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Hyperthermia Enhances CD95-Ligand Gene Expression in T Lymphocytes

Marco Cippitelli,2,3*† Cinzia Fionda,3*† Danilo Di Bona,§ Mario Piccoli,* Luigi Frati,*‡ and Angela Santoni*†

Hyperthermia represents an interesting therapeutic strategy for the treatment of tumors. Moreover, it is able to regulate several aspects of the immune response. Fas (APO-1/CD95) and its ligand (FasL) are cell surface proteins whose interaction activates apoptosis of Fas-expressing targets. In T cells, the Fas-Fas-L system regulates activation-induced cell death, is implicated in diseases in which lymphocyte homeostasis is compromised, and plays an important role during cytotoxic and regulatory actions mediated by these cells. In this study we describe the effect of hyperthermia on activation of the fas-L gene in T lymphocytes. We show that hyperthermic treatment enhances Fas-L-mediated cytotoxicity, fas-L mRNA expression, and fas-L promoter activity in activated T cell lines. Our data indicate that hyperthermia enhances the transcriptional activity of AP-1 and NF-κB in activated T cells, and this correlates with an increased expression/nuclear translocation of these transcription factors. Moreover, we found that heat shock factor-1 is a transactivator of fas-L promoter in activated T cells, and the overexpression of a dominant negative form of heat shock factor-1 may attenuate the effect of hyperthermia on fas-L promoter activity. Furthermore, overexpression of dominant negative mutants of protein kinase Cε (PKCε) and PKCθ partially inhibited the promoter activation and, more importantly, could significantly reduce the enhancement mediated by hyperthermia, indicating that modulation of PKC activity may play an important role in this regulation. These results add novel information on the immunomodulatory action of heat, in particular in the context of its possible use as an adjuvant therapeutic strategy to consider for the treatment of cancer.

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available on its complex regulatory activity. In fact, triggering of Fas-L is required for CTLs to achieve an optimal proliferation (24–26), and an activated Fas receptor may induce phenotypic and functional maturation of dendritic cells together with specific secretion of proinflammatory cytokines and a preferential local T cell polarization toward a Th1 phenotype (27). Furthermore, Fas ligation may contribute to T cell development and proliferation, suggesting important connections between cell proliferation and apoptosis (28, 29).

In this report we describe the effect of hyperthermia on activation of the fas-L gene in activated T lymphocytes and the regulatory actions of this treatment on the human fas-L promoter. We show that hyperthermia may enhance fas-L mRNA expression, promoter activity, and effector function in T cell lines such as the 2B4.11 T cell hybridoma and Jurkat cells. Progressive deletion analysis has identified a minimal promoter fragment spanning nucleotides from −195 bp immediately 5’ of the translational start site, which is still sensitive to hyperthermia treatment. This correlates with an increased expression and activity of specific transcription factors, such as c-Jun, FosB, and NF-kB, important regulators located in this position. Moreover, overexpression of a constitutive active form of heat shock factor-1 (HSF-1) significantly enhances fas-L promoter activity, and a dominant negative mutant of this transcription factor is able to decrease the effect of hyperthermia in activated T cells. Furthermore, cotransfection of dominant negative mutants of PKCe and PKCθ may partially inhibit overall fas-L promoter activity and significantly decrease the specific enhancement mediated by hyperthermia.

The data described in this study provide novel findings on the complex effects of heat on the regulation of the immune response and indicate that the fas-L gene is a novel molecular target, adding to the variety of immunoregulatory mechanisms mediated by hyperthermia. The physiological and therapeutical implications of these observations are discussed below.

Materials and Methods

Cell lines, reagents, and treatments

Jurkat cells and 2B4.11 murine T hybridoma cells were maintained as described previously (30). PMA and ionomycin were purchased from Sigma-Aldrich. Anti-CD3 e-chain mAb (clone 145-2C11) was purchased from BD Pharmingen. CAY10410 (9,10-dihydro-15-deoxy-Δ7,12,15-PGF2α) was purchased from Cayman Chemical. For hyperthermia, culture flasks were sealed with Parafilm (American National Can) and immersed in a water bath at the indicated temperature and time. Control cells were left in the incubator (sealed with Parafilm) at 37°C. After hyperthermia treatment, cells were returned to the incubator (37°C) for recovery and appropriate stimulation.

Cytotoxicity assay

Wild-type and Fas-transfected L1210 cells (10⁶ cells; provided by Dr. Hueber, Institute of Signdaling, Developmental Biology, and Cancer Research, Nice, France) were labeled with 0.2 μCi of ⁵¹Cr (Amersham Biosciences) at 37°C. Target cells (2 × 10⁵) were cocultured with 2B4.11 effector cells in 200 μl of complete medium in U-bottom, 96-well plates for 4 h. 2B4.11 effector cells were previously activated with 20 ng/ml PMA and 0.5 μg/ml ionomycin in the absence of pretreatment or after pretreatment at 42°C for 1 h or 39.5°C for 4 h, followed by a 0.5-h recovery period at 37°C in complete medium. Cells were then harvested, washed twice in complete medium, and cocultured with ⁵¹Cr-labeled target cells. After 4 h, 100 μl of supernatant was removed from each well and counted in a gamma counter for determination of ⁵¹Cr release. Maximal and spontaneous release were determined by incubating ⁵¹Cr-labeled target cells with 1% Nonidet P-40 or medium alone, respectively. The percent specific killing was calculated as 100% (experimental ⁵¹Cr release − spontaneous ⁵¹Cr release)/(maximal ⁵¹Cr release − spontaneous ⁵¹Cr release). All determinations were made in triplicate, and E/T cell ratios ranged from 80:1 to 20:1, as indicated.

Northern blot analysis

Total RNA was extracted from 2B4.11 hybridoma T cells by TRizol (Invitrogen Life Technologies). Equal amounts of RNA (15 μg/lane) were fractioned on a 1.5% agarose-formaldehyde gel. The specific mRNA was detected by hybridization of Nitryan membranes (Schleicher & Schuell) with a 3²P-labeled cDNA probe specific for the murine fas-L gene. The RNA-containing membranes were prehybridized for 20 min and hybridized for 2 h at 65°C with QuikHyb hybridization solution (Stratagene). The membranes were then washed twice in 2× SSC containing 0.1% SDS and twice in 0.1× SSC containing 0.1% SDS at 60°C (20 min each time) and exposed to X-OMAT AR films (Eastman Kodak) at −70°C with intensifying screens.

Plasmid constructions

The human Fas-L promoter luciferase (Luc) reporter pFasL-486, the distal NFAT binding mutant (ΔNFAT-Dist), and the response element 3′Fas-L regulatory element (RE3/FLRE) binding mutant were provided by Dr. G. A. Koretzky (University of Pennsylvania, Philadelphia, PA) (31, 32). pGL3-Fas-Luc has been already described (33). The different deletions of the human Fas-L promoter −453 Fas-L(PGL2), −237 Fas-L(PGL2), and −195 Fas-L(PGL2) were provided by Dr. C. V. Paya (Mayo Clinic, Rochester, MN) (34). The CMV-β-galactosidase (β-gal) plasmid pCMV-β-gal (Stratagene) and the Rous sarcoma virus (RSV)-Gal expression vector have been previously described (30). The expression vector for human constitutively activated HSF1 S303A/S307A double mutant (pcDNA3-HSF1-S303A/S307A) was provided by Dr. R. I. Morimoto (Northwestern University, Evanston, IL). To measure the expression vector for HSF1 dominant negative (deletion of aa 453–529, the C-terminal sequences including the C-terminal region element, which is part of the transcription activation domain) (35), the appropriate deletion fragment was generated by RT-PCR according to standard methods from an HSF1 expression vector containing the human HSF1 gene and cloned in the pRCSv expression vector (Invitrogen Life Technologies, Carlsbad, CA). The first primers were used for amplification: HSF1-1 forward, 5′-ATCGGAATTCCTGGAGATTGCAGATGACTTGCGCCTGG-3′; and HSF-1 reverse, 5′-CATCTAGACTCCTCGGGGACAGAAGCTC-3′.

Luc reporter vectors for the transcription factors AP-1 (pAP1-TA-Luc), c-Myc (pMyc-TA-Luc), NF-κB (pNF-κB-TA-Luc), and the control vector containing the minimal TATA box from the herpes simplex virus thymidine kinase promoter pTA-Luc, were purchased by BD Clontech. The dominant negative (KR) expression vectors for human PKCθ (K-409R), rat PKCe (K-363R), and the control vector (pEFneo) were provided by Drs. G. Baier and F. Ueberall (University of Innsbruck, Innsbruck, Austria). The cDNA for murine Fas-L was provided by Dr. R. De Maria (Instituto Superior di Santa `, Rome, Italy).

DNA transfections

Transfections of Jurkat cells were conducted using the DEAE-dextran method as described previously (30). To decrease variations in the experiments due to different transfection efficiencies from one experiment to the next, each transfection was conducted in single batches, which were then separated into different drug treatment groups. A CMV-β-Gal expression vector was cotransfected to normalize DNA uptake. After 24 h, cells were treated with different combinations of stimuli, and after an additional 24 h, cells were harvested, and protein extracts were prepared for the Luc and β-Gal assays as described previously (30). Protein concentration was quantified by the bicinchoninic acid (BCA) method (Pierce). Luciferase activity was measured using the luciferase assay system (Promega) following the manufacturer’s instructions. β-Gal activity was determined as described previously (30).

EMSA

Nuclear proteins were prepared as described previously (30). The protein concentration of extracts was determined using the BCA method (Pierce). Nuclear proteins (10 μg) were incubated with radiolabeled DNA probes in a 20-μl reaction mixture containing 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 0.5 mM DTT, 1–2 μg of poly(dI-dC), and 4% Ficoll. Nucleo-protein complexes were resolved as described previously (30). Oligonucleotides were purchased from Invitrogen Life Technologies. Complementary strands were annealed and end-labeled as described previously (30). Approximately 3 × 10⁷ cpm of labeled DNA was used in a standard EMSA reaction. The following double-strand oligonucleotides were used as specific labeled probes: human Fas-L proximal, 5′-AAAGAGAAGACGACGAGGTGGTTTCTCCCT-3′; human Fas-L/AP-1 proximal, 5′-GGCTGGCGCTGACTCACCCAGTCGCT-3′, and octamer (human histone H2b), 5′-aggCTTCACCTTATTGCAATACGGAGAT-3′.
Western blot analysis

For Western blot analysis, nuclear proteins were prepared as described previously (33). Protein concentration of nuclear extracts was determined by the BCA method (Pierce). Forty micrograms of nuclear extract was run on 12% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and blocked in 3% milk in TBST. Immunoreactive bands were visualized on the nitrocellulose membranes, using HRP-coupled goat anti-rabbit or goat antimouse Iggs and the ECL detection system (Amersham Biosciences) following the manufacturer’s instructions. Abs against RelA, Oct-1, c-Jun, and FosB were purchased from Santa Cruz Biotechnology.

Results

Hyperthermia enhances Fas-L-dependent target lysis mediated by activated 2B4.11 T cells

To investigate on the functional effect of hyperthermia on Fas-L expression/function in activated T cells, cytotoxicity assays were performed using the 2B4.11 T cell hybridoma in the presence of 51Cr-labeled, Fas-transfected, L1210-Fas+ target cells. As shown in Fig. 1A, 2B4.11 T cells activated by a combination of PMA and ionomycin after pretreatment at 42°C for 1 h, followed by a recovery period at 37°C for 0.5 h (indicated as heat shock (HS) treatment), significantly increased specific killing compared with the cytotoxicity elicited by 2B4.11 cells treated at normal temperature (37°C). This result was also confirmed in a different setting of mild hyperthermia (pretreatment of cells at 39.5°C for 4 h), as shown in Fig. 1C.

The effects of HS and mild hyperthermia on L1210-wild-type (L1210-WT) target cells (used in these experiments as a negative control for Fas-L-mediated killing) are shown in Fig. 1, B and D. Treatment of 2B4.11 cells with HS or mild hyperthermia in the absence of PMA and ionomycin stimulation was unable to induce any specific killing, at least in this experimental system. Thus, hyperthermia enhances Fas-L-mediated cytotoxicity in activated 2B4.11 T cells.

Hyperthermia increases fas-L gene expression and promoter activation in 2B4.11 T cells

We investigated whether hyperthermia could affect induction of the fas-L gene in activated T cells. Total RNA was isolated from 2B4.11 T cells 5 h after activation and analyzed for fas-L mRNA expression by Northern blot assay. As shown in Fig. 2A, fas-L mRNA was induced by PMA plus ionomycin stimulation and was significantly increased by HS pretreatment, indicating that fas-L is a molecular target of hyperthermia in T cells. Hyperthermia without PMA and ionomycin was unable to induce fas-L mRNA in this experimental system. The effect of hyperthermia was also confirmed by stimulating 2B4.11 T cells with plate-bound anti-CD3 and was verified in mild hyperthermia, as shown in Fig. 2, B and C, respectively.

To determine whether one of the mechanisms involved in the hyperthermia-mediated increase in fas-L gene activation could be a direct effect on the transcriptional activity of its promoter, transient transfection assays were performed in Jurkat cells. As shown in Fig. 2D, the activity of a human fas-L promoter fragment, consisting of 486 bp immediately 5’ of the translational start site, was induced by PMA plus ionomycin treatment and was significantly enhanced by HS or mild hyperthermia in Jurkat cells. HS or mild hyperthermia in the absence of PMA and ionomycin stimulation was unable to activate this promoter, at least in this experimental system. Thus, fas-L mRNA expression and promoter activation are enhanced by hyperthermia in activated T cells.

Hyperthermia enhances fas-L gene expression in activated T cells: promoter analysis

To gain further insight into the mechanism(s) involved in fas-L promoter enhancement, we investigated the possible presence of a fas-L promoter region(s) involved in the hyperthermia-mediated action on this gene. We analyzed the activity of specific internal mutations and progressive deletions of the fas-L promoter by transient transfection assays in Jurkat cells. Transfection of fas-L promoter constructs bearing internal mutations that abrogate the binding of important transactivators, such as NF-AT (ΔNFAT-Dist.) or early growth response factors (ΔRE3/FLRE), considerably decreased activation after stimulation with PMA and ionomycin as previously reported (31, 32, 36). However, in this context hyperthermia was still able to significantly increase residual promoter activation compared with the wild-type fas-L promoter reporter (Fig. 3, A–C). Progressive deletion of these enhancer elements could delineate a minimal promoter fragment spanning nucleotides from −195 bp immediately 5’ of the translational start site, which was still sensitive to the hyperthermia treatment (Fig. 3, D–F). These data suggest that signaling events triggered by hyperthermia can augment fas-L promoter activation by mechanisms that cooperate at the level of the enhancer activity of its first −195 bp immediately 5’ of the translational start site.

**FIGURE 1.** Effect of hyperthermia on Fas-L-mediated target lysis. 51Cr-labeled L1210-Fas+ cells (A and C) and the control cell line L1210-WT (B and C) were incubated for 5 h with 2B4.11 effector cells, previously activated as indicated in the figure, to induce Fas/Fas-L-dependent lysis. Effector cells were activated for 5 h by a combination of PMA and ionomycin after pretreatment at 42°C for 1 h or 39.5°C for 4 h, followed by a 0.5-h recovery period at 37°C (indicated in this report as HS and mild hyperthermia, respectively). One experiment representative of three is shown.
A number of studies have shown that the fragment from −195 to +1 bp of the human fas-L promoter contains several important enhancer elements that cooperate for the transcription of this gene. Among these, a major transcription initiation site at −181 bp from the first ATG (34), a NF-κB/SP-1 binding site located −150 bp from the first ATG (37), and a noncanonical c-Myc binding element localized in a position originally described as a putative TATA box sequence (38) have been described. Moreover, an AP-1 binding site in the proximity of the noncanonical c-Myc binding element at position −16 bp from the first ATG (37, 39) has been recently identified (Fig. 4A).

To determine whether the effect of hyperthermia on the fas-L promoter activation could involve an altered activity of these transactivators, we transfected Luc multicopy reporter vectors driven by NF-κB, c-Myc, and AP-1 transcription factors in Jurkat cells. As shown in Fig. 4, B–D, the PMA- plus ionomycin-induced activity of these reporter vectors was differently modulated by hyperthermia. The transcriptional activity of AP-1 and, to a lesser extent, of NF-κB was significantly increased by hyperthermia (Fig. 4, B and D). On the contrary, the multicopy reporter vector driven by c-Myc was unaffected, similar to the basal activity of the control vector containing only the minimal TATA box from the herpes simplex virus thymidine kinase promoter (Fig. 4, C and E). Thus, hyperthermia differently affects the enhancer activity of the fas-L promoter transactivators AP-1 and NF-κB, but not c-Myc, in activated T cells.

Hyperthermia enhances AP-1 and NF-κB transcription factor expression/nuclear translocation in activated T cells

To investigate the mechanisms involved in hyperthermia-mediated increase in AP-1 and NF-κB transcriptional activity, mobility shift and Western blot assays were performed on nuclear extracts prepared from activated Jurkat cells treated with hyperthermia. As shown in Fig. 5, A and B, activation of Jurkat cells induced specific FasL-NF-κB and AP-1 binding complexes that were significantly enhanced by hyperthermia. As a control for equal protein loading, the same amount of nuclear extract was tested in the presence of an octamer factor(s)-specific probe. The DNA binding of octamer-1 was unaltered in hyperthermia-treated cells (Fig. 5C).

We also confirmed these results by Western blot assay using Abs specific for NF-κB/RelA, c-Jun, and FosB, on these nuclear extracts.
extracts. As shown in Fig. 5, D–F, activation of Jurkat cells induced RelA nuclear translocation together with c-Jun and FosB expression. Hyperthermic treatment significantly increased c-Jun and FosB expression and, to a lesser extent, RelA nuclear translocation, in agreement with the data relating to the transcriptional activity and DNA-binding activity of these transactivators. As shown in these figures, the same filters were stained with an Ab specific for the octamer-1 transcription factor as a control for equal protein loading. Thus, hyperthermia increases the expression and nuclear translocation of AP-1 and NF-H9260B in activated T cells.

Hyperthermia enhances fas-L gene expression in activated T cells: role of HSF-1

Hyperthermic stress has been shown to activate protective HS response genes in many different experimental models, including T cells (40–44). This evolutionarily conserved phenomenon, called cellular stress response, is mediated by a family of transactivators, the HSFs. They are endowed with heat-inducible DNA-binding activity and transactivation (40, 41) and regulate the induction of a set of proteins, the HSPs, that is able to increase stress tolerance (we also confirmed these pathways in our cell lines; data not shown). Although four closely related hsf genes have been described (hsf-1 to hsf-4), HSF-1 is the primary transactivator responsible for the HS response (40, 41).

To investigate whether HSF-1 could directly modulate the activity of the fas-L promoter, we cotransfected an expression vector encoding a constitutively active mutant of human HSF-1 in Jurkat cells. As shown in Fig. 6A, the expression vector encoding the constitutively active mutant of the human HSF-1 significantly increased fas-L promoter activity at normal temperature, suggesting that HSF-1 is able to cooperate for transactivation in this context. Interestingly, the internal promoter mutation that abrogates binding of early growth response factors (∆RE3/FLRE) was more responsive to HSF-1 overexpression, whereas the mutant that abrogates NF-AT binding at the distal site (∆NFAT-Dist.) lost responsiveness to HSF-1 (Fig. 6, B and C). Progressive deletion of these enhancer elements could confirm that the

**FIGURE 3.** The fas-L promoter activation is enhanced by hyperthermia. Promoter analysis. A–F, Jurkat cells were cotransfected with 15 μg of the indicated Luc reporter vector plus 4 μg of pEQ176 CMV-β-Gal expression vector as described in Materials and Methods. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with 20 ng/ml PMA and 0.5 μg/ml ionomycin (P/I) in the absence or the presence of HS treatment for 24 h. Cells were then harvested, and protein extracts were prepared for Luc and β-Gal assays. Results are expressed as relative Luc activity normalized to protein concentration as well as to β-Gal activity produced by the internal control plasmid and represent the mean ± SE from at least four experiments.
distal NF-AT binding site in this promoter represents a critical enhancer element for HSF-1-mediated transactivation in activated T cells (Fig. 6, D–F). Moreover, the overexpression of a deleted form of HSF-1 that constitutively binds HSE enhancers, but is unable to transactivate even under stress conditions (35, 45), could partially abrogate the effect of hyperthermia on fas-L promoter activation (Fig. 7). These data indicate that activation of HSF-1 plays a role in fas-L promoter regulation mediated by hyperthermia in activated T cells and identify the distal NF-AT binding site as an important element for this mechanism.

Hyperthermia enhances fas-L gene expression in activated T cells: role of PKC

Hyperthermia has been shown to affect a number of immune functions, including lymphocyte homing and activation (3–5, 7, 8). In particular, hyperthermia treatment results in a notable reorganization of the cytoskeleton in T lymphocytes, with HSP70, the cytoskeletal protein spectrin, the adaptor protein receptor for activated C kinase 1, and different PKC isoform colocalization in cytoplasmic aggregates and at the uropod (4, 5). Moreover,
heat exposure can increase PKC activity in T lymphocytes, indicating that PKCs may be involved in hyperthermia-induced signal transduction events, leading to lymphocyte activation and function (3–5). Among the PKC isoforms involved in this context, PKC\textsuperscript{θ} and PKC\textsuperscript{ε} are particularly interesting because they are important regulators of the \textit{fas-L} gene and promoter in activated T lymphocytes (46–48). To investigate whether the hyperthermia-mediated increase in \textit{fas-L} promoter activation could also involve PKC activity, transient transfection assays were performed in Jurkat T cells using expression vectors encoding dominant negative mutants of PKC\textsuperscript{θ} and PKC\textsuperscript{ε}. As shown in Fig. 8A, hyperthermia significantly enhanced activation of the human \textit{fas-L} promoter in Jurkat cells. Overexpression of a dominant negative mutant of PKC\textsuperscript{θ} partially inhibited promoter activation as previously described (46–48) and, more importantly, could significantly reduce the enhancement mediated by hyperthermia. To verify that the action of these dominant negative vectors was...
for the maintenance of immune privilege, in the induction and regulation of fas-L expression in activated T lymphocytes. In particular, regulation of the pharmacological intervention applied to the modulation of cytokine actions of several antineoplastic drugs (2), a great deal of attention has focused on the ability of heat to regulate the immune system actions of radio-/chemotherapy protocols (2, 51, 52).

over the last few years, our laboratory has investigated the molecular mechanisms involved in normal physiological regulation and pharmacological intervention applied to the modulation of cytokine expression in activated T lymphocytes. In particular, regulation of the fas-L gene has been studied in these cells (30, 33), given its relevance for the maintenance of immune privilege, in the induction and regulation of organ-specific autoimmune diseases, and for T lymphocyte-mediated cytotoxicity, a mechanism by which Ag-specific effector cells may eliminate different targets, such as tumor cells, via Fas-mediated mechanisms (19, 20, 22, 23).

In this study, the effect of hyperthermia on fas-L gene activation has been investigated in human and murine T cell lines. We have shown that hyperthermia may enhance Fas-L-mediated cytotoxicity, fas-L mRNA expression, and promoter activity in 2B4.11 T cell hybridoma and Jurkat cells. These effects have been observed using different modalities of T cell activation (PMA plus ionomycin, plate-bound anti-CD3) and with two experimental setting of hyperthermia (HS, 42°C for 1 h; mild hyperthermia, 39.5°C for 4 h). Our data also indicate that a minimal promoter fragment spanning nucleotides from −195 bp immediately 5‘ of the translational start site is still responsive to hyperthermia; this correlates with increased expression and transcriptional activity of specific transcription factors such as c-Jun, FosB, and NF-κB, important regulators of the fas-L gene, located in this position.

Many cellular effects of hyperthermia have been described. High temperature may induce multiple actions on cell structure and physiology, the most remarkable of which are the pathways involved in activation of the stress response, resulting in the induction of a particular set of proteins, the HSPs. HSPs may cooperate together with accessory co-chaperones to protect the cell from dangerous stress, regulating different aspects of cell homeostasis, such as altered protein folding, or interacting with various signaling molecules to form active complexes (40, 41). This evolutionarily conserved phenomenon is mediated by the activation of a pivotal transcriptional factor called HSF-1, which is endowed with heat-inducible, DNA-binding activity and transactivation, in many different experimental
models, including T lymphocytes (42) (we have confirmed this pathway in our cell lines; data not shown). In this regard, the overexpression of a constitutively active mutant of human HSF-1 could significantly increase fas-L promoter activity at normal temperature in our cotransfection assays, suggesting that HSF-1 is able to cooperate for transactivation in this context. Moreover, the overexpression of a dominant negative HSF-1, unable to transactivate even under stress conditions, could partially abrogate the effect of hyperthermia on fas-L promoter activation. These results indicate that activation of HSF-1 plays a role in fas-L promoter regulation mediated by hyperthermia in activated T cells.

Hyperthermia may also affect the stability and fluidity of cellular membranes and may induce various changes in cytoskeletal organization in different cell types that may affect important functions, such as signaling events, cell shape, and migration (2, 56). In this regard, fever-range whole body hyperthermia may induce different changes in lymphocyte physiology indicative of an activation condition, such as reorganization of the cytoskeleton and cellular redistribution of several PKC isoforms with increased enzymatic activity (3–5).

Among the PKC isoforms involved in this effect, PKCθ and PKCε are particularly interesting because they are important regulators of the fas-L gene and promoter in activated T lymphocytes (46–48). In addition, in T cells these two PKC isoforms are differentially redistributed to the membrane or associated with the cytoskeleton after hyperthermia, and this correlates with increased PKC activity associated with these cellular fractions, suggesting a possible role in heat-induced signal transduction events leading to lymphocyte activation and function (4, 5). Our cotransfection assays have shown that overexpression of dominant negative mutants of the PKCθ and PKCε isoforms partially inhibited promoter activation as previously described (46–48) and, more importantly, could significantly reduce the specific enhancement mediated by hyperthermia. These data indicate that PKC may play an important role in this regulation. Interestingly, increased fas-L promoter activity correlates with increased expression and activity of transcription factors, such as AP-1 and NF-κB, which are critical transactivators regulated by PKC (57–60) and are located in the minimal promoter fragment (from −195 bp), still sensitive to hyperthermia.

The thermal component of fever and the HS response may act in the context of different pathologies in several ways. Besides its direct action on the viability of the pathogens, hyperthermia may increase their immunogenicity via HSP production or differentially regulate important pathways of the immune response (61). The experiments described in this report, showing that hyperthermia increases fas-L gene expression in activated T cells, add further evidence of the multiple regulatory effects of heat on the immune system; they may help in our understanding of the complex pathways that operate during adjuvant hyperthermia treatments of malignant disease (2, 51, 52). Various laboratories have already shown that triggering of death receptors, such as Fas/CD95 or TNF receptor type 1, may inhibit activation of the HSF-1/hsp70-mediated stress response; they activate protein phosphatase (PP) 1/PP2a- and PP2b-specific phosphatases that inhibit HSF-1 hyperphosphorylation and DNA-binding activity, thus increasing the sensitivity of the cell to heat-induced apoptosis (62, 63). In contrast, hyperthermia may activate caspase-8 and down-regulate FLIP levels, which sensitizes different cell lines to Fas/CD95-mediated apoptosis (64). Our observation, that hyperthermia increases fas-L gene expression in activated T cells could well fit with these regulatory pathways that involve death receptor signaling, stress response to heat, and modulation of apoptosis. Appropriate

schedules of hyperthermia treatment (or hyperthermia together with radio-/chemotherapeutics) might render specific target cells more susceptible to Fas-mediated apoptosis and, in parallel, increase fas-L gene expression and action in activated CTL. This might, in turn, further sensitize targets to heat by inhibiting the HSF-1/hsp70 stress response via Fas receptor signaling. Associated with these effects is the ability of elevated temperatures to induce immature dendritic cells to mature phenotypically and functionally, both in vitro and in vivo (also in the absence of any exogenous inflammatory stimuli), and this correlates with the improved immunostimulatory ability of these cells (13–15). These observations indicate that enhanced dendritic cell activation and function might represent an additional strategic use of heat to enhance the potency of immunotherapy schedules or to improve the activity of vaccines consisting of relatively weak Ags, such as cancer vaccines (65). In this context, the ability of Fas-L to elicit an optimal proliferation of CTLs (24–26) and the effect of an appropriate Fas receptor triggering on the phenotypical and functional maturation of dendritic cells should also be considered (27).

In summary, this study has identified fas-L as a novel molecular target of hyperthermia that extends knowledge about the immunoregulatory actions of heat. Additional work will be necessary to further characterize the effects of hyperthermia on the molecular mechanisms of T cell activation and to support these data using nontransfected systems and/or in vivo models to improve the potential for thermal therapy in the clinic, particularly in the context of cancer immunotherapy.

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References


Apoptosis and Fas-L transcription

Hypothermia and Fas-L transcription

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