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Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway

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Abstract

Dendritic cells (DC) are key components of innate and adaptive immune responses. The identity of endogenous signals that activate DC is a crucial and unresolved question. We report here that heat shock proteins (HSP), the most abundant and conserved mammalian molecules, constitute such an internal signal. Necrotic but not apoptotic cell death leads to release of HSP gp96, calreticulin, hsp90 and hsp70. HSP stimulate macrophages to secrete cytokines, and induce expression of antigen-presenting and co-stimulatory molecules on the DC. The HSP gp96 and hsp70 act differentially, and each induces some but not all molecules. HSP interact with these antigen-presenting cells through the highly conserved NF-κB pathway. As HSP are intracellular, abundant and soluble, their presence in the extra-cellular milieu and the consequent activation of antigen-presenting cells (APC) constitutes an excellent mechanism for response to cell death. As HSP are conserved from bacteria to mammals, the ability of HSP to activate APC provides a unified mechanism for response to internal and external stimuli.

Introduction

Our previous studies have shown that microgram quantities of heat shock protein (HSP)–peptide complexes can immunize rats, mice, frogs (see 1) and humans (2), and elicit specific T cell responses. The mechanism through which HSP–peptide complexes elicit T cell responses has been elucidated partially (3). HSP interact with HSP receptors on antigen-presenting cells (APC) such as macrophages and dendritic cells (DC) (4,5); the HSP–peptide complexes are taken up into non-acidic compartments, and the peptides are processed and re-presented by the MHC I molecules of the APC. The HSP–APC interaction lies therefore at the heart of the unusually potent immunogenicity of the HSP–peptide complexes.

During our analysis of the HSP–APC interaction, we have become aware of a novel aspect of HSP function. We observe that HSP stimulate macrophages to elaborate cytokines and induce expression of higher levels of co-stimulatory molecules on the DC. These phenomena occur with the involvement of the NF-κB pathway. The new properties of HSP, as reported here, are reminiscent of those of bacterial products such as lipopolysaccharides (LPS) or of certain cytokines, which are secondary activators of DC. HSP are shown here to be the first products of autologous (mammalian) origin which are primary activators of DC. Our observations suggest a key role for HSP–APC interaction in the maintenance and potentiation of innate immunity, in addition to the previously documented role of such interaction in eliciting adaptive responses to the HSP-chaperoned peptides.

Methods

HSP, antibodies and LPS antagonist

hsp90, hsp70 and gp96 were purified simultaneously from C57BL/6 mouse liver as described (6). Antibodies against

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CD80 or B7-1 (clone 16-10A1), CD86 or B7-2 (GL1), CD40 (3/23), CD11b (M1/70), CD11c (HL3) and MHC II (clone AF6-120.1) for FACS analysis were purchased from PharMingen (San Diego, CA). LPS antagonist Rslp was obtained from Dr Niloufer Qureshi.

Assay of LPS content

The LPS content was measured by the limulus amebocyte lysate (LAL) assay (LAL kit QCL-1000; Biowhittaker, Walkersville, MD).

Preparation of necrotic and apoptotic cells

Cells were frozen and thawed through four cycles of liquid nitrogen–room temperature treatments in order to mimic necrosis. Cells were irradiated (7500 rad) in order to initiate apoptosis.

Generation of bone marrow-derived DC

Femurs and tibia of C57BL/6 mice were removed. The marrow was flushed out from the bones with media and leukocytes obtained were cultured as described (7).

Cytokine assay

Cells (5×10^4 or 3×10^4 as indicated) were incubated for 20 h at 37°C in complete medium with 5% FCS, or with increasing quantities of HSP, in 96-well, flat-bottom plates. Supernatants were harvested and assayed by ELISA for tumor necrosis factor (TNF)-α, IL-12, IL-1β, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN-γ. IL-1β, TNF-α, GM-CSF and IFN-γ kits were purchased from Endogen (Woburn, MA), and the IL-12 kit was purchased from R&D Systems (Minneapolis, MN).

Preparation of nuclear extracts and electrophoretic mobility shift assay

APC were washed with PBS (LPS-free) and re-suspended in cold lysis buffer [buffer A: 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μM aprotinin, 1 μM pepstatin and 14 μM leupeptin] with 0.1% NP-40 and incubated on ice for 30 min. Nuclei were pelleted at 14,000 r.p.m. for 2 min at 4°C. Proteins were extracted from the nuclei in a hypertonic buffer [buffer C: 20 mM HEPES (pH 7.9), 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μM aprotinin, 1 μM pepstatin and 14 μM leupeptin] on ice for 30 min (mixing frequently by vortexing). Nuclear debri was pelleted at 14,000 r.p.m. for 5 min at 4°C. Supernatant was collected and protein concentration was measured by Bradford assay. The standard DNA-binding reaction was performed using κB DNA probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′), as described (8).

Results

Necrotic but not apoptotic cells release HSP

Cell death can be achieved in a variety of ways, popularly classified into two: apoptotic and necrotic. We have asked which of these two forms of death can result in release of the major HSP: hsp70, hsp90, calreticulin (CRT) and gp96. E.G7 cells were subjected to a freeze–thaw procedure as a necrosis-mimetic or were irradiated as a form of apoptosis-mimetic process, as described in Methods. Cells were checked for necrosis visually under the microscope, and for apoptosis by externalization of phosphatidyl serine (as detected by staining with Annexin V) and degradation of PARP by caspases (data not shown) (9). The supernatants of the treated cells were collected immediately after treatment or 24 h after treatment by either method, and analyzed by SDS–PAGE and immunoblotting with antibodies to the four HSP. It was observed that necrotic but not apoptotic death led to release of all four HSP (Fig. 1). No HSP were detected in the supernatants of apoptotic cells even 24 h after death.

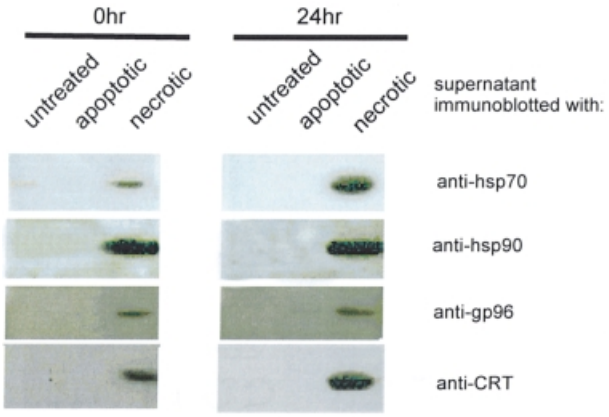

Fig. 1. Necrotic but not apoptotic cell death leads to release of HSP. E.G7 cells were subjected to a freeze–thaw procedure (as mimicking necrosis) or irradiated (causing apoptosis). Supernatants of necrotic or apoptotic cells (1×10^6 cell equivalents) were collected immediately after or 24 h after treatment, resolved on SDS–PAGE and blotted. Blots were probed with antibodies to the HSP indicated.

HSP stimulate macrophages to secrete cytokines

Three major HSP, hsp90 and gp96 (of the hsp90 family) and hsp70, were tested. hsp90 and hsp70 are cytosolic proteins, whereas gp96 is localized to the endoplasmic reticulum. Approximately 30–100 μg gp96, 200 μg hsp70 and 400 μg hsp90 can be isolated in purified form from $2–5\times10^8$ cells. Altogether, these three HSP constitute the most abundant soluble components (>2% of the total protein) of the mammalian cells. Homogenous preparations of the three HSP were obtained from livers of C57BL/6 mice as described in Methods (Fig. 2A) and were identified by immunoblotting with respective mAb (data not shown). Peritoneal cells from mice previously injected i.p. with pristane were positively selected for $CD11b^+$ cells as described in Methods and then cultured *in vitro* with increasing quantities of gp96 for 20 h at 37°C. Supernatants were harvested and tested for the presence of IL-1β, TNF-α, GM-CSF and IL-12, and as a negative control, IFN-γ, by ELISA (Fig. 2B). Treatment with anti-CD11b antibody during or after positive selection did not result in activation of the cells. gp96 was found to stimulate in a titratable manner secretion of all the cytokines tested, except IFN-γ (data not shown). Similar results were obtained with hsp90 and hsp70, but not with control proteins histone, ovalbumin and insulin (Fig. 2B). Although gp96 was the most potent inducer of the four cytokines at comparable protein quantities, it is the least

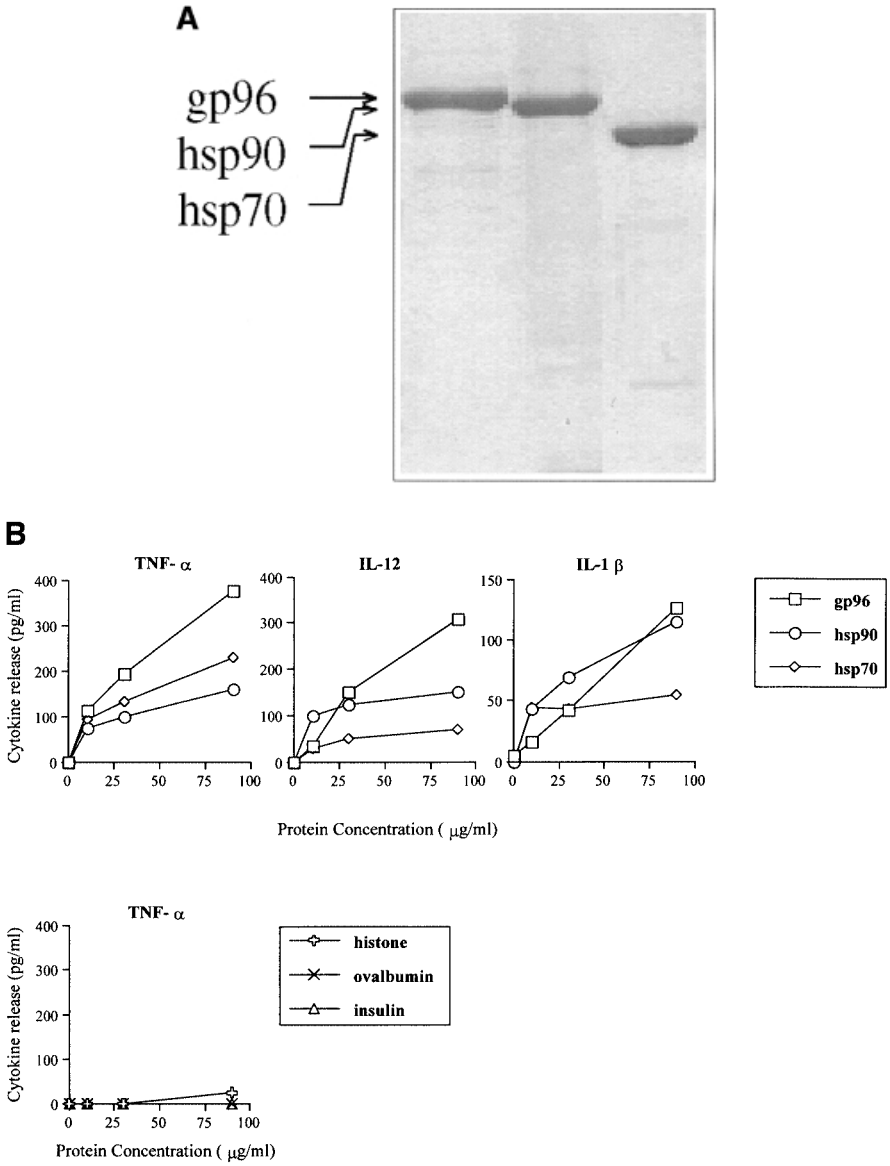

Fig. 2. HSP stimulate macrophages to secrete cytokines. (A) SDS–PAGE analysis of purified preparations of gp96, hsp90 and hsp70. The HSP were purified from livers of C57BL/6 mice, as described in Methods, and had undetectable levels of LPS; 2 μg of each HSP preparation was applied to each lane. (B) Peritoneal cells obtained from C57BL/6 mice previously injected i.p. with pristane were positively selected for $CD11b^+$ cells. Cells (5×10^4) were incubated for 20 h at 37°C in complete RPMI 1640 medium with 5% FCS, or with increasing quantities of homogenous preparations of gp96, hsp90 or hsp70 purified from livers of C57BL/6 mice or control proteins histone, ovalbumin and insulin as indicated, in the same medium. Supernatants were harvested and assayed by ELISA for TNF-α, IL-12 and IL-1β. Only the TNF-α data are shown for the control proteins; similar negative data were observed for IL-12 and IL-1β for the control proteins.

abundant among the HSP tested. hsp90 therefore appears to be the most significant stimulator on a per cell equivalent basis when one considers that it is the most abundant among the HSP.

The ability of HSP to stimulate APC does not derive from contaminating LPS

Because LPS is a known and potent stimulator of APC, HSP were purified by the deliberate use of Good Manufacturing Practices of the Food and Drug Administration and the resulting HSP preparations were shown to be free of detectable levels of LPS (<0.02 EU in absolute quantity) by the LAL assay. The possibility of contribution of LPS was tested by two independent parameters. Activation of APC by LPS, particularly at low concentrations of LPS, is dependent upon the presence of the LPS-binding protein (LBP) normally present in serum (10,11). gp96 preparations were tested for their ability to stimulate secretion of IL-1β by macrophages in medium with and without serum. The activity of gp96 was found to be entirely serum-independent. In contrast, the activity of LPS in a similar assay was highly sensitive to the presence of serum (Fig. 3A). As yet another test for

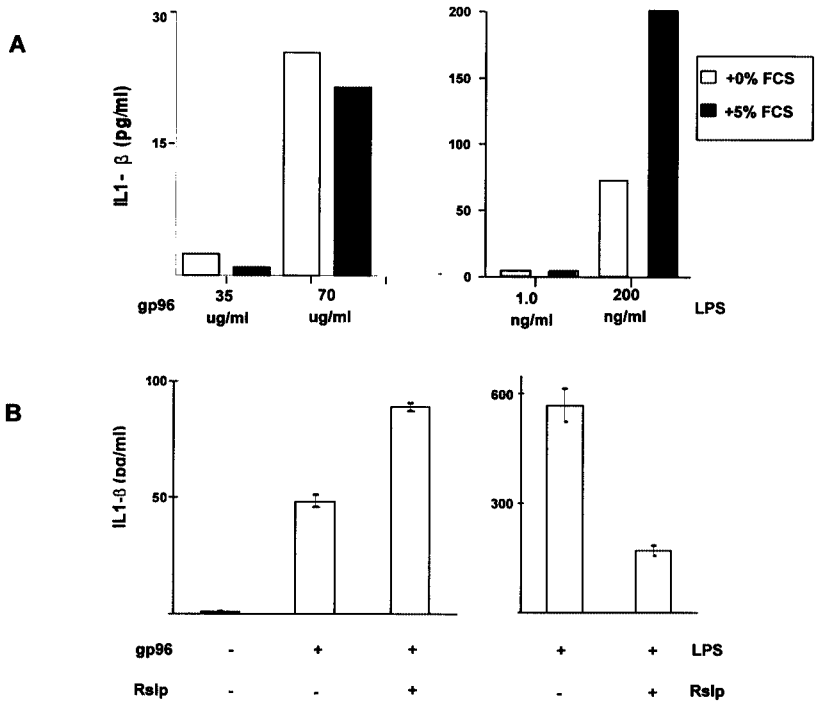

Fig. 3. The APC-stimulating activity of gp96 does not derive from contaminating LPS. (A) CD11b$^+$ cells (3×10^4), isolated from C57BL/6 mice were incubated in complete RPMI 1640 medium with or without 5% FCS (as a source of LBP) as indicated, or treated with gp96 or LPS at the indicated amounts in the above media for 20 h at 37°C. Supernatants were harvested and assayed for IL-1β by ELISA. The lower levels of IL-1β released by stimulation with gp96 in this experiment (as compared to the experiment in Fig. 2) are a consequence of the fact that a smaller number of CD11b$^+$ cells/well were used here. (B) The LPS antagonist Rslp, derived from *R. spheroides* (2 μg/ml), was added to cytokine secretion assays of LPS (2 μg/ml) or gp96 (90 μg/ml) as indicated.

potential contamination of HSP by LPS, an antagonist (competitive inhibitor) of LPS, derived from *Rhodopseudomonas spheroides*, was used (14). This inhibitor (Rslp) diminished the ability of LPS but not gp96 to stimulate secretion of IL-1β by >75%. In fact, the activity of gp96 was greater in presence of Rslp (Fig. 3B). Other points of distinction were observed between LPS and HSP and these are indicated elsewhere (see Fig. 5 and attendant text). Other control experiments were performed to rule out the possibility of contaminating LPS being responsible for the activity observed by us. These included boiling and trypsin treatment of the HSP preparations and LPS. In each case tested, the APC-stimulatory activity of HSP preparations was sensitive to both treatments, while the corresponding activity of LPS preparations was resistant to both (data not shown).

HSP stimulate DC to express antigen-presenting and co-stimulatory molecules

The effect of HSP on maturation of DC was examined. Homogenous, LPS-free preparations of the HSP gp96 and hsp70 were obtained from livers of C57BL/6 mice. Bone marrow-derived DC, obtained by culturing in GM-CSF-containing medium (7), were pulsed with gp96 or hsp70, or with LPS (as a positive control) or serum albumin (as a negative control). The pulsed DC were tested for surface expression of MHC II, B7-1, B7-2 and CD40 molecules. LPS induced expression of all markers tested, except B7-1. We attribute this result to a relatively high proportion of B7-1$^+$ cells in the starting DC culture. gp96 (400 μg/ml) was observed to induce a high degree of expression of MHC II and the co-stimulatory molecule B7-2, but not B7-1 nor CD40 (Fig. 4). hsp70 (400 μg/ml), on the other hand, elicited a modest stimulation of surface expression of B7-2 but not B7-1, nor MHC II and CD40. The complete lack of stimulation of CD40 expression by gp96 or hsp70 led us to test this phenomenon more extensively and at a range of concentrations of the HSP (40–400 μg/ml); however, CD40 expression was not induced at any concentration tested. Serum albumin (400 μg/ml), in the same buffer as the HSP, did not induce expression of any of the markers tested.

Activation of APC by HSP activates translocation of NF-κB

The mechanism through which gp96 interacts with APC was investigated, with reference to the activation of the NF-κB pathway, shown previously (12) to be a key transcriptional regulator for several cytokines and other immunologically important molecules. This pathway has also been shown to be activated in response to LPS and to be involved in the maturation of DC (13). Primary cultures of CD11c$^+$ cells were pulsed with gp96 or LPS and cells were harvested at various time intervals. Nuclear extracts from the samples were used for binding to NF-κB-specific oligomers and were resolved by native PAGE. It was observed that gp96 activates the transduction pathway and does so with a kinetics distinctly different from that of LPS (Fig. 5). The nuclear translocation of NF-κB is seen in gp96-treated DC as early as 15 min after

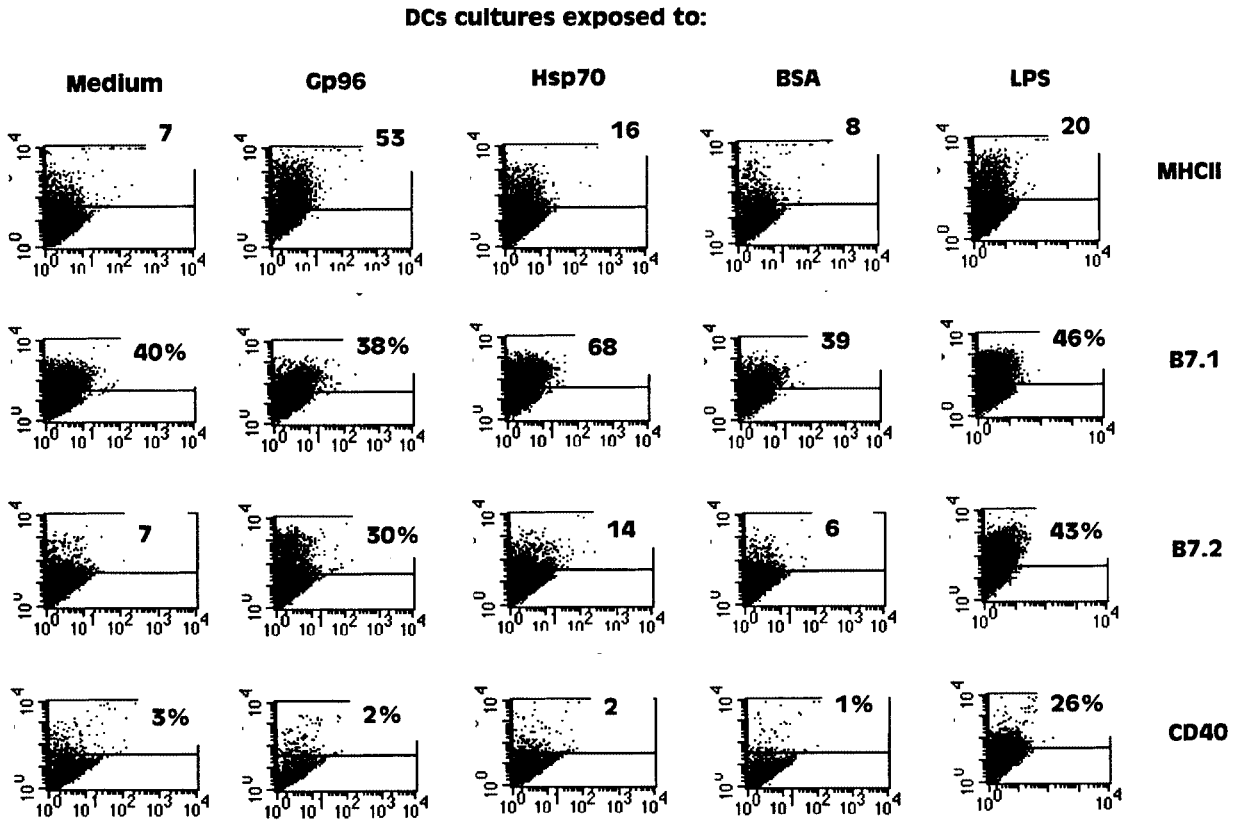

Fig. 4. HSP stimulate CD11c$^+$ cells to express antigen-presenting and co-stimulatory molecules. Bone marrow-derived DC cultures were exposed to the medium, HSP (400 μg/ml), albumin (400 μg/ml) or LPS (2 μg/ml) for 20 h, harvested and analyzed for expression of the cell-surface molecules indicated. GM-CSF was not present in the DC cultures when they were treated with medium alone, gp96, LPS or albumin. The percentages shown are CD11c$^+$ cells that are also positive for the indicated surface markers. Mean fluorescence values for medium, with respect to MHC II, B7-1, B7-2 and CD40 are 27, 35, 25 and 17. Albumin-treated cells have almost identical values. For gp96-treated cells, the corresponding values are 54, 33, 35 and 13. For hsp70-treated cells, the corresponding values are 29, 62, 28 and 16. For LPS-treated cells, the values are 30, 49, 50 and 25. Cells were analyzed by flow cytometry using the FACScan (Becton Dickinson, La Jolla, CA). Live cells were gated based on FSC/SSC profiles.

pulsing and the signal diminishes to background levels by 120 min. In contrast, the translocation in LPS-treated DC has a slower initiation kinetics. The differences in the kinetics of translocation of NF-κB between gp96 and LPS as seen here is not a function of the quantities of either agent. Exposure of DC to graded quantities of each shows the same differences in kinetics (data not shown). In addition to providing a key glimpse into the mechanism through which HSP activate APC, these studies show the extent to which the effects of LPS and HSP on APC are similar yet distinct.

Necrotic but not apoptotic lysates deliver a maturation signal and cause translocation of NF-κB

In view of the data shown in Fig. 5, and in view of the recent demonstration by Gallucci *et al*. (14) and Sauter *et al*. (15) that necrotic but not apoptotic cells mediate maturation of DC, we tested whether exposure of DC to necrotic or apoptotic cells leads to translocation of NF-κB to the nucleus. Cultures of immature DC were exposed to necrotic or apoptotic E.G7 cells (prepared as described in Methods), and were monitored for expression of MHC II, B7-1, B7-2 and CD40. Exposure of DC to necrotic but not apoptotic cells elicited expression of each of the maturation markers on the DC (Fig. 6A). Interestingly, although purified gp96 or hsp70 did not stimulate expression of CD40 (Fig. 4), the necrotic lysate did (Fig. 6A). The possible significance of this observation is commented upon in Discussion. The DC cultures exposed to medium, or necrotic or apoptotic lysates were also tested for translocation of NF-κB. Consistent with the observations in Fig. 5, necrotic but not apoptotic supernatants mediated translocation of NF-κB to the nucleus (Fig. 6B). While these observations do not prove that the HSP in the necrotic lysates are the causative agents of this translocation, they are definitely consistent with that observation.

Discussion

HSP are intracellular molecules and the physiological relevance of their ability to activate APC may not be immediately obvious. However, being the most abundant, soluble, *intra*cellular molecules, the presence of HSP in the extracellular milieu would act an excellent message alerting the APC to physical damage of the surrounding cells, whether as a consequence of bacterial and viral infections or

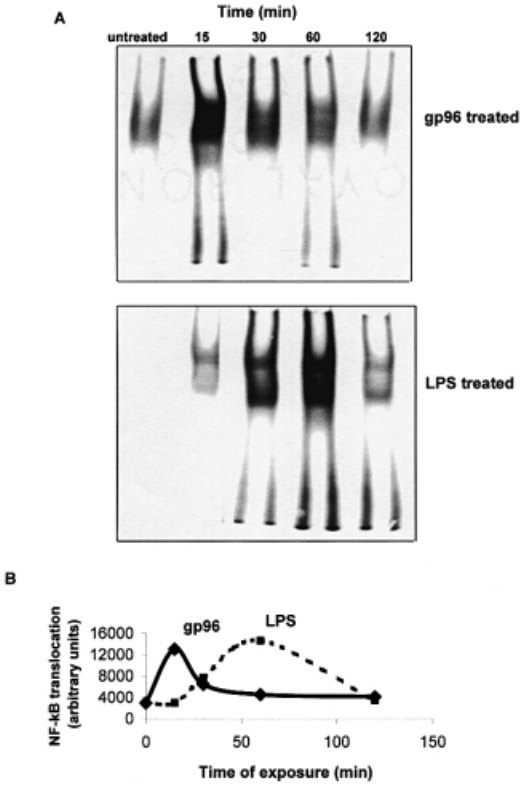

Fig. 5. gp96 HSP interacts with APC through the NF-κB signal transduction pathway. (A) DC (1×10^6 cells) were pulsed with gp96 (100 μg/ml) or LPS (4 μg/ml) for the indicated time points. Nuclear extracts of unpulsed or pulsed cultures were prepared and were used in binding to NF-κB-specific oligomer as described in Methods. The complexes were resolved by native PAGE and autoradiographed. (B) The data from (A) were quantitated by scanning the gels under linear conditions of exposure and plotted.

mechanical injury. The ability of this signal to activate APC can therefore be easily imagined to confer an immunological and hence survival advantage to the organism. The co-segregation of immunogenicity of a variety of cancers with higher levels of expression of inducible hsp70, without any preceding change in the antigenic repertoire of the cancers, is a case in point (16,17). Conversely, the lack of such a signal may provide a mechanism for discrimination between the presence of antigen with and without 'danger', as proposed by Matzinger (18). The quantities of HSP shown here to be necessary to stimulate APC *in vitro* are well within the range expected to be released locally as a result of cell lysis *in vivo*. Typically, 1 g of tissue yields ~30 μg gp96, 200 μg hsp70 and 400 μg hsp90. These recoveries are somewhere in the range of 25%. Thus 1 g of tissue contains ~2.5 mg HSP. Considering that the tissue lysis *in vivo* can be reasonably assumed to happen not in solution but in a semi-liquid physical state, lysis of as little as 1 mg of cells (~10^5–10^6 cells, depending on the cell type) will lead to release of ~2 μg HSP in a volume of ~1–2 μl or less. That is a concentration of 1–2 mg/ml—a higher concentration than that used in our studies *in vitro*. Considerations of quantity are therefore compatible with a role *in vivo* of HSP in activation of APC. Asea *et al*. (19) have reported recently that far lower concentrations of hsp70 than used in our studies are able to mediate stimulation of human monocytes. We are unable to detect any activity, either cytokine secretion or maturation of DC, at the concentrations of hsp70 used by Asea *et al*. The differences may be attributable to the differences in purity of our hsp70 preparations (see Fig. 2A), possible differences between human and murine APC or functional differences between the APC populations used by us.

The HSP gp96 and hsp70 are not identical in their activity: gp96 induces MHC II and B7-2 but not B7-1, while hsp70 induces B7-1 but not MHC II nor B7-2. However, in view of the variability of B7-1 expression in our DC cultures (see Fig. 4 versus Fig. 6), the lack of effect of either HSP on B7-1 expression needs further examination. Neither HSP induces CD40, thus indicating that the maturation signal delivered by each is only partial. While these studies were under submission, Asea *et al*. (19) and Ohashi *et al*. (20) have reported on the ability of hsp70 and hsp60 respectively to stimulate monocytes or macrophages. Those results, together with our demonstration of release of gp96, hsp90, CRT and hsp70 by necrotic but not apoptotic death, and of the ability of gp96 and hsp70 to deliver a partial maturation signal to DC, support our original thesis (1,21,22) of a wider and general role for HSP as endogenous stress signals to APC.

Examination of the levels of cytokines released by macrophages or of the extent of induction of the maturation markers on DC by stimulation with HSP, shows that the HSP stimulate the APC to a modest degree, as compared with the stimulation conferred by LPS. For this reason, we have tested our observations repeatedly and have found them to be consistent. We wish to infer from this that the endogenous activators of DC (HSP in this instance) are much slower activators than external activators such as LPS for a physiological reason: the lower 'specific activity' of endogenous signals allows for a more regulated activity, as the response to an internal signal might have to be far more modulated and more titratable, than that to an external signal. While our data show unambiguously the APC-stimulatory activity of HSP, they are silent on the question of other non-HSP activators of APC, which might be present in necrotic lysates. Other internal APC activators might indeed exist. Studies involving the APC-activating ability of necrotic lysates depleted of HSP should be revealing in this regard and are in progress.

The HSP are clearly more ancient and more ubiquitous than LPS, as even the earliest forms of life contain HSP, while LPS is unique to Gram-negative bacteria. We suggest that the HSP-mediated and LPS-mediated activation of APC represent utilization of similar mechanisms for similar objectives, although starting from different points. The mechanism of response to external danger may indeed be modeled on the more primitive mechanism of response to cellular disintegration that is usually signaled by the release of internal molecules such as HSP. Janeway has suggested that pattern recognition receptors present on APC serve to act as sentinels of the immune system against primordial non-self and its descendants (23). HSP are highly conserved between self and non-self organisms. In that light, the possibility that the ligands for the HSP which are responsible for the HSP–APC interaction may serve as pattern recognition receptors while at the same

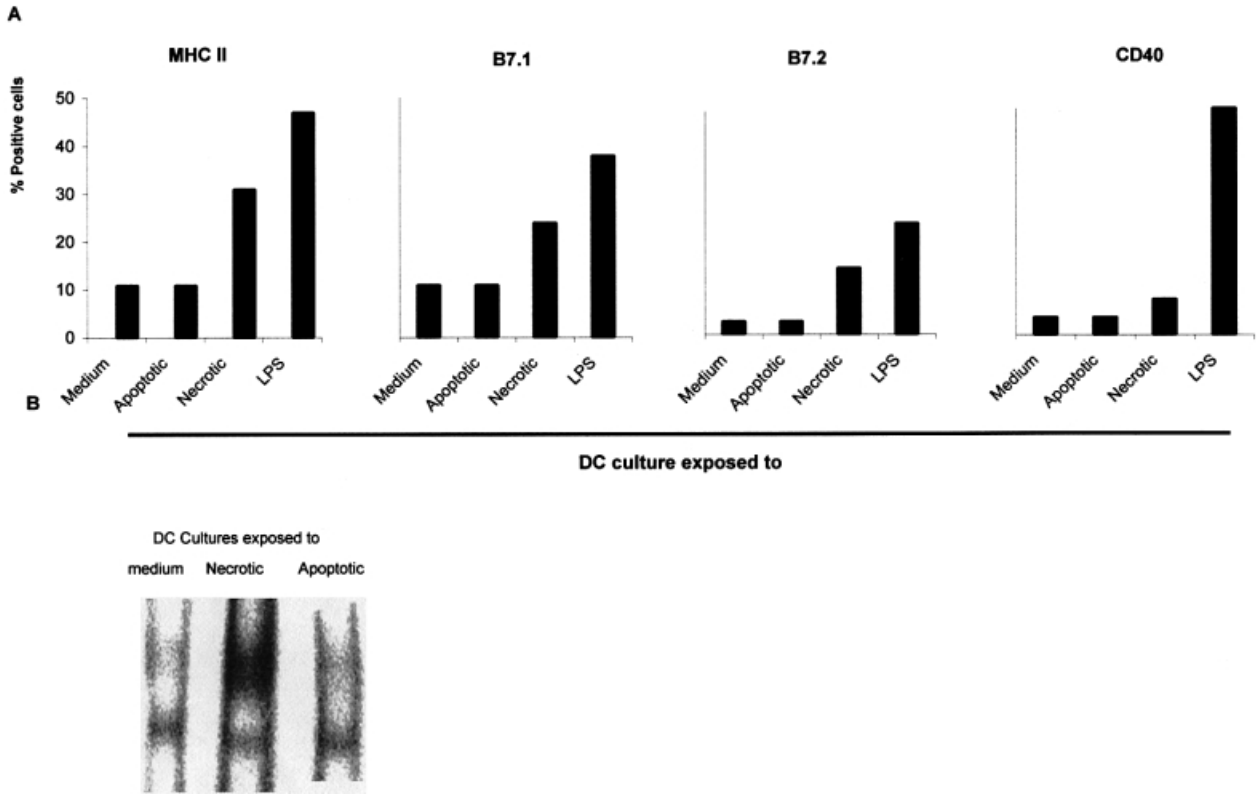

Fig. 6. Exposure of DC to necrotic but not apoptotic cells leads to maturation of DC and to nuclear translocation of NF-κB. Cultures of immature DC (2×10^6) were pulsed with medium, or 10^6 cell equivalents each of necrotic or apoptotic E.G7 cells, or LPS (as a positive control) for 20 h. DC cultures were monitored for expression of surface markers as indicated (A). Mean fluorescence values for MHC II expression are 30 (medium), 31 (apoptotic supernatant), 60 (necrotic supernatant) and 66 (LPS). Corresponding values for B7-1 are 28, 30, 30 and 71. For B7-2, they are 22, 23, 30 and 39, and for CD40, they are 15, 17, 19 and 32. (B) DC cultures exposed to medium, or to necrotic or apoptotic E.G7 cells for 15 min and analyzed for translocation of NF-κB as described in Fig. 5.

time acting as sentinels of internal danger is attractive, and helps reconcile the differences between the self/non-self and danger models of immune response.

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Abbreviations

APC	antigen-presenting cell
CRT	calreticulin
DC	dendritic cell
GM-CSF	granulocyte macrophage colony stimulating factor
HSP	heat shock protein
LAL	limulus amebocyte lysate
LBP	LPS-binding protein
LPS	lipopolysaccharide
TNF	tumor necrosis factor

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