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Primary Tumor Tissue Lysates Are Enriched in Heat Shock Proteins and Induce the Maturation of Human Dendritic Cells

Selin Somersan,* Marie Larsson,* Jean Francois Fonteneau,* Sreyashi Basu,† Pramod Srivastava,† and Nina Bhardwaj2*∗

Upon exposure to lysates or supernatants of necrotic transformed cell lines, human dendritic cells (DCs) undergo maturation. In contrast, DCs exposed to apoptotic transformed cell lines or necrotic lysates of primary cells remain immature. Analysis of supernatants of necrotic transformed cell lines showed them to be enriched in the heat shock proteins (hsp)70 and gp96, in contrast to supernatants of primary cells. Likewise, cells from a variety of primary human tumors contained considerably higher levels of hsp than their normal autologous tissue counterparts. Of the majority of human tumors enriched in hsps (hsp70 and/or gp96), their corresponding lysates matured DCs. The maturation effect of tumor cell lysates was abrogated by treatment with boiling, proteinase K, and geldanamycin, an inhibitor of hsps, suggesting that hsps rather than endotoxin or DNA were the responsible factors. Supporting this idea, highly purified, endotoxin-depleted hsp70, induced DC maturation similar to that seen with standard maturation stimuli LPS and monocyte conditioned medium. These results suggest that the maturation activity inherent in tumor cells and tissue is mediated at least in part by hsps. The release of hsps in vivo as a result of cell injury should promote immunity through the maturation of resident DCs. The Journal of Immunology, 2001, 167: 4844–4852.

Dendritic cells (DCs) are bone marrow-derived APCs that play a central role in initiating adaptive and innate immune responses. In peripheral tissues, they exist in an immature state, where they acquire Ags via multiple mechanisms, e.g., phagocytosis, receptor-mediated endocytosis, and macropinocytosis. When exposed to pathogenic and inflammatory stimuli, they migrate to draining lymph nodes and undergo maturation. When mature, DCs down-regulate their ability to acquire Ags, but increase their T cell-stimulatory capacity through up-regulation of costimulatory and MHC molecules, increased expression and durability of peptide-MHC complexes, development of immunoregulatory molecules, and capacity to synthesize cytokines such as IL-12, IL-15, and IL-18 (reviewed in Ref. 1). Several factors induce DC maturation. They include microorganisms (bacteria, Ref. 2; viruses, Ref. 3; yeast, Ref. 4), CD40 ligand on activated T cells (5, 6), cytokines (e.g., TNF-α, IL-1β), bacterial and viral products (LPS, Refs. 7 and 8; unmethylated CpG DNA sequences, Ref. 9; dsRNA, Refs. 3 and 10), and nucleotides (ATP, UTP) either alone or in conjunction with TNF-α (11). Recently, we showed that lysates or supernatants of necrotic transformed lines also induced DC maturation (12). Maturation was characterized as up-regulation of costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and DC-costimulatory molecules (HLA-DR, CD40, CD86), development of DC-maturation restricted markers such as CD83 and CD8T cells. Only factors released by necrotic transformed cells rather than primary cells such as monocytes or T cells were effective in this regard. Notably, neither apoptotic cells nor their culture supernatants induced maturation. Similar distinctions between necrotic and apoptotic passaged cell lines have been made with murine DCs (13). However, the factor(s) responsible for this effect have yet to be fully characterized. We and others have shown that heat shock proteins (hsps), conserved molecular chaperones within all cells, are released by necrotic but not apoptotic murine lines (14, 15). Necrotic cell lysates induce the activation of murine CD11c+ cells, including the nuclear translocation of NF-kB and the partial up-regulation of MHC class II and costimulatory molecules. Purified gp96 and hsp70 stimulate peritoneal macrophages to produce IL-1β, TNF-α, and IL-12 and induce maturation of murine DCs (14). Injection of gp96 into mice leads to migration of CD11c+ cells in draining lymph nodes (16). Furthermore, highly purified gp96 induces the maturation of human DCs (17). These results suggest that the release of hsps during cell fragmentation may be a critical determinant of APC activation.

To ascertain whether this phenomenon has physiological relevance, and is not simply a feature of transformed cell lines, we analyzed tissue lysates from human tumors for their potential to mature DCs. Strikingly, lysates from several types of tumors induced DC maturation, including heightened T cell stimulatory activity. In contrast, the majority of the primary normal tissues lacked potent stimulatory capacity. Tumor tissues were enriched in hsps (both hsp70 and gp96), whereas considerably lower levels were detected in their autologous normal tissue counterparts. The maturation capacity of necrotic transformed lines and primary cells also correlated directly with their hsp content. Our results

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3 Abbreviations used in this paper: DC, dendritic cell; hsp, heat shock protein; MCM, monocyte conditioned medium; LAMP, lysosome-associated membrane glycoprotein; B-LCL, EBV-transformed B lymphocytes; hsc70, constitutive hsp70; LAL, limulus amebocyte lysate; MP, influenza matrix protein peptide.
support the hypothesis that cell injury in vivo leads to the activation of APCs, but that the source and nature of the cells may be critical determinants in this effect.

Materials and Methods

Isolation of PBMCs

Leukocyte-enriched buffy coats were obtained from New York Blood Center (New York, NY). Alternatively, blood from healthy donors was used. PBMCs were separated by density on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). PBMCs were enriched in monocytes and T cells by rosetting with neuraminidase (Calbiochem-Novabiochem, La Jolla, CA)-treated SRBCs as described before (18).

DC cultures

Human DCs were generated by culturing monocyte-enriched fractions or adherent PBMCs with 100 IU/ml GM-CSF (Immunex, Seattle, WA) and 300 µg/ml IL-4 (Scherling-Plough, Kenilworth, NJ) for 5 days in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 20 µg/ml gentamicin (Life Technologies, Gaithersburg, MD), 1 mM HEPES (Mediatech), and 1% human plasma. The cytokines were added to the DCs at days 0, 2, or 4. On days 5 or 6, nonadherent cells were transferred to new plates, and cultures were incubated with apoptotic or necrotic cells or tissue lysates. LPS (Sigma, St. Louis, MO) and monocyte conditioned medium (MCM) (19) were used as standard maturation stimuli. DCs were collected for analysis of maturation markers on days 7 and 8.

Primary tissues

Tumor tissues or their corresponding normal tissues were obtained from the Massachusetts General Hospital Tumor Bank (Boston, MA) as frozen specimens. On the day of use, they were thawed and weighed. The tissues were brought up to same weight (where normal and tumor tissues have the same gram per milliliter concentrations) or protein (where normal and tumor tissues have the same milligram per milliliter protein concentration evaluated by Bradford assay; Bio-Rad, Hercules, CA) concentrations in RPMI 1640. The tissues were then minced using sterile scalpels. Repetitive freeze-thaw cycles were performed for lysate preparation.

Cell lines

The human cell lines EBV-transformed B lymphocytes (B-LCL) and HeLa cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FCS (Mediatech). Mouse thymoma line EL-4 was grown in DMEM (Mediatech) and 10% heat-inactivated FCS (Mediatech). All media were supplemented with 20 µg/ml gentamicin (Life Technologies) and 1 mM HEPES (Mediatech). All cell lines were tested for mycoplasma.

Induction of apoptosis and necrosis

UV-triggered apoptosis was induced using a 60-mJ UVB lamp (Derma Control; Kinetic Biomedical Services, Southampton, PA) calibrated to provide 2 mJ/cm²/s. Cells were incubated for 8 h at 37°C for apoptosis to occur. Necrosis was induced by repetitive freezing (dry ice/ethanol) and thawing (37°C waterbath) cycles. Supernatants were prepared by spinning these apoptotic or necrotic cells at 14,000 rpm for 30 min.

Western blots

The supernatants of the cells (as prepared above) were run on a 10% SDS-PAGE gel. The gel was transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk, after which it was stained with Abs to constitutive hsp70 (hsc70), inducible hsp70, both constitutive and inducible hsp70 (StressGen Biotechnologies, Victoria, British Columbia, Canada), gp96 (Neomarkers, Fremont, CA), or actin (Sigma). All HRP-conjugated secondary Abs were purchased from Sigma. The membrane was used for detecting ECL-Plus (Amersham Pharmacia Biotech, Piscataway, NJ). The analysis was performed by either exposing the membrane to Kodak (Rochester, NY) film followed by development of the film, or by Storm imaging system and the ImageQuant analysis program (Molecular Dynamics, Sunnyvale, CA).

Quantitative analysis

Using ImageQuant volume analysis, a standard curve with bovine hsc70 as the control protein (StressGen Biotechnologies) was made. Using regression analysis by Excel, the values for hsc70 content in different dilutions of cell supernatants were plotted. From the equation of the trendline of this graph the numbers for hsc70 content for 100,000 cells was obtained.

FACS analysis

On day 7 or 8, DCs were harvested for analysis. Surface staining was performed by mAbs to CD83, CD40 (Beckman Coulter, Fullerton, CA), CD86, HLA-DR (BD Pharmingen, San Diego, CA), MHC I (Sigma), CD91 (PROGEN Biotechnik, Heidelberg, Germany). Intracellular staining was performed for maturation marker DC-LAMP (Beckman Coulter). For this, cells were fixed with 4% paraformaldehyde for 10 min at room temperature followed by washing two times with PBS. A third wash was done with 0.1% saponin (Calbiochem, La Jolla, CA) in PBS. Cells were incubated with DC-LAMP or IgG1 as isotype control (BD Pharmingen) at room temperature for 30 min. After washing away the excess Ab, a goat anti-mouse secondary Ab (BioSource International, Camarillo, CA) was added at a 1/200 dilution for 30 min at room temperature. Samples were analyzed on a BD Biosciences (Mountain View, CA) FACScan using CellQuest software.

ELISPOT assay

Milliliter (Millipore, Bedford, MA) 96-well plates were coated overnight at 4°C with 5 µg/ml anti-IFN-γ mAb (Mabtech, Stockholm, Sweden). The next day the Ab was washed away, and the plate was blocked with RPMI 1640 containing 5% pooled human serum for 1 h at 37°C. Cells were added to the wells and incubated for 14–20 h at 37°C. The cells were washed with PBS containing 0.05% Tween 20, and 1 µg/ml biotin-conjugated anti-IFN-γ mAb was added for 2 h. The plates were washed with 0.1% Tween 20 and incubated with avidin-biotinylated HRP H for 1 h at room temperature. The assay was developed with the addition of stable diaminobenzidine for 5 min at room temperature. The spots formed were counted with a stereomicroscope (Stemi 2000 stereomicroscope; Carl Zeiss, New York, NY).

Purified hsp70

Purified, LPS-free hsp70 was obtained from a fibrosarcoma line and provided by Antigenics (Boston, MA).

Inhibition assays

For proteinase K (Amersham) treatment, EL4 lysates, LPS (20 ng/ml final concentration) or medium alone were either untreated or pretreated with 10 µg/ml proteinase K for 45 min at 37°C. Geldanamycin (Life Technologies) was added at a final concentration of 0.05 µg/ml to the cultures at the same time as other stimuli.

Results

Necrotic tumor cell lysates contain high levels of hsps and induce DC maturation

Immature DCs generated from blood monocytes by culture in GM-CSF and IL-4 for 5–6 days express low-moderate levels of costimulatory and adhesion molecules but lack the maturation-restricted markers CD83 (20–23) and DC-LAMP (24). In confirmation of previous studies, coculture of these cells with LPS (a standard maturation stimulus) and necrotic lysates of transformed cell lines (the murine EL4 thymoma line) induced their maturation, as determined by DC-LAMP expression (Fig. 1A, left panel), and heightened T cell-stimulatory activity (Ref. 12; and data not shown). Optimal maturation was achieved with a ratio of at least 1–2 necrotic cell equivalents per DC. In contrast, exposure to apoptotic EL4 cells failed to induce substantial maturation. Furthermore, the effect was restricted to transformed cells, as necrotic lysates of primary human monocytes were ineffective (Fig. 1A, right panel).

Hsps are molecular chaperones that have multiple roles including the activation of APCs such as macrophages and maturation of murine and human DCs. We showed recently that hsps are released by necrotic but not apoptotic transformed cell lines (14). Based on these findings, we hypothesized that hsps levels would be elevated in transformed cell lines, but not primary cells, and high levels would correlate with DC maturation capacity. Therefore, we compared the levels of hsps released by necrotic tumor cell lines vs...
primary cells. Necrosis was induced by repetitive freeze/thaw cycles, and supernatants of lysates were collected for hsp analysis by immunoblotting. As controls, cells were induced to undergo apoptosis by UV irradiation. Two abundant and well studied members of the hsp family, namely hsp70 and gp96, were evaluated. Both hsp family members were detected in supernatants of necrotic but not apoptotic transformed lines including B-LCL, EL4, and HeLa cells (Fig. 1B). Interestingly, the hsp70 was primarily of the constitutive (hsc70) rather than the inducible form (Fig. 1C). Hsps were not destroyed during the process of apoptosis, as they were detected within apoptotic cells after they were lysed (Fig. 1D). In fact, these lysates also induced DC maturation (data not shown), further supporting the argument that hsps play a role in this effect.

On a per cell basis monocytes lacked appreciable levels of hsps (Fig. 1B). This is consistent with our hypothesis that intracellular contents of primary cells lack DC maturation potential because of a relative paucity of hsps. If this were the case, one would expect primary cells expressing high levels of hsps to possess DC maturation capacity. In addition to monocytes several other primary cells were tested, including T cells, SRBCs, and immature DCs. On a per cell level, only immature DCs had comparable levels of gp96 and hsc70 to EL4 cells (Fig. 1E).
As predicted, only DCs and not T cells, monocytes, or SRBCs induced the maturation of immature DCs, reflected by the up-regulation of DC-LAMP (Fig. 1E, right panel). Even addition of up to a 5-fold excess of monocyte lysates failed to induce maturation of immature DCs. Altogether, the data suggest a correlation between the hsp content of a cell and its capacity to mature DCs.

We next quantified the hsc70 content in primary vs transformed necrotic cell supernatants by storm image analysis using bovine hsc70 as a standard. The two transformed lines tested had high levels of hsc70 (B-LCL: 1.1 µg/10⁵ cells; EL4: 0.92 µg/10⁵ cells, Fig. 1F). Similar levels were detected within supernatants of necrotic immature DCs. In contrast, monocytes had <1 ng/10⁵ cells. It is important to keep in mind that these measurements estimate the minimal amount of hsps present in the cells for two reasons. First, only supernatants were evaluated, and it is likely that residual hsps remain within the membranous components of lysates. Second, only a single hsp family member was enumerated. Nevertheless, this quantitative data correlated with what was observed visually in Fig. 1, B and C.

**FIGURE 2.** The maturation induced by necrotic EL4 lysates is not due to mycoplasma or endotoxin contamination and is inhibited by proteases and hsp inhibition. A, Immature DCs were cultured with EL4 lysates or LPS, after which they were analyzed for maturation by DC-LAMP expression (right panel). Supernatants of these cocultures lacked endotoxin by LAL assay (left panel). B, EL4 lysates or LPS were either boiled or untreated, after which they were cocultured with DCs. DC maturation was analyzed by expression of DC-LAMP. C, After pretreatment with proteinase K, EL4 lysates, LPS (20 ng/ml final concentration), or medium alone were added to immature DCs. As an additional condition, LPS was added together with the proteinase K-treated EL4 lysates to ensure that the effects of proteinase K were not due to toxicity. D, Geldanamycin, a hsp90 specific inhibitor was added to cocultures of immature DCs and EL4 cell lysates, LPS (20 ng/ml), or medium alone.
Evidence that hsps in transformed cell lines are the factors that induce DC maturation

Because both mycoplasma and endotoxin are known inducers of DC maturation (25), it was important to exclude their presence in our cell lines. PCR analysis failed to detect mycoplasma DNA, eliminating the possibility of its presence and thus its effect on DC maturation in lysates of transformed cell lines. Endotoxin was an unlikely contributor because none was detected in EL4 lysates by limulus amebocyte lysate (LAL) assay (Fig. 2A). Boiling abrogated the maturation effect caused by necrotic lysates of EL4 cell lines (Fig. 2B), but did not affect LPS-induced maturation, further ruling out endotoxin contamination. Treatment with proteinase K, an unspecific proteolytic enzyme, inhibited the maturation effect of tumor cell lysates (Fig. 2C). As expected, proteinase K did not affect maturation induced by LPS. Altogether, these data suggest that proteins rather than DNA or bacterial LPS are responsible for DC maturation.

Geldanamycin, a benzoquinoid antibiotic from Streptomyces hygroscopicus, is an inhibitor of hsp90 family proteins. It binds the amino-terminal domain and regulates conformation of other hsp90 domains (26, 27). At 0.05 μg/ml, geldanamycin specifically inhibited maturation of DCs by tumor cell lysates, but not LPS (Fig. 2D). This effect is unlikely to be due to geldanamycin toxicity because it did not block LPS-treated DCs from maturing.

**FIGURE 3.** Endotoxin-free purified hsp70 induces DC maturation. A. Endotoxin-free purified hsp70 (195 μg/ml) or PBS alone was added to immature DCs for 2 days, after which DCs were analyzed for maturation markers by FACS (left panel) or for enhanced function (right panel). For the latter, DCs were pulsed with 100 ng of MP and added to a MP-specific CD8+ T cell clone at a ratio of 1:5 DC-T cells. Results are shown as the number of IFN-γ producing spot-forming cells per 5000 T cells. B. After 2 days of coculture with 200 μg/ml purified hsp70, DCs were analyzed for up-regulation of MHC-I, HLA-DR, CD40, CD86, CD83, and DC LAMP.

**Table 1.** Tumor tissues are enriched in hsps relative to their normal counterparts and release factors that induce DC maturation

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<th>Tissue Source (n = 22)</th>
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<th>gp96</th>
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<td>T &gt;&gt;N</td>
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</tbody>
</table>

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Endotoxin-free, purified hsp70 induces human DC maturation

Purified gp96 induces the maturation of human DCs (17); however, the effects of hsp70 on human DCs have not yet been evaluated. Endotoxin-free hsp70 was purified from fibrosarcoma lines by GMP standards. Hsp70 (60–200 μg/ml) was added to cultures of immature DCs. After 2 days the DCs were analyzed for up-regulation of maturation markers and for their capacity to stimulate Ag-specific T cells. In three separate experiments, hsp70 consistently induced maturation (Fig. 3A, left panel), which approached that seen with the standard maturation stimuli MCM and LPS. The DCs were also mature by functional criteria. Flu16 is a CD8+ T cell clone that recognizes influenza matrix protein peptide (MP) with high avidity. This clone preferentially recognizes low doses of MP on mature rather than immature DCs (28). DCs matured with hsp70 and pulsed with MP stimulated IFN-γ production from Flu16 by ELISPOT assay comparably to MCM- and LPS-matured DCs, and significantly better than immature DCs. Hsp70-matured DCs also up-regulated several additional markers associated with mature cells, including CD83, MHC class I and II molecules, CD86, and CD40 (Fig. 3B).

Recently CD91, the α2 macroglobulin receptor was identified as a receptor for gp96, hsp70, and calreticulin on murine macrophages and DCs (29, 30). However, its expression and modulation have not been characterized on human DCs. We found that CD91 was expressed at high levels on human DCs. Upon receipt of a maturation stimulus, CD91 expression was down-regulated (Fig. 3C). These findings show that human DCs, like their murine counterparts, express at least one of the known receptors for hsp binding.

**Lysates of primary tumor tissues induce DC maturation**

Based on our findings above, we hypothesized that tumor cell lysates, but not lysates from normal tissues, would induce DC maturation. Biopsy samples were obtained from 22 specimens derived from seven different sources of tissues (Table I). Autologous healthy tissue was also available in most cases. Each cancerous and healthy tissue was minced and subjected to repetitive freezing and thawing. Lysates were analyzed for hsp70 and gp96 levels either on a weight basis or by total protein content and/or tested for their ability to induce DC maturation.

Fig. 4 shows the hsp content and maturation capacity of four human tumor and normal tissue specimens of different origins. When evaluated on a weight basis, melanoma tissue released substantially higher levels of both gp96 and hsp70 than its healthy tissue counterpart. Only lysates from the former induced the maturation of DCs comparably to LPS (Fig. 4A). Similar observations were made when we compared hsp content and maturation capacity based on total protein released following repetitive freezing and thawing cycles (Fig. 4B). Boiling of lysates from necrotic tissues led to the depletion of immunoreactive hsp70, concomitantly with abrogation of a maturation effect (Fig. 4C). These results are consistent with our hypothesis that hsps within transformed cells are delivering a maturation signal to DCs.

Overall, 91 and 78% of tumors had 2- to 1000-fold higher amounts of hsp70 and gp96 than normal counterparts, respectively.
transformed cell lines released factors that induced the maturation of DCs. In previous studies we showed that necrotic but not apoptotic tumor tissue lysates induced DC maturation, as monitored by the expression of the DC-restricted marker DC-LAMP. We considered an effect positive if $>35\%$ of the DCs expressed DC-LAMP above background staining with a control isotype Ab. Only 3 of 15 normal tissue lysates induced maturation. Of the two for which hsp analysis was performed, one had similar hsp70 levels to those in the tumor tissue, whereas the other had 3-fold higher levels of hsp70, but undetectable levels of gp96. In no tissues analyzed was maturation ever seen when hsp levels were undetectable. These data show a correlation between the DC maturation capacity of transformed tissue lysates and their levels of hsps.

### Discussion

In previous studies we showed that necrotic but not apoptotic transformed cell lines released factors that induced the maturation of human DCs (12). In this study we expand these findings to a more physiological situation, and demonstrate for the first time that primary human tumors, but not primary tissues, also contain factors that promote DC maturation. Hsps are the most likely candidates for this effect for several reasons. Hsp70 and gp96 levels were significantly higher in supernatants of frozen and thawed tumor tissues and transformed cell lines than necrotic healthy tissues and primary cells. Their maturation effect was directly correlated with their relative hsp content. Maturation of DCs was abrogated by boiling and proteinase K treatment of necrotic cell/tissue lysates, which simultaneously destroyed hsp detection by immunoblotting. Furthermore, in the presence of geldanamycin, an inhibitor of the hsp90 family proteins, maturation was also blocked. Finally, highly purified, endotoxin-free hsp preparations directly induced the maturation of DCs (Fig. 3A, and Refs. 14, 16, and 17). It would have been ideal to show that depletion of hsps in our tumor cell lysates abrogated the maturation effect on DCs. Attempts to deplete individual hsps and, consequently, maturation activity were not successful, probably due to residual hsp family members. Also, depletion heightens the chance of endotoxin contamination, further complicating the interpretation of these results.

We excluded other candidates that might contribute toward DC maturation such as mycoplasma, endotoxin, nucleotides, and DNA. All cell lines were mycoplasma-free by PCR. Endotoxin contamination was ruled out by LAL assay of supernatants of necrotic cells or tissues. Nucleotides and DNA would be expected to be resistant to proteinase treatment and, therefore, are probably not involved. In addition, mammalian DNA lacks the unmethylated CpG sequences of prokaryotic DNA, which are responsible for inducing maturation of DCs. Recently, Fadok et al. (31) showed that proteases liberated during cell lysis induced human macrophages to secrete pro-inflammatory cytokines. However, these effects were restricted to neutrophils, not transformed cell lines. Because neutrophils contain high levels of serine proteases and elastases, these factors are unlikely to be involved in the DC maturation seen in our studies. Therefore, we conclude that the biologic effect of factors within tumor cell lysates and supernatants on DCs is primarily due to hsps, although the possible contribution of other factors is certainly acknowledged.

We consider the failure of apoptotic tumor cells to mature DCs to be due to the retention of hsps within cell bodies. The upstream pathway by which hsps mature human DCs is currently under investigation. At least one receptor for hsps, CD91, has been characterized. Here we show for the first time that this receptor is present on immature human DCs and is down-regulated upon maturation. This finding is consistent with those of Singh-Jasuja et al. (17) and Kuppner et al. (32), who showed reduced binding of hsps to mature murine and human DCs. Interestingly, Basu et al. (30) did not see a down-regulation of gp96 binding to its receptor on mature murine DCs. These contradicting results may be due to differences between the systems and cell sources used. Whether the maturation effect of hsps and tumor cell factors proceeds through CD91 is currently under investigation.

The concentration of hsps is critical for their capacity to mature DCs. Up to 200 pg/ml purified hsps was required to induce human DC maturation. Supernatants of tumor cell lysates contained up to 10 pg of hsp70 per 10^6 cells, or 5–10 pg/ml in culture medium. But these levels are an underestimate because we do not take into account other hsp families or cell membrane-associated hsps in lysates. Although we concentrated on two of the most abundant members of the hsp family in our studies, we hypothesize that all family members play a role in this effect, especially considering the fact that many of these bind the same receptor: CD91 (30). How then might one explain the fact that a complete inhibition of maturation is observed with geldanamycin? Geldanamycin blocks the hsp90 family of hsps. If we assume that a critical total hsp concentration has to be reached for maturation to ensue, geldanamycin could decrease total active hsps to below this hypothetical critical level.

DCs were the only primary cells tested containing similarly high levels of hsps and which upon lysis released maturation-inducing factors. This may be due to the cytokine treatment and culture period required to generate DCs from monocytes or, less likely, a property inherent to DCs. The inability of lysates from other primary cells to induce DC maturation can be explained by their relatively low levels of hsps (<1–10 ng/10^6 monocytes). However, our data should not be interpreted to mean that only necrotic transformed cells or DCs release quantities of hsps that lead to maturation. In a tissue environment the differences between cells may become less apparent given that there is minimal “liquid.” As tissue necrosis occurs, and hsps are released, the concentrations released by primary cells in vivo may approach very high levels (14). It has been estimated that 1 mg of tissue (10^5–10^6 cells) will release ~2 μg of total hsps in a volume of 1–2 μl or 1–2 mg/ml, a concentration that should lead to DC maturation.

Differential expression of hsps (especially hsp70 and hsp27) was previously described in malignant vs normal human tissue, e.g., cervical cancer, breast cancer, pancreatic carcinoma, and leukemias (33, 35). This pattern of hsp expression was correlated with...
malignant transformation in experimental models (34, 36). Transformation of cells with c-myc (37, 38), c-mycb, H-ues, H-ues/p53, T Ags of SV40 and polyoma virus (39), and adenovirus E1A (40–42) up-regulates hsp70. Complexes of c-myc and the CCAAT-binding factor/NF-Y protein have been shown to regulate the expression of hsp70 through binding to transcriptional enhancer regions (43). Therefore, hsp5 may play a regulatory role in malignant cells either as a chaperone for cellular and oncogene products or in survival and anti-apoptotic mechanisms (reviewed in Refs. 44 and 45). This might explain our novel finding that tumor cell lines express mostly constitutive rather than inducible hsp70.

The expression of hsps in tumors can be manipulated to induce immunogenicity to the tumor. Indeed, in murine tumor models, using suicide gene transfer, the induction of nonapoptotic but not apoptotic death in vivo correlated with up-regulation of hsp70 and immunogenicity. Gene transfer of hsp70 into B16 and CMT93 melanoma cells further enhanced tumor immunogenicity. The authors (46) concluded that increased levels of hsps together with a nonapoptotic death provide a functional signal to the immune system to break tolerance to tumor Ags (44). Our results show that lysates of human tumor tissues provide such a signal by maturing DCs, presumably through hsps. In animal models, it has been shown that cell lines or their lysates contain endogenous adjuvants that stimulate T cell responses when coinjected with particulate or soluble Ags (13, 47). These factors are constitutively present in the cells and are not being induced, because treatment with a protein synthesis inhibitor emetine did not abrogate the effect observed. The adamant activity within the cytosol was enhanced by induction of stress, e.g., UV irradiation, Fas ligand, possibly due to hsp up-regulation. Our findings, in conjunction with these, support the assumption that immune responses are stimulated by signals released by damaged cells (13).

Tumor cells generally do not activate Ag-specific T cells. Many reasons have been put forward to explain the poor immunogenicity of most tumors, e.g., production of inhibitory factors that suppress an effective immune response, lack of costimulatory molecules, loss of MHC class I molecules, and Ag escape. Instead, Ags associated with these cells must be acquired and presented by professional APCs, an effect probably mediated via hsps (Refs. 48 and 49). The CCAAT-binding factor/NF-Y protein have been shown to regulate the expression of hsp70, and these observations suggest that interventions that induce tumor cell death in situ would simultaneously release Ags to and induce the maturation of tumor resident DCs, perhaps through hsp-mediated effects. Our study suggests another possibility for the up-regulation. Our results show that immune responses are stimulated by signals released by damaged cells (13).

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