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Cell Surface Targeting of Heat Shock Protein gp96 Induces Dendritic Cell Maturation and Antitumor Immunity

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gp96 is a residential heat shock protein of the endoplasmic reticulum that has been implicated in the activation of dendritic cells (DCs) for the initiation of adaptive immunity. By genetic targeting of gp96 onto the cell surface, we demonstrate that direct access of gp96 to DCs induces their maturation, resulting in secretion of proinflammatory cytokines IL-1β, IL-12, and chemokine monocyte chemotactant protein-1 and up-regulation of the expression of MHC class I, MHC class II, CD80, CD86, and CD40. Furthermore, surface expression of gp96 on tumor cells renders them responsive via a T lymphocyte-dependent mechanism. This work reinforces the notion that gp96 is an endogenous DC activator and unveils that the context in which Ag is delivered to the immune system, in this case surface expression of gp96, has profound influence on immunity. It also establishes a principle of bridging innate and adaptive immunity for cancer immunotherapy by surface targeting of an intracellular heat shock protein. The Journal of Immunology, 2001, 167: 6731–6735.

The context in which Ags are delivered to the immune system plays an important role in the initiation of the adaptive immune response (1, 2). It is now clear that antigenic stimulation alone (signal 1) is insufficient for the induction of productive immunity as characterized by priming and expansion of naive T or B lymphocytes (3). Such a process is also critically dependent on the engagement of lymphocytes with a family of costimulatory molecules (signal 2) delivered by the activated dendritic cells (DCs). Therefore, the proper context for generating an Ag-specific immune response is when Ag delivery coincides with activation of DCs (4). Two broad classes of DC activators have been unveiled: exogenous molecules typified by bacterial product LPS (5) and endogenous activators, including the ubiquitously present intracellular heat shock proteins (HSPs) or stress proteins (6–13) and type I IFN induced after virus infection (14, 15).

gp96 is a prototypical HSP that can bind to the surface of APCs in a receptor-dependent manner (16–18). One of its receptors has been identified as CD91 (19). The binding of gp96 to its receptor(s) induces DC maturation (11–13) and facilitates the transfer of gp96-associated peptides from the extracellular compartment to the endoplasmic reticulum (ER) (22–24). Because of the above immunological properties of gp96 and the importance of the context of Ag delivery on immunity, we reasoned that surface targeting of gp96 on tumor cells would have profound immunological consequences. In this study, we have indeed conducted such directed surface expression of gp96 by genetic and cellular engineering, and we report that it has a dramatic effect on both innate and adaptive immunity. We show that direct access of gp96 to the immune system induces robust maturation of DCs in vitro and primes tumor-specific T cells in vivo. Our data lay further support for the notion that gp96 is one of the endogenous DC activators. Strikingly, it reveals the importance of the context during which Ag is delivered, in this case cell surface expression of HSP gp96, in the initiation of antitumor immune responses.

Materials and Methods

Mice, cell lines, and Abs

All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained by the Center for Laboratory Animal Care at the University of Connecticut Health Center (Farmington, CT) using standard guidelines. Parental tumor cell lines Meth A and CT-26 were obtained from Dr. P. K. Srivastava (University of Connecticut School of Medicine, Farmington, CT). Abs were from BD PharMingen (San Diego, CA) except where indicated.

Construction of vectors for surface expression of gp96

The KDEL-minus murine gp96 cDNA was obtained by RT-PCR of mRNA isolated from normal BALB/c mouse liver and cloned into an EcoRI and SacII double-digested dDisplay vector (Invitrogen, Carlsbad, CA). The hemagglutinin tag was removed from the original vector. The sequence of the construct was verified.

Transfection and confocal microscopy

Meth A and CT-26 were transfected by electroporation and LipofectAMINE (Life Technologies, Carlsbad, CA), respectively, and selected by G418. gp96 surface-expressing cells were identified by FACS using Abs against gp96 (Charles River Breeding Laboratories, Wilmington, MA) or myc tag (Invitrogen) and were subcloned by limiting dilution. Surface expression was also confirmed by confocal microscopy after fixation and staining with rabbit anti-gp96 Ab, followed by an Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) and containing with FITC-conjugated anti-KDEL mAb. Optical sections were obtained by laser confocal microscopy using an LSM 410 microscope (Zeiss, Oberkochen, Germany).

DC culture, in vitro maturation assay

Syngeneic bone marrow-derived immature DCs were prepared after 6-day culture in the presence of GM-CSF (20 ng/ml; BD PharMingen) following
the published protocol (22). Typically, $5 \times 10^5$ day +6 immature DCs were incubated with LPS (0.1 $\mu$g/ml), or $2 \times 10^4$ live tumor cells with or without cell surface gp96 in the presence or absence of polymyxin B (10 $\mu$g/ml; Sigma-Aldrich, St. Louis, MO) for 20 h in a 12-well plate. In some experiments, tumor cells and DCs were separated by a 0.4-$\mu$m Trans-well filter (Millipore, Bedford, MA). Cytokine production was measured by ELISA kits (monocyte chemotactant protein-1 (MCP-1) from R&D Systems, Minneapolis, MN; IL-1$\beta$ and IL-12 from Endogen, Woburn, MA).

**Tumor rejection assay**

The immunogenicity of tumor cells was tested by inoculating them intradermally into the naive syngeneic mice and athymic nude mice. Tumor growth was monitored twice a week and recorded using vernier calipers, measuring both the longitudinal and the transverse diameters. Average diameters (mm) of the two axes were plotted. For the cross-protection experiment, $5 \times 10^5$ Meth A-96tm or Meth A-neo was injected into the left flank, and $1 \times 10^5$ Meth A-WT was injected in the right flank. In some experiments, mice were injected with a mixture of Meth A-96tm ($5 \times 10^5$) and Meth A-WT ($1 \times 10^5$). Tumor growth kinetics was compared with Meth A-WT alone. There were five mice in each group.

**ELISPOT**

BALB/c mice were immunized intradermally with $5 \times 10^5$ live tumor cells with or without surface gp96 expression. Seven days after the immunization, the total splenocytes (2×10$^5$ cells/well) were obtained and incubated with irradiated (100 Gy) Meth A-WT ($5 \times 10^5$ cells/well) in 96-well IP- multiscreen plates (Millipore) precoated with 100 $\mu$L of mouse anti-mouse IFN-γ Ab (10 $\mu$g/ml, clone R4–6A2; BD PharMingen). This was followed by sequential washing, incubation with biotinylated anti-IFN-γ (XMG1.2) mAb, and an HRP-based secondary Ab (Vector Laboratories, Burlingame, CA). The spots were developed with 3-amino-9-ethylcarbazole and $H_2$O$_2$ (Sigma-Aldrich), counted with a Zeiss ELISPOT reader, and reported as the number of IFN-γ spots per 1×10$^6$ cells.

**Results and Discussion**

**Surface expression of gp96**

Gp96 resides normally in the lumen of ER due to the presence of an ER retention signal, KDEL, at its carboxyl terminus. In stress and other conditions, both secretion (23) and surface expression of gp96 have been reported (24, 25). To study the direct impact of extracellular gp96 on both innate and adaptive immunity, we targeted gp96 onto the cell surface of Meth A fibrosarcoma and CT-26 colon carcinoma. If gp96 indeed possesses intrinsic immunological properties, we reasoned that cell surface expression of gp96 would have measurable immunological consequences. The construction of the expression vector for cell surface gp96 (96tm) was achieved by deletion of the KDEL sequence followed sequentially by washing, incubation with biotinylated anti-IFN-γ (XMG1.2) mAb, and an HRP-based secondary Ab (Vector Laboratories, Burlingame, CA). The spots were developed with 3-amino-9-ethylcarbazole and $H_2$O$_2$ (Sigma-Aldrich), counted with a Zeiss ELISPOT reader, and reported as the number of IFN-γ spots per 1×10$^6$ cells.

**FIGURE 1.** Expression of gp96 on the cell surface. A. Schematic representation of wild-type gp96 and 96tm protein domain organization. SP, Signal peptide. B. Confocal fluorescence microscopy after staining with anti-gp96 Ab and secondary Ab conjugated with Alexa Fluor 594, followed by costaining with FITC-conjugated mAb against MHC class I (k$\beta$). C. FACS analysis of surface expression of gp96 after staining with an irrelevant Ab (green) and Abs against myc tag (red) and gp96 (black).

**Gp96 surface expression leads to activation of DCs in vitro**

We next examined whether the presence of surface gp96 allows tumor cells to mature DCs. Murine bone marrow-derived immature DCs were cocultured for 20 h with tumor cells with or without cell surface expression of gp96. The 96tm-expressing tumor cells, but not control transfectants, activated DCs efficiently as evidenced by up-regulation of costimulatory molecules CD40, CD80, and CD86 as well as MHC class I and MHC class II (Fig. 2A). Furthermore, we found that the activation of DCs by 96tm, but not neo transfectants, occurred even when cells had undergone radiation-induced apoptosis (data not shown), indicating that the effect was not dependent on active proliferation of 96tm-expressing cells. All culture media were endotoxin tested to be free of LPS. As expected, DC maturation was not blocked by addition of polymyxin B, which inhibited the effect of LPS completely (Fig. 2B). To rule out possible effect from other diffusible agents such as mycoplasma, cell debris, or DNA fragments, DCs and tumor cells were separated by a 0.4-$\mu$m Trans-well filter during coculturing. The maturation effect was abrogated (Fig. 2B), indicating a requirement for direct contact between DCs and 96tm-expressing tumor cells. In addition, matured DCs secreted proinflammatory cytokines IL-12, IL-1$\beta$, and chemokine MCP-1 after coculturing with 96tm (Fig. 2C). The profile of cytokine induction by LPS and 96tm is clearly different.

**DC maturation as a result of surface expression of gp96**

Gp96 was confirmed in both Meth A and CT-26 using multiple transfectants. Furthermore, using a nonreplicating adenovirus vector, we found that direct expression of 96tm on DCs, but not a control protein β-galactosidase, led to DC activation (data not shown). Our data do not exclude the possibility that other molecules, as a result of gp96 surface expression, are involved in the activation of DCs. Formal proof awaits detailed structure-function analysis of gp96 and the availability of conformation-specific Ab or compound against the receptor-binding site of gp96. Nevertheless, our result, in conjunction with the findings that soluble gp96 activates DCs in vitro (11, 13), matures and promotes DC trafficking to the draining lymph nodes in vivo (12), argues strongly for the idea that gp96
itself is a strong DC activator. Moreover, we showed that the induction of DC maturation occurred regardless of whether gp96 surface-expressing cells were live or apoptotic. Thus, DC activation reported by others as being induced by necrotic but not apoptotic cell lysate (15, 26) is perhaps not so much due to the mode of cell death, but instead is likely dependent upon whether HSPs are accessible to DCs. This explanation is consistent with the finding that HSPs are released when cells undergo necrosis, but not apoptosis (11).

Surface targeting of gp96 increases immunogenicity of tumor cells

We next studied the immunogenicity of gp96 surface-expressing tumor cells. We hypothesized that direct access of gp96 to the immune system via surface expression on tumor cells facilitates the interaction of tumor cells with DCs, leading to enhanced priming of tumor-specific T cells. Meth A-WT grows progressively in BALB/c mice. Intradermal injection of $1 \times 10^5$ live Meth A-WT or Meth A-neo led to progressive tumor growth in all the mice. By contrast, injection of up to $5 \times 10^6$ live gp96 Meth A-96tm induced efficient T cell-mediated tumor rejection (Fig. 3, A and B). By depletion of various cellular components, we found that tumor-specific protection is dependent on CD8$^+$ cells (data not shown). Furthermore, a single immunization with Meth A-96tm, but not with Meth A-WT or Meth A-neo significantly induced the expansion of Meth A-specific IFN-γ-producing T cells without any further in vitro stimulation (Fig. 3E). We also demonstrated that the immunity elicited by Meth A-96tm is cross-protective against the parental Meth A. The injection of Meth A-96tm mixed with Meth A-WT led to rejection of both, in contrast to progressive growth kinetics of Meth A-WT alone (Fig. 3C). Inoculation of Meth A-96tm to the left flank and Meth A-WT to the right flank of the animals resulted in regression of not only Meth A-96tm, but also the contralateral Meth A-WT (Fig. 3D). The successful rejection of parental tumor cells both proximally and distally as a result of immunization with gp96 surface-expressing tumors demonstrated that the 96tm gene targeting approach has therapeutic potential.

Thus, we have demonstrated that forced surface expression of gp96 on tumor cells stimulates DC maturation in vitro and induces efficient T cell priming and tumor rejection in vivo. Because gp96 can be displayed on the cell surface during stress and under certain physiological conditions (23–25, 27), it is perhaps reasonable to speculate about the immunological significance of surface gp96. We would like to suggest that the extracellular targeting (secretion and cell surface expression) of intracellular HSPs such as gp96 have physiological bearings (Fig. 4). Indeed, it was found that tumor cells secreting a gp96-1g fusion protein had increased immunogenicity (28). We are actively studying the basis for the dynamics and plasticity of subcellular localization of gp96 and the influences of “ectopic” trafficking of gp96 on immunity in both physiological and pathophysiological conditions.
Our finding is the first report of the use of HSPs targeted onto the cell surface for potential cancer immunotherapy. Our data, together with those of others (28, 29), should stimulate a new wave of experiments in designing or discovering pharmacological agents that promote HSP export for cancer therapy in the future.

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References


FIGURE 3. Surface expression of gp96 on tumor cells leads to tumor rejection and enhanced T cell priming. A. Rejection of Meth A-96tm (●) but not Meth A-neo (○) by wild-type BALB/c mice. B. Progressive growth of both Meth A-96tm (●) and Meth A-neo (○) in nu/nu BALB/c mice. C. Injection of the mixture of Meth A-96tm with Meth A-WT (●) led to rejection of both, compared with progressive tumor growth of Meth A-WT mixed with Meth A-neo (○). D. Simultaneous injection of Meth A-96tm (●) but not Meth A-neo (○) to the left flank of the mice resulted in rejection of Meth A-WT injected at the right flank of the same mice. E. Day 7 splenocytes from mice immunized with PBS, Meth A-WT, Meth A-neo, and Meth A-96tm were assayed directly ex vivo for the frequency of IFN-γ-producing cells by ELISPOT in response to media, Meth A, or CT-26 without further in vitro stimulations. Three independent experiments were performed with similar results.

FIGURE 4. A model for the roles of cell surface gp96 in immunity. Surface expression of gp96 leads to maturation of DCs, as supported by this work (shaded). Soluble gp96 can also be released after pathological cell death. gp96 in this fashion serves essentially as an endogenous adjuvant.


