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***Xenopus*, a unique comparative model to explore the role of certain heat shock proteins and non-classical MHC class Ib gene products in immune surveillance**

Jacques Robert, Ana Goyos, and Hristina Nedelkovska

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

Jacques Robert: jacques_robert@urmc.rochester.edu

Abstract

The heat shock proteins (HSPs) gp96 and hsp70 can elicit potent anti-tumor responses and as such have significant clinical potential. Besides cytotoxic CD8 T cell (CTLs) effectors, evidence suggests that natural killer (NK) cells and other less well-characterized cell types also play a critical role in HSP-mediated anti-tumor responses. Owing to their high degree of phylogenetic conservation, we have proposed that HSPs are ancestral agents of immune surveillance; and postulated that their immunological properties, if important, should have been conserved during evolution. We are investigating this issue using a unique non-mammalian comparative tumor-immunity model in the frog *Xenopus*, which allows us to focus on the relationship between HSPs, classical MHC class Ia, and non-classical MHC class Ib molecules. In addition to a transplantable lymphoid tumor in genetically defined cloned *Xenopus*, we are generating transgenic frogs with inducible or knocked-down (RNAi) gene expression.

Keywords

Comparative immunology/evolution; Tumor immunity; NK cells; CTLs; Antigen presentation

Introduction

Heat shock proteins are among the most abundant intracellular proteins known. They are evolutionarily ancient and highly conserved molecular chaperones constituting several multigenic superfamilies [reviewed in 1]. HSPs, which are produced by all cell types from prokaryotes to eukaryotes, perform essential biological functions under normal as well as stressful physiological conditions. These functions include: preventing protein aggregates under physical stress, serving as molecular chaperones in protein transport between cell organelles, and contributing to the folding of nascent and altered proteins [1]. Increasing evidence suggests that some of these HSPs (e.g., hsp70, gp96) are key players in immune responses [reviewed in 2, 3].

The unique ability of HSPs to evoke potent anti-tumor immune responses relies on two main properties: (1) their peptide-dependent capacity to chaperone and elicit adaptive cytotoxic T lymphocytes (CTL) responses against antigenic peptides through receptor-mediated endocytosis by antigen presenting cells (APC) and re-presentation of chaperoned peptides by APC's classical MHC class Ia (class Ia) molecules; and (2) their peptide-independent capacity to stimulate APCs to produce pro-inflammatory cytokines and chemokines, and to up-regulate costimulatory molecules. In the case of HSP-mediated anti-tumor responses, class Ia-restricted CTLs are thought to be the main effector cells and as such, have been the main focus of most studies. However, increasing evidence suggests that natural killer (NK) cells [4, 5] and other less well-characterized cell types may also play a critical role [6–8]. Defects in class Ia expression occur frequently in malignancy; this is usually considered indicative of a poor prognosis because of the critical role played by class Ia molecules in the interaction between tumor cells and the host immune system. In this regard, increasing evidence suggests that certain non-classical MHC class Ib (class Ib) molecules, play an important role in immune recognition of class Ia low or negative tumor cells through their interaction with non-T-cell inhibitory or triggering receptors expressed by NK and T cells [reviewed in 9]. Therefore, the ability of HSPs to generate immune responses that are not class Ia-restricted but possibly involve class Ib molecules clearly merits further exploration.

To investigate the possible involvement of class Ib molecules in HSP-mediated immune responses, we are taking an evolutionarily approach using a unique non-mammalian comparative tumor-immunity model that we have developed in the frog *Xenopus*. The immune system of *X. laevis* is fundamentally similar to that of mammals and is one of the best defined outside of mammals [reviewed in 10]. It is characterized by T and B lymphocytes with rearranging B- and T-cell receptors, respectively (BCR; TCR) [11, 12]; cytokines [13]; and by class Ia and class II genes [14, 15]. MHC-restricted cytotoxic and helper T cell responses have been identified in adults [16–18]. Importantly, the comparative *Xenopus* model includes several different MHC-defined clones produced gynogenetically from diploid eggs of *X. laevis* × *X. gilli* interspecies hybrids [19]. Two of these clones (LG-6 and LG-15) display identical heterozygous (*a/c*) MHC haplotype, but differ from each other by multiple minor histocompatibility (H) loci [16, 20]. Skin grafts transplanted between adults of these clones are rejected significantly slower (30–50 days at ~21°C) than are MHC-disparate allografts (20 ± 2 days at ~21°C) [18], and this rejection is thymus-dependent [21]. In addition, a thymic lymphoid tumor line originally derived from a spontaneous thymic tumor in a LG-15 frog is perfectly transplantable in both LG-15 and LG-6 clones [22, 23]. The 15/0 tumor does not express class Ia and class II mRNA or protein, and is highly tumorigenic (e.g., as little as 100 cells injected in a LG-15 adult grow into a solid tumor within 1 month). This system allows us to study immunity to a tumor that has down-regulated class Ia, in a syngeneic as well as in an allogeneic host.

Our approach is to investigate the potential immunological role of HSPs from an evolutionary perspective using the frog *Xenopus*. We postulate that given the high degree of phylogenetic conservation of the structure of gp96 an hsp70, their role in immune responses, if fundamental, should have been conserved during evolution. Our *Xenopus* model may

contribute significantly in understanding the potential of HSPs to stimulate effector cells other than conventional CTLs and assessing their biological relevance.

Conserved ability of HSPs to elicit adaptive CD8 T cell responses

Immune response against minor H-Ag (gp96 and hsp70)

To study the phylogenetic conservation of HSP T-cell adjuvant properties in our *Xenopus* model, we assessed the ability of HSPs to generate a MHC-restricted CD8 T-cell mediated response against chaperoned exogenous antigenic peptides encoded for by *Xenopus* minor H genes. Immunization of LG-6 frogs with either gp96 or hsp70 purified from LG-15 tissues induced an accelerated rejection of LG-15 skin graft typical of a secondary response [18]. We have shown that this response is specific to the antigen chaperoned by HSPs (e.g., gp96 purified from an irrelevant donor and hsp70 stripped of its peptides by ATP treatment does not accelerate skin graft rejection). This same immunization protocol generates CTLs that, in vitro, specifically kill minor H-Ag-disparate PHA-blasts but not cognate or MHC-disparate targets. Moreover, anti-minor H-Ag effector function (accelerated rejection of minor H-Ag-incompatible grafts) is transferred with the adoptive transfer of splenocytes from a frog that had been immunized with gp96-minor H-Ag complexes [24]. The use of CFSE staining to follow transferred cells has further revealed that splenic effectors primed with gp96-minor H-Ags accumulate in the spleen of recipients when the same minor H-Ags are presented in the context of a skin allograft. Taken together these data strongly support the idea that HSPs play a fundamental role in immunity.

Interaction of HSPs with CD91 and minor H-Ag cross-presentation

In mammals, the re-presentation or cross-presentation of antigenic peptides chaperoned by HSPs in the context of class Ia by APCs involves receptor-mediated endocytosis by CD91, the receptor for $\alpha 2$ -macroglobulin ($\alpha 2$ -M; [25, 26]). We have characterized the *Xenopus* homolog of CD91, which displays a high sequence identity ($>65\%$) with other CD91 homologs and contains the additional distinctive cytoplasmic NPXY motif [27]. We have shown that CD91 is expressed at the surface of *Xenopus* peritoneal leukocytes (PLs) and can bind gp96 [28]. On average, approximately 15–30% of PLs exhibit surface binding of gp96, or of the CD91 natural ligand $\alpha 2$ -M, or are stained by an anti-CD91 Ab. In addition, gp96 surface binding is inhibited by molar excess of unlabeled gp96 or the CD91 ligand $\alpha 2$ -M, by anti-CD91 antibody and by the specific CD91 antagonist receptor-associated protein (RAP). Surface binding followed by internalization of gp96 was confirmed by fluorescent microscopy.

To functionally characterize the receptor-mediated cross-presentation of antigens chaperoned by gp96, we took advantage of the minor H-Ag-mismatched system between LG-6 and LG-15 clones. Adoptive transfer of PLs pulsed with as little as 800 ng of gp96 chaperoning minor H-Ags, but not minor H-Ag-free gp96, induced potent CD8 T-cell infiltration and antigen-specific accelerated rejection of minor H-locus disparate skin grafts [28]. Inhibition of gp96-CD91 interaction by pre-treatment with anti-CD91 antibody and RAP impairs both CD8 T-cell infiltration and acute skin graft rejection. These data provide

evidence of the conserved ability of gp96 to facilitate cross-presentation of chaperoned antigens by interacting with CD91.

To extend our study to hsp70, we decided to produce and purify larger quantities of recombinant hsp70. Since hsp70 mediated responses, both anti-minor H-Ag and antitumor, are peptide dependent [6, 18] we had to ensure that our recombinant hsp70 is bound to endogenous antigenic peptides. We were able to stably transfect the 15/0 tumor cell line with a construct that contained a His tagged *Xenopus* hsp72 (rec-hsp72, the inducible hsp70) under the transcriptional control of the *Xenopus* EF-1 α promoter and produce rec-hsp72 protein using NiNTA agarose. Using this strategy we produced a protein that carries both anti-minor H and anti-tumor antigenic peptides since the 15/0 tumor cell line originated from a LG-15 clone. A functional study of the rec-hsp72 showed that this protein is as efficient as gp96 purified from tumor tissue in acceleration of skin graft rejection (Table 1). Briefly, PLs pulsed with 1 μ g of either rec-hsp72, gp96 or APBS were adoptively transferred into LG-6 recipients, which were then grafted with LG-15 skin. The accelerated graft rejection of individuals primed with the rec-hsp72 suggests that this protein carries anti-minor H-Ag and is able to elicit a potent peptide-dependent CTL response against the minor H-Ag disparate LG-15 skin graft. The successful purification of functional rec-hsp70 proteins is an important tool because in addition to obtaining large quantities of protein it will also allow us to purify different members of the hsp70 family, such as the inducible hsp72 and the constitutively expressed hsp73, separately. Making this distinction will allow us to see whether different hsp70 members can recruit different effector cells (CD8 versus CD4 T cells) and which molecules they interact with to facilitate antigen cross-presentation.

Conserved ability of HSPs to elicit anti-tumor responses

Our original study demonstrated that both gp96 and hsp70 are able to generate potent immune responses against the 15/0 thymic tumor in *Xenopus* [6]. Interestingly, the absence of MHC class Ia expression by 15/0 tumor raised the question about the type of effectors mediating this response. In vivo and in vitro studies suggest that in *Xenopus*, anti-15/0 tumor responses elicited by HSPs involve NK- as well as classical MHC class Ia-unrestricted CD8 T cell effectors [29, 30].

Hsp-mediated NK cell anti-tumor responses

Convergent evidence supports the ability of gp96 in *Xenopus* to stimulate anti-tumor NK cell effectors. The stimulation of a sustained NK cell response by gp96 is revealed by the partial inhibition of killing of 15/0 targets by pre-incubating the effectors with the anti-NK mAb 1F8 [30]. Preliminary results suggest that the gp96-mediated anti-tumor response is abrogated in vivo (no delay in tumor appearance) by anti-NK mAb treatment [29]. More definitive evidence comes from the fact that gp96 purified either from tumor or normal tissue generates strong CD8-negative cytotoxic effectors (i.e., CD8 T cells are depleted by a *Xenopus*-specific anti-CD8 mAb) that kill class Ia-negative tumor cells (15/0 and B3B7) but not class Ia⁺ allotargets [30]. Interestingly, in vitro re-stimulation with tumor cells is required to obtain a potent NK cell killing. This suggests that further differentiation and/or expansion of these NK cell effectors is required.

Hsp-mediated MHC class Ia-unrestricted anti-tumor CD8 T cells

Immunization of *Xenopus* with gp96 purified from 15/0 tumor generates potent anti-tumor immunity in the absence of class Ia restriction. Interestingly, investigation into the antitumor effector cells generated by this immunization scheme demonstrates that, in addition to the NK cell effectors mentioned above, there is a population of splenic CD8 T cells which can specifically kill this class Ia-negative tumor [30]. We therefore termed these effector cells classical class Ia-unrestricted CTL (CCU-CTL). CCU-CTLs are obtained from a purified CD8⁺ subpopulation of splenocytes (>95% purity) only after immunization and in vitro stimulation with irradiated 15/0 tumor or tumor-derived gp96. This CD8⁺ subpopulation co-express CD5 (a pan-T cell marker in *Xenopus*) but does not express the 1F8 NK-associated cell marker. Furthermore, unlike typical NK cell killing, the cytotoxicity of anti-15/0 CCU-CTLs is directed specifically against class Ia-negative 15/0 tumor targets. In addition, anti-15/0 CCU-CTLs do not kill cognate class Ia⁺ LG-15 splenic lymphoblasts, or the class Ia-negative lymphoid tumor B3B7 (derived from another *Xenopus* MHC-disparate inbred strain). Conversely, CCU-CTLs generated by immunization with gp96 derived from B3B7 tumor cells and in vitro stimulation with irradiated B3B7 cells, specifically kill B3B7 but not 15/0 tumor targets.

We have also provided in vivo evidence, using adoptive cell transfer, of the involvement of CD8 T cells in HSP-mediated anti-tumor responses [24, 29]. Accumulation and cell proliferation occurred when CFSE stained CD8 T cells primed by 15/0 tumor-derived gp96, but not LG-15 liver-derived gp96, were transferred to an isogenetic recipient bearing the class Ia-negative 15/0 tumor. Furthermore, the transfer of such primed anti-tumor splenocytes into naïve recipients prior to tumor challenge delayed the appearance of tumors. Attempts to assess the importance of hsp-generated CCU-CTLs in vivo by using Ab inhibition showed that whereas anti-CD8 mAb treatment caused increased growth of transplanted 15/0 tumor, no marked effect was obtained by treatment with the anti-NK 1F8 mAb. These data strongly suggest that gp96 is able to generate, besides conventional CTLs, *bonafide* Ag-specific CD8 T cell effectors that kill 15/0 tumor cells in the absence of class Ia expression.

Involvement of non-classical MHC class Ib molecules in HSP-mediated anti-tumor responses

Increasing evidence suggests that in mammals certain class Ib molecules play an important role in immune responses against class Ia low or negative tumor cells [9, 31]. Although 15/0 tumor cells do not express class Ia, they do express *Xenopus* non-classical MHC class Ib (XNC) genes [22, 32] as well as β 2-microglobulin (β 2-m; [33]). An estimated 20 XNC genes, grouped by sequence similarity into nine subfamilies, have been characterized in *X. laevis* [34]. To investigate the involvement of XNC gene products in *Xenopus* anti-tumor responses, we generated, for the first time in ectothermic vertebrates, stable tumor transfectants expressing short hairpin RNA (shRNA) to silence either XNCs directly or β 2-m to prevent XNC surface expression [35]. In the absence of anti-*Xenopus* anti-class Ib or β 2-m specific Abs, we verified that the silencing of β 2-m and XNC mRNA does indeed result in reduced cell surface protein expression by transiently transfecting 15/0 parental cells or shRNA transfectants with a *Xenopus* β 2-m expression construct containing a His-

tag. We then monitored the cell surface expression of recombinant $\beta 2$ -m and complexed XNC molecules by immunoprecipitation with an anti-His mAb. In several experiments using moderate washing conditions, we co-precipitated the $\beta 2$ -m (14–15 kD) and a 38–40 kD molecule whose size is in the range of class Ia molecules that vary between 38 and 44 kD. Since 15/0 cells do not express class Ia molecules this co-precipitated band is likely a putative XNC molecule. In contrast to 15/0 parental cells, neither $\beta 2$ -m nor putative XNC molecules were detected at the surface of cells expressing anti- $\beta 2$ -m shRNA. The defect was limited to the $\beta 2$ -m complex since expression of other proteins (e.g., CD8) remained unaffected. The down-regulation of recombinant $\beta 2$ -m was also detected by Western blot. Interestingly, $\beta 2$ -m was also undetectable at the surface of XNC-silenced transfectants, even though intracellular $\beta 2$ -m protein expression was not markedly changed. Although, we cannot directly monitor the decrease of XNC protein due to the lack of specific Abs, these results suggest that as a consequence of the absence or suboptimal expression of XNC proteins, $\beta 2$ -m cannot form any complex and its loading at the cell surface is prevented.

Importantly, both types of 15/0 transfectants are more resistant to CCU-CTL killing while more susceptible to NK-like cell killing in vitro. Tumor transplantation experiments further demonstrate that the 15/0 transfectants are also more tumorigenic in vivo [35]. This study provides in vitro and in vivo evidence of the evolutionary conservation of class Ib gene involvement in Hsp-mediated anti-tumor responses. By RACE PCR, we have found that 15/0 tumor cells express three known XNC subfamilies (XNC 4, 6, and 8), as well as two novel subfamilies that we have named XNC10 and XNC11 (unpublished data). Which of these XNC gene products are interacting with CCU-CTLs and NK cells and which molecules on the effector cells are the putative XNC ligands remains to be determined.

The interaction of class Ib gene products with anti-tumor CD8 T cells in a species evolutionarily distant from mammals such as *Xenopus* provides further support for the critical role of class Ib molecules in adaptive immunity. In addition, our results strongly suggest that association of $\beta 2$ -m with XNC gene products, implied by the nucleotide sequence conservation in the $\alpha 3$ domain of known class Ia and Ib cDNAs [34] and by our immunoprecipitation experiments, is indeed required for optimal expression of surface class Ib molecules.

Transgenesis and in vivo knockdown approach

Recently, we decided to develop a more powerful reverse genetic strategy to further explore the relationship between HSPs and class Ia as well as class Ib molecules in vivo. This approach consists in generating transgenic cloned frogs with modulated expression of class Ia and class Ib. We have established three different techniques for generating transgenic MHC-defined isogenetic clones. These include the recently developed Φ C31 integrase [36], the Sleeping Beauty transposase [37] and the I-Sce meganuclease [38, 39] techniques that efficiently mediate the insertion of plasmid DNA into the *Xenopus* genome at low copy number. We have been largely successful in generating GFP transgenic clones (Fig. 1) with transgenesis efficiency comparable to results reported with outbred (OB) *Xenopus* [40].

Importantly, we also devised a strategy to silence class Ia and Ib genes in vivo using shRNA technology. We designed shRNAs targeting either class Ia or class Ib genes directly or β 2-m and cloned them under the control of the human U6 Pol III promoter into the different vectors for transgenesis. Generation of transgenic clones with down-regulated expression of class Ia and class Ib will allow us to further explore the requirement and significance of these molecules in vivo in HSPs-mediated anti-tumor responses. Currently, we are working on producing stable transgenic founder lines.

Conclusion and perspectives

Our studies in *Xenopus* so far have revealed that the immunological properties of HSPs have been conserved during evolution for more than 350 millions years, the time that separates modern amphibians and mammals from their common ancestors. Additionally, our studies suggest a fundamental and conserved role of class Ib molecules in anti-tumor immunity. As summarized in Fig. 2, class Ib genes may represent good indicators of the arms race between tumors and the host immune system. While a tumor attempts to escape immune surveillance and control by the immune system by down-regulating class Ia genes, it may become vulnerable to attack by NK cells. The expression of class Ib molecule at the tumor cell surface can therefore be viewed as a way to prevent NK cell killing through interaction with inhibitory NK receptors. On the other hand, the activation of CD8 T cell effectors interacting with class Ib molecules (CCU-CTLs) can represent an adaptation of the host immune system against tumor escape from NK cell killing. Finally, the ability of HSPs to stimulate CCU-CTL responses constitutes additional evidence of the intimate and complex role of HSPs in immune surveillance. The comparative tumor-immunity model we have developed in *Xenopus* provides a unique opportunity to further investigate this complex system.

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Fig. 1.

Generation of transgenic *Xenopus* with the “Sleeping Beauty” transposase. Dejellied eggs from outbred (OB) or LG-6 isogenetic cloned animals were co-injected with 10^{-2} ng of transposase mRNA and 15 pg of vector containing a GFP reporter transgene under the control of the elongation factor $\alpha 1$ promoter in 10nL volume. Control eggs (C) were dejellied but not injected. Live larvae were screened for EGFP expression with a fluorescence stereomicroscope at pre-metamorphic stage (st. 56, 1-month old). Arrows: thymus

Fig. 2.

Model of the involvement of HSPs and class Ib molecules in immune surveillance and anti-tumor responses. **A** During the priming phase (adapted from [2]), HSP-peptide complexes released in the extracellular compartment from infected or stressed cells (e.g., apoptosis, cell lysis) are internalized by APCs through receptor-mediated endocytosis (e.g., CD91). (1) Antigenic peptides channeled into the class Ia presentation pathway specifically activate CD8 T cells. (2) HSPs internalized by the same receptors or interacting with other receptors (e.g., Toll-like receptors) stimulate production of inflammatory cytokines and chemokines and up-regulate co-stimulatory molecules. (3) HSPs are proposed to also stimulate class Ib-mediated response by a yet unknown mechanism that is likely to be Ag-specific, and involve a CD8 T cell subset. **B** During the effector phase, tumor targets that have down-regulated surface class Ia molecules to escape recognition and lysis by conventional CTL, can still be killed by NK cells unless they express surface class Ib molecule that can bind inhibitory NK receptors. We propose that HSPs generate CCU-CTL effectors that can recognize and kill tumors expressing these class Ib molecules

Table 1

Cross-presentation by gp96 and rec-hsp72

	Number of frogs	Average time in days ($\pm$ SD) for complete skin graft rejection by LG-6 recipient
LG-6 recipients ^b		
Secondary ^a	3	22.7 $\pm$ 12.5 ^c
APBS + PLs ^b	3	63.3 $\pm$ 16.6
LG-15 gp96 + PLs	3	21.7 $\pm$ 12.5 ^c
LG-6 recipients ^b		
APBS + PLs	3	70.3 $\pm$ 4.1
15/0 gp96 + PLs	3	27.0 $\pm$ 2.1 ^c
15/0 rec-hsp72 + PLs	3	24.3 $\pm$ 1.2 ^c

^aAfter complete rejection of a first LG-15 skin graft, LG-6 frogs were grafted a second time with a LG-15 skin

^bPLs cells (3×10^5) were pulsed with 1 μ g of gp96/minor H- or rec-hsp72/15/0-Ag complexes, washed, and adoptively transferred into recipients 1 week before skin transplantation

^cStudent *t* test: *P* = 0.001 compared to APBS-controls