CD95-mediated alteration in Hsp70 levels is dependent on protein stabilization

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Abstract Engagement of death receptors induces caspase activation and apoptosis. A recent study reported altered protein expression, including increased Hsp70 levels during CD95-mediated apoptosis. Here, we examined the mechanism underlying increased Hsp70 levels in cells challenged with a monoclonal antibody directed against the CD95 receptor. Levels of Hsp70 were found to increase in a dose-dependent manner, occurring independently of either heat shock factor 1 activation or the accumulation of Hsp70 messenger ribonucleic acid (mRNA), suggesting the involvement of posttranslational modifications. Inhibition of translation and de novo protein synthesis by cycloheximide resulted in Hsp70 protein levels diminishing over time in control cells, whereas its level remained constant during CD95 signaling. In addition, death receptor activation through exposure of cells to tumor necrosis factor-related apoptosis-inducing ligand did not alter Hsp70 levels. These findings demonstrate that receptor-specific signaling through the CD95 increases the stability of Hsp70 protein, rather than mRNA, when compared with control cells. The results describe a novel mechanism of heat shock protein accumulation, where increased protein stability and reduced turnover, is the mechanism by which Hsp70 accumulates in cells during CD95-mediated apoptosis.

INTRODUCTION

One of the most prominently studied signaling pathways involved in the initiation phase of apoptosis is mediated by the CD95 receptor, a widely expressed membrane-associated death receptor (Itoh et al 1991). Engagement of the CD95/Fas/Apo-1 receptor, either by its ligand or by cross-linking antibodies, results in receptor oligomerization and recruitment of the adaptor protein, Fas-activated death domain, which along with procaspase-8, forms the death-inducing signaling complex at the plasma membrane. Within this complex, auto-activation of procaspase-8 occurs, which in turn triggers activation of the caspase cascade by its ability to proteolytically cleave and activate downstream effectors, including caspase-3, caspase-6, and caspase-7 (Ashkenazi and Dixit 1998). Once activated, these caspases systematically disassemble the cell by

targeting and cleaving key intracellular substrates (Fischer et al 2003).

Although the engagement of the CD95 receptor typically results in caspase activation and apoptosis, evidence suggests that CD95 can also trigger the activation of several other intracellular signaling pathways, resulting in a variety of cellular responses. For instance, CD95 receptor ligation has been associated with the activation of nuclear factor- κ B (Ponton et al 1996) and Jun kinase pathways (Latinis and Koretzky 1996) as well as upregulation of the protooncogene, *c-fos* (Siegmond et al 2001). More recently, a study investigating proteome changes occurring during CD95-mediated apoptosis identified heat shock proteins (Hsps) as proteins whose levels are altered during this process (Gerner et al 2000).

Hsps encompass several families of cytoprotective proteins, including Hsp27, Hsp70, and Hsp90. They function within the cell as molecular chaperones involved in the transport, folding, and assembly of polypeptides (Parsell and Lindquist 1993). Under conditions of environmental and physiological stress, Hsps are rapidly synthesized

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and are shown to confer resistance to more severe stress and cell death (Samali and Orrenius 1998; Parcellier et al 2003). Besides preventing protein aggregation, Hsps can directly interfere with the apoptotic machinery in a variety of ways. For example, Hsp27 can inhibit activation of caspase-3, through sequestration of procaspase-3 and cytochrome *c* (Concannon et al 2001). Alternatively, Hsp70 has an antiapoptotic effect when it binds Apoptotic Protease Activating Factor-1 (Beere et al 2000; Saleh et al 2000). Furthermore, Hsp90 has been shown to inhibit apoptosome formation through direct binding of APAF-1 (Pandey et al 2000; Sreedhar and Csermely 2004). Induction of Hsps is mainly mediated at the transcriptional level, involving the conversion of a transcriptionally inactive heat shock factor 1 (HSF1) monomer to a hyperphosphorylated trimer, capable of binding deoxyribonucleic acid (DNA) and transducing the various Hsp genes (Morimoto 1998). In addition to HSF1-mediated transcriptional control, Hsp70 accumulation by messenger ribonucleic acid (mRNA) stabilization on various stress conditions has also been reported (Theodorakis and Morimoto 1987; Kaarniranta et al 2002).

The aim of this study was to investigate whether engagement of the CD95 receptor with a specific monoclonal antibody resulted in a typical heat shock response. In our model, we show that the engagement of the CD95 receptor with agonistic monoclonal antibodies induces an atypical heat shock response in which only an increase in Hsp70 levels is apparent. This response does not occur through the classical HSF1-mediated stress response but rather through a novel mechanism involving increased stability of the Hsp70 protein.

MATERIALS AND METHODS

Cell culture and treatments

Jurkat cells were maintained in Roswell Park Memorial Institute medium 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2–3 days. For induction of CD95 signaling, cells were seeded at a density of 5×10^5 /mL in fresh medium. Anti-CD95 monoclonal antibody (clone CH-11, MBL Inc, Nagoya, Japan) was added to the medium at 25 ng/mL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (IQ Products, Groningen, Netherlands) at 25, 100, or 125 ng/mL for the indicated time periods. For heat shock experiments, cells were seeded at 1×10^6 /mL of medium in screw-cap flasks, which were then sealed with parafilm and immersed in a water bath at 42°C ($\pm 0.5^\circ\text{C}$) for 1–2 hours, as described previously (Samali

and Cotter 1996). After heat shock, cells were allowed to recover under normal culture conditions for varying time periods. To inhibit protein synthesis, cells were treated with 10 $\mu\text{g}/\text{mL}$ cyclohexamide.

Western blotting

Cells were washed once in phosphate-buffered saline (PBS) and lysed in whole-cell lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES] pH 7.5, 350 mM NaCl, 1 mM MgCl₂, 0.5 mM ethylenediamine-tetraacetic acid [EDTA], 0.1 mM ethylene glycol-bis (aminoethylether) tetracetic acid, 1% NP-40, 0.5 mM dithiothreitol [DTT], 100 μM phenylmethylsulfonyl-fluoride [PMSF], 1 $\mu\text{g}/\text{mL}$ pepstatin, 2.5 $\mu\text{g}/\text{mL}$ aprotinin, 10 μM leupeptin). Protein concentration in lysates was determined by means of the Bradford method, using bovine serum albumin as the standard. Equal amounts of protein (15–25 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5–10% gels, before being transblotted onto nitrocellulose. The resulting blots were probed with either mouse monoclonal antibodies to Hsp70, Hsp90, or Hsp27; a rat monoclonal antibody against Hsc70 (StressGen Biotechnologies Corp., Victoria, Canada); or rabbit polyclonal antibodies to human HSF1 (Holmberg et al 2000) or β -actin (Sigma-Aldrich, Dublin, Ireland). Horseradish peroxidase-conjugated secondary antibodies (Pierce) were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc, Rockford, IL, USA). Densitometry was performed using AlphaEaseFC software from Alpha Innotech, San Leandro, CA, USA.

Electrophoretic mobility shift analysis

Cells were washed once in ice-cold PBS and snap frozen in liquid nitrogen. Whole-cell extracts were prepared by adding 50 μL of buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) to the frozen pellet, and samples were allowed to swell on ice for 5 minutes. A self-complementary oligonucleotide corresponding to the proximal heat shock element of the human *hsp70* promoter (5' GCC TCG AAT GTT CGC GAA GTTT TC) was end-labeled with 20 μCi [γ -³²P] adenosine triphosphate (ICN, Doornik, Belgium) using T4 polynucleotide kinase (Promega, Southampton, UK). Ten micrograms of whole-cell extract was incubated with the labeled oligonucleotide for 20 minutes in binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 12.4 $\mu\text{g}/\text{mL}$ poly dIdC). Protein-DNA complexes were resolved on a native 4% polyacrylamide gel using Tris-Borate-EDTA as running buffer, at 150 V for 2 hours. The gel was dried

under vacuum onto Whatmann 3-mm filter paper and autoradiographed.

Northern blot analysis

Total RNA was extracted using Trizol (Invitrogen, De Schelp, The Netherlands), according to the manufacturer's instructions. Subsequently, 10 µg of total RNA was separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran SuPerCharge, Schleicher/Schuell, Keene, NH, USA) by overnight capillary transfer. The membrane was cross-linked using a Stratagene UV Stratalinker 1800 and prehybridized at for 1 hour at 42°C in UltraHyb solution (Ambion, Huntingdon, Cambridgeshire, UK). Complementary DNA probes for human Hsp70 and glyceraldehyde 3 phosphate dehydrogenase generated by reverse transcription-polymerase chain reaction (RT-PCR), were labeled with 50 µCi [α -³²P] deoxyadenosine triphosphate using the Highprime labeling kit (Roche, Welwyn Garden City, Hertfordshire, UK) and hybridized to membrane overnight at 42°C. Blots were then washed twice at 50°C in standard saline citrate (SSC) buffer (300 mM NaCl, 30 mM sodium citrate pH 7.0) containing 0.1% SDS, followed by 2 washes in a 1:20 dilution of SSC buffer containing 0.1% SDS and subsequently autoradiographed at -70°C.

Reverse transcription-polymerase chain reaction

RT was carried out with 2 µg total RNA and oligo (dT) 12-18 (Invitrogen) using SUPERScript II (Invitrogen) according to the manufacturer's instructions. The Hsp70-specific sequence was amplified during 35 cycles of 30 seconds denaturing, 1 minute annealing, and 1 minute extension, with the following primers: forward 5'-TGA ACTACAAGGGCGAGA-3'; reverse 5'-ATGGACGTGT AGAAGTCGA.

Flow cytometry

The binding of annexin V-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, CA, USA) to externalized phosphatidylserine (PS) was used to determine the extent of apoptosis in cultures. After treatment with anti-CD95 antibody, cells were washed twice in PBS and resuspended in 100 µL of binding buffer (10 mM HEPES pH 7.40, 140 mM NaCl, 2.5 mM CaCl₂) containing 5 µL annexin V-FITC and 10 ng propidium iodide, for 15 minutes in dark at room temperature, before flow cytometric analysis. Cells were analyzed using a FACScalibur flow cytometer and Cell Quest software (BD Biosciences, Dublin, Ireland), with a total of 10 000 events acquired per sample.

RESULTS

Engagement of the CD95 receptor increases cellular Hsp70 levels

In initial experiments, we investigated the ability of the CD95 agonist antibody to initiate apoptosis, as assessed by annexin V binding to externalized PS, a hallmark of apoptotic morphology in Jurkat cells. Flow cytometric analysis revealed that 25 ng/mL of the antibody induced a significant degree of apoptosis within 8 hours of treatment as assessed by the increased binding of annexin V-FITC (Fig 1A). To determine whether the engagement of the CD95 receptor results in a heat shock response, the expression pattern of Hsps was determined at various time points by Western blotting. As shown in Figure 1B, CD95 ligation also resulted in a time-dependent increase in Hsp70 levels. Densitometric analysis revealed that the expression of Hsp70 increased up to 3-fold, 8 hours after CD95 ligation. In contrast, however, the expression level of Hsp90 and of Hsp27 remained unchanged (data for Hsp27 not shown). It is worth noting that Hsp27 is not detectable in our model, under normal culture conditions. However, robust expression of Hsp27 does occur after heat shock. Similarly, detected levels of Hsp90 protein also remained unchanged (Fig 1B). Finally, the ability of Jurkat cells to upregulate Hsp70 expression in response to heat shock was also demonstrated (Fig 1B). This upregulation was much more pronounced than that induced by CD95 signaling (3-fold vs 11-fold). The data presented is representative of 3 independent experiments. Taken together this data suggests that CD95-mediated apoptosis induced an atypical heat shock response in which only the levels of Hsp70 were raised.

To determine whether the accumulation of Hsp70 was specific for the CD95 pathway, the expression of Hsp70 after ligation of another death receptor, the TRAIL receptor, was examined. As demonstrated in Figure 1C, increased expression of Hsp70 was not apparent after treatment with the TRAIL ligand. These results suggest that the accumulation of Hsp70 is a CD95-specific response, not common to signaling through another tumor necrosis factor-death receptor family member.

Hsp70 accumulation occurs independently of altered gene expression

The induction of Hsps under stress conditions is primarily regulated at the transcriptional level through the activation of HSF1, which in turn can be activated by the CD95 receptor-binding DAXX protein (Boellmann et al 2004). HSF1 is a member of the heat shock family of transcription factors, which includes HSF1-4 and is considered to be the major regulator of Hsp70 (Pirkkala et al 2001). We, therefore, examined its DNA binding and ap-

Fig 1. Engagement of the CD95 receptor results in caspase activation and Hsp70 accumulation. Cells were incubated with anti-CD95 antibody (25 ng/mL) for indicated time periods. (A) Levels of apoptosis in control and CD95-treated samples were analyzed by annexin V–propidium iodide (PI) staining using flow cytometric analysis. (B) Western blot analysis of Hsp70 and Hsp27 expression in control and CD95-treated samples. As a positive control, cells were heat shocked for 1 hour at 42°C, followed by 2 hours recovery at 37°C. Densitometry of Hsp70 bands was performed and fold increases were expressed relative to actin levels. (C) Cells were incubated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (125 ng/mL) for up to 8 hours. Hsp70 levels were assessed by Western blotting. In all Western blots, equal loading was confirmed using β -actin antibody.

pearance of the active phosphorylated form during CD95-mediated apoptosis. Band shift analysis revealed the absence of HSF1 DNA-binding activity at any time point investigated (Fig 2A). In contrast, a significant DNA-binding activity was evident when cells were heat shocked at 42°C, thereby demonstrating a functional HSF1 DNA-binding activity in our cell system. Further-

more, Western blot analysis revealed the presence of the active hyperphosphorylated form of HSF1 only in the heat shocked sample and not in the CD95-treated samples, further confirming that HSF1 is not functioning to drive Hsp70 transcription during CD95-mediated apoptosis (Fig 2B). Despite this absence of HSF1 activation, Western blot analysis clearly demonstrates increased lev-

els of Hsp70 protein after 5 hours of anti-CD95 treatment (Fig 2C). Hsp70 has been shown to accumulate independently of HSF1 activation in response to heat shock or mechanical stress, in a mechanism that involves increased stability of the Hsp70 mRNA transcript (Kaarniranta et al 1998). However, Northern blot analysis and RT-PCR revealed that after engagement of CD95, the steady state level of Hsp70 mRNA did not increase for the duration of the experiment (Fig 3). Without a visible increase in the levels of Hsp70 mRNA, it would appear that the accumulation of Hsp70 protein does not involve increased stability of the mRNA transcript.

Increased stability of Hsp70 protein during CD95 apoptosis

Because the accumulation of Hsp70 was found to be independent of gene expression, we examined whether the stability of the Hsp70 protein was altered during CD95-mediated apoptosis. Using the translational inhibitor cycloheximide, to inhibit *de novo* protein synthesis, we tracked the expression levels of Hsp70 for 8 hours. Western blot analysis revealed that levels of Hsp70 expression in control cells decreased after 2 hours of cycloheximide treatment and were undetectable by 8 hours (Fig 4A). In contrast, the levels of Hsp70 during CD95-mediated apoptosis remained constant for the 8 hours investigated (Fig 4B). Therefore, during CD95 signaling the half-life of Hsp70 protein is dramatically increased. As the turnover rate of Hsp70 is significantly reduced, this allows for its levels to increase to above the levels seen in control cells.

DISCUSSION

Activation of CD95 signaling is an important biological pathway essential for deletion of autoreactive T cells, downregulation of the immune response, and maintenance of sites of immune privilege (Scaffidi et al 1999). Therefore, any deviation from this central pathway, which antagonizes the death signal, may result in excessive cell proliferation and tumorigenesis.

This study demonstrates a novel mechanism by which Hsp70 levels increase in Jurkat cells after treatment with the anti-CD95 antibody. Although the accumulation of Hsp70 during CD95-mediated apoptosis has been reported previously, the mechanism behind this has not been fully elucidated (Gerner et al 2000; Parr et al 2001).

We have shown that engagement of the CD95 receptor initiated apoptosis, while also increasing the cellular levels of Hsp70. However, the expression levels of the other major inducible Hsps, Hsp90, and Hsp27 remained unchanged. This is in contrast to the reported induction of Hsp27 and reduced Hsp90 expression, after treatment of Jurkat cells with anti-CD95 (Gerner et al 2000) and may

Fig 2. Examination of HSF1 activity during CD95 signaling. Cells were treated with anti-CD95 antibody (25 ng/mL) for indicated time periods. (A) Whole-cell extracts derived from control, CD95-treated, and heat shocked (HS) cells were prepared and analyzed for HSF1 deoxyribonucleic acid (DNA)–binding activity by gel mobility shift assay. (B) Duplicate extracts were probed with HSF1 antibody (pHSF1 = phosphorylated HSF1). (C) Western blot analysis showing Hsp70 levels in anti-CD95-treated samples. Hsc70 was used as the loading control.

be due to their use of a low serum (1%) concentration. To decipher the mechanism by which Hsp70 accumulates in response to CD95, we examined the expression of HSF1 and Hsp70 mRNA. Electrophoretic mobility shift assays and Northern blot analysis revealed that altered Hsp70 levels occurred independent of both HSF1 activation and increased Hsp70 mRNA levels, although both were inducible in response to heat shock. Our results indicate involvement of posttranslational regulation in the accumulation of Hsp70. Inhibition of *de novo* protein synthesis, by cycloheximide, decreased Hsp70 protein levels quite rapidly in cells not exposed to the anti-CD95 antibody. In sharp contrast, Hsp70 levels remained constant during CD95-mediated apoptosis, in the presence of cycloheximide. Taken together, these data suggest that the stability of the Hsp70 protein increases dramatically dur-

Fig 3. Hsp70 messenger ribonucleic acid (mRNA) levels remain constant during CD95 signaling. Jurkat cells were treated with the CD95 antibody (25 ng/mL) for up to 8 hours, or were heat shocked through incubation at 42°C for 1–2 hours, followed by 2 hours recovery at 37°C. Total RNA was extracted and Northern blot analysis performed, using a probe specific for Hsp70. Equal loading of the blot was confirmed using a probe for the house keeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Reverse transcription–polymerase chain reaction (RT-PCR) analysis of Hsp70 (bottom panel) also demonstrated no increase in mRNA levels during the course of the experiment.

Fig 4. Increased stability of Hsp70 protein during CD95-mediated apoptosis. De novo protein synthesis was inhibited using cycloheximide (10 µg/mL). Hsp70 protein levels were assessed in anti-CD95-treated cells by Western blotting. Uniformity of sample loading was confirmed by probing for β-actin.

ing CD95-mediated apoptosis. Several reports in the literature have suggested that in addition to posttranscriptional control, posttranslational regulation of Hsp70 can occur (Twomey et al 1993; Kaarniranta et al 2002). Twomey et al (1993) have suggested that elevated Hsp70 expression in the peripheral blood mononuclear cells, seen in a subset of lupus patients, may be mediated through increased protein stability. However, this study is the first to provide direct evidence that increased stabilization of Hsp70 can occur. The exact posttranslational changes resulting in the increased stability of Hsp70 protein require further clarification. However, some evidence suggests that Bag-1 isoforms may regulate the chaperone activity of Hsp70 (McClellan and Frydman 2001) and that it, therefore, may also be involved in stabilizing the protein.

Because it has been previously demonstrated that the induction of the stress response and apoptosis are mutually exclusive (Samali et al 1999), the finding that CD95-mediated apoptosis results in increased levels of Hsp70

is intriguing. Induction of Hsp70 may be a protective strategy aimed at inhibiting the induction of apoptosis through CD95. However, this seems unlikely, given that overexpression of Hsp70 was shown to have little effect on CD95-mediated apoptosis (Tran et al 2003) and, in some cases, has even been found to enhance the process (Liossis et al 1997; Creagh and Cotter 1999). Furthermore, the increase in Hsp70 expression is of several times less magnitude than that during heat shock. This increase is unlikely to be sufficient to adequately inhibit the activation of caspases, although it may in part have a role to play in the formation of malignancies associated with increased resistance to CD95-mediated apoptosis.

In summary, our results show that the accumulation of Hsp70 during CD95-mediated apoptosis occurs independently of increased gene expression and rather through a novel mechanism whereby the stability of the Hsp70 protein is increased.

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