit a cellular response to environmental stress.

Since IL-1, a stress response cytokine also triggered by UV-light (34, 44), is known to induce IL-6 production, we investigated 1. whether IL-1 secretion is reduced in hsp70 overexpressing cells and 2. whether the signal threshold of the IL-1 signal with respect to induction of IL-6 release is altered in cells with enhanced hsp70 concentrations. IL-1 secretion was found to be reduced significantly after UVA-treatment when hsp70 is overexpressed. Therefore, the suppressed IL-6 release could be partially due to hsp70 mediated suppression of IL-1. However, this seems to be unlikely, because the response to exogenous recombinant IL-1α is markedly decreased in hsp70 overexpressing cells demonstrated by a profound reduction of released IL-6 (Fig. 5 *B*). Seemingly, hsp70 overexpression affects not only IL-6 but also other stress inducible factors like IL-1, suggesting a more general phenomenon.

UV-light as well as oxidative conditions are known to induce lipid peroxidation (45) thereby possibly generating leaky cell membranes. As monitored by trypan blue exclusion this effect was efficiently suppressed in WN113 cells compared with WN10× (data not shown). The explanation for our finding, that the IL-6 release which is reduced by hsp70 overexpression is due to a leakage of the cell membrane leading to a release of the cellular IL-6 pool can be excluded by (*a*) the observation that IL-6 induced by UVA, which in our hands did not cause extensive membrane damage, is suppressed by hsp70 overexpression and (*b*) by the reduced sensitivity of WN113 cells to IL-1α which acts through a receptor mediated mechanism.

It has been reported that cytotoxicity of TNF for WEHI-S cells is inhibited by hsp70 overexpression, but that the TNF signal to the transcription machinery seems to be unaffected (46). Moreover, TNF mediated activation of NFκB is not suppressed in the presence of high hsp70 concentrations (47). Accordingly, we could show that IL-1–induced NFκB-like binding activity is unaffected by hsp70 overexpression, suggesting a mode of hsp70 action beyond the transcriptional control, at least beyond this particular signaling pathway which is necessary for IL-6 transcription (42, 43). This hypothesis is also supported by the finding that induction of c-*fos* mRNA by UVB-light was not affected by hsp70 overexpression.

The functions of hsp70 have been attributed to protein folding, translocation through membranes and protein degradation, but also to the translation machinery (48), all involving necessarily the binding of peptides to hsp70 (reviewed in 9). The members of the 70-kD family of hsps share ATPase activity which is thought to be necessary for the release of associated peptides (49). Taking this into account, one could speculate that hsp70 could bind to nascent IL-6, e.g., for proper folding and translocation. When hsp70 is overexpressed, IL-6 could decreasingly dissociate from the IL-6/hsp70 complex due to the lack of sufficient ATP. Since the secretion of both IL-6 and IL-1 is affected by hsp70 overexpression, this phenomenon could be considered more general: by a similar mechanism certain components of the secretory pathway could be bound to hsp70 and could thereby loose their biological activity.

In summary, the present study demonstrates (*a*) that hsp70 overexpression renders cells tolerant against irradiation with UVB as well as against menadione or hydrogen peroxide mediated oxidative stress. (*b*) We demonstrate the link between UV-irradiation and oxidative stress and that hsp70 is not able to prevent UV-mediated ROI formation. (*c*) We found that the normal stress response is not impaired by hsp70 overexpression, but that the tolerance threshold appears to be shifted to higher doses of these deleterious environmental conditions. Accordingly, IL-6, a cytokine released in response to these stress types, was found reduced when hsp70 is overexpressed and the signal threshold for IL-6 release induced by UVA, UVB, or oxidative stress appears to be increased in the presence of high cellular hsp70 concentrations. This observation is not confined to IL-6 since the same phenomenon was observed for UVA-mediated IL-1 release. Furthermore, the response threshold of cells bearing high hsp70 concentrations to exogenous IL-1 is increased compared with wild-type cells. Taken together, hsp70 appears to exert an important influence on the regulation of stress associated, immunological cytokines, further supporting a pivotal role of hsp70 in modulating the cellular response to adverse environmental conditions like UV-light.

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