Virally Induced Lytic Cell Death Elicits the Release of Immunogenic GRP94/gp96*

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Necrotic cell death yields the release of cellular components that can function in the initiation of cellular immune responses. Given the established capacity of the endoplasmic reticulum molecular chaperone GRP94 (gp96) to elicit CD8+ T cell activation, we have investigated the cellular fate and antigenicity of GRP94 in differing scenarios of cell death. Virally induced cell death or mechanical cell death, elicited by freeze/thaw treatment of cell suspensions, yielded GRP94 release into the extracellular space; apoptotic cell death occurring in response to serum deprivation did not elicit GRP94 release. To assess the antigenicity of GRP94 released following virally induced cell death (lethal infection of cells with rVV ES-OVA258–265, a recombinant, ovalbumin epitope-expressing vaccinia virus) or mechanical cell death (freeze/thaw of ovalbumin-expressing cells), tissue culture supernatant fractions were pulsed onto antigen-presenting cells, and antigen re-presentation was assayed as activation of an ovalbumin-specific T cell hybridoma. For both cell death scenarios, released GRP94 elicited a dose-dependent, ovalbumin-specific, hybridoma activation. In contrast, calreticulin derived from rVV ES-OVA258–265-infected cell extracts did not stimulate B3Z activity. These data identify GRP94 as an antigenic component released upon pathological, but not apoptotic, cell death and provide an assay system for the identification of cellular components of related activity.

The endoplasmic reticulum molecular chaperone GRP94 (gp96) can elicit both prophylactic and therapeutic CD8+ cytotoxic T lymphocyte responses against host tissue-derived antigens (1–5). As a chaperone, GRP94 interacts with nascent (poly)peptides undergoing structural maturation in the endoplasmic reticulum (ER),1 the site of peptide loading onto nascent major histocompatibility (MHC) class I molecules (6, 7). This (poly)peptide binding activity, viewed with respect to the subcellular localization of GRP94, provides insight into the derivation of GRP94 immunogenicity; it resides in the recipient compartment for peptides transported by the transporter associated with antigen presentation (TAP) (7). Furthermore, antigen-presenting cells (APCs) can internalize GRP94-peptide complexes from the extracellular space by both receptor-mediated and bulk flow mechanisms (8–10). Recent evidence indicates that GRP94-peptide complexes internalized via the receptor-mediated pathway gain access to the MHC class I antigen presentation pathway, yielding re-presentation of GRP94-associated peptides on APC MHC class I molecules, an example of a process referred to as cross-priming or cross-presentation (8, 10, 11).

Although it is clear that GRP94-peptide complexes can elicit cytotoxic T lymphocyte responses, a finding of substantial therapeutic implications, it is not known whether GRP94 can function in a physiological context to regulate cellular immune responses. The physiological setting of greatest relevance to chaperone-elicited immune responses is that of cell death (broadly speaking, necrosis and apoptosis). Although a precise terminology for unambiguously distinguishing necrosis and apoptosis continues to evolve, the principal physiological difference between the two forms of cell death is that apoptosis is an essential physiological mechanism in growth, differentiation, and tissue remodeling, whereas necrosis is a pathological end point (12, 13). Importantly, necrotic cell death results in inflammation and stimulation of the host immune system (14–16). The contribution of the inflammatory response to the regulation of cellular immune responses is a central element of the “danger hypothesis,” which predicts that the immune system of the host responds to antigens in the context of a physiological danger signal (15). In this view, the initiation of a cellular immune response requires both the antigen and inflammation-induced co-stimulatory factors that stimulate APC cell maturation and antigen presentation. Indeed, recent work indicates that necrotic cells and their supernatants can induce maturation of dendritic cells, as well as provide antigens for re-presentation to CD8+ T cells (16–19). At present, the full identity of the necrotic cell components that elicit APC activation and serve as the source of antigenic component(s) necessary for the elicitation and maintenance of a cellular immune response remains to be determined (19, 20).

In this study, we report that lethal viral infection and mechanical cell death elicited the release of GRP94 into the extracellular medium, whereas apoptotic cell death did not. GRP94 released as a consequence of viral infection was structurally intact, it could be internalized by APCs, and its bound peptides re-presented for T cell activation. These data extend recent findings that necrotic cell extracts stimulate APC maturation and re-presentation and establish GRP94 as a candidate physiological mediator of necrosis-dependent immune responses (16, 18, 19).

MATERIALS AND METHODS

Cells—EL4 and E.G7-OVA are murine thymomas, E.G7-OVA being EL4 cells stably transfected with the gene encoding chicken ovalbumin (21). B3Z is a CD8+ T cell hybridoma that expresses LacZ in response to activation of T cell receptors specific for the SIINFEKL peptide (OVA-immunodominant peptide) in the context of H-2Kb MHC class I.

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1 The abbreviations used are: ER, endoplasmic reticulum; APC, antigen-presenting cell; MHC, major histocompatibility complex; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
molecules (22). P815 is a murine mastocytoma cell line, H-2d haplotype, expressing the P1A tumor antigen. RAW309 Cr.1, a K\(^{\text{K}}\)R murine macrophage cell line, was obtained from ATCC (ATCC TIB-69). All cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum.

**Cell Death-dependent Release of Heat Shock Proteins**—To analyze GRP94 release following mechanical (nonphysiological) cell death, cultures of E.G7-OVA cells were killed by freeze/thaw treatment (16, 18), the addition of digitonin to 0.005%, or the addition of 5 \(\mu\)M ionomycin. For the latter two conditions the cells were incubated as such for 6 h at 37 °C. In experiments addressing mechanical cell death, 1 \(\times\) 10\(^6\) cells were used in the preparation of cell extracts. Following the indicated treatment, cell suspensions were centrifuged (15 min, 50,000 rpm, Beckman TL100.2 rotor), and the resulting supernatant was retained as the soluble fraction. Tissue culture supernatant extracts were subjected to ammonium sulfate fractionation (40% w/v), and the glycoprotein fraction remaining in the 40% ammonium sulfate supernatant was purified by adsorption onto concanavalin A-Sepharose beads (Amer sham Pharmacia Biotech). Bound glycoproteins were eluted from the concanavalin A beads with SDS-PAGE sample buffer (0.5 \(\times\) Tris, 5% SDS) and analyzed by immunoblot for GRP94 content. The anti-GRP94 antibody (DU120) was prepared by contract service with Cocalico Biologicals (Beamstown, PA).

For the induction of pathological cell death, P815 cells were infected with rVV ES-OVA\(_{\text{MHC-I}}\), a recombinant vaccinia virus encoding the modified ovalbumin epitope MIINFEKL in a signal sequence-bearing minigenic construct (23), at a multiplicity of infection of 5 for 1 h in balanced salt solution, 0.1% bovine serum albumin (BSS/BSA). BSS/BSA medium was then exchanged into Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and the cultures were maintained for 48 h at 37 °C. 48 h postinfection, cell cultures were centrifuged as above, and the supernatant fraction was recovered. The ER chaperone content of the cell pellet was released by sequential extraction of the cell pellet with 0.05% digitonin and 10 mM CHAPS detergents. The supernatant fraction was subjected to ultracentrifugation (Beckman TL100 rotor, 60,000 rpm, 20 min) prior to fractionation. For the supernatant fractions, fractionation was performed by gel filtration chromatography on a Sephacryl S-300 column, with elution performed in phosphate-buffered saline. Cell pellet detergent extracts were initially fractionated by MonoQ anion exchange chromatography and subsequently by gel filtration chromatography, as previously described (24).

Apoptotic cell death was elicited by transferring EL4 cell cultures into serum-free Dulbecco’s modified Eagle’s medium. Following transfer, aliquots of the cell suspension were removed at the indicated time points for analysis of GRP94 release in the medium (as above), cell death, as assayed by vital dye (trypan blue) staining, and apoptotic state, as assayed by fluorochrome isothiocyanate-annexin V staining (Phar mingen) (according to the manufacturer’s protocol). For fluorochrome isothiocyanate-annexin V staining, cells were postfixed in 2% paraformaldehyde and assayed by fluorescence-activated cell sorter analysis.

**OVA Re-presentation Assays**—OVA-specific immunogenicity was determined in a re-presentation assay using the K\(^{\text{K}}\)R macrophage cell line RAW309 and the OVA-specific T cell hybridoma B3Z. Typically, fractions derived from the different tissue culture supernatants were added to cultures of 5 \(\times\) 10\(^5\) RAW309 cells and 10\(^5\) B3Z cells, in a total volume of 1 ml of tissue culture medium. These findings confirm recent data identifying the release of GRP94 into the soluble fraction following freeze/thaw-elicited cell death and identify a physiological sce-
GRP94 Release Accompanies Pathological Cell Death

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**Immunogenicity of Soluble GRP94: Mechanical/Chemical Cell Death**—Following identification of GRP94 in the supernatant fraction of killed cell cultures, a series of experiments were performed to assess the immunogenicity of these GRP94 fractions. For experiments with GRP94 derived from cells killed through freeze/thaw treatment, E.G7-OVA cells were used. E.G7-OVA expresses ovalbumin (43 kDa), itself an immunogenic protein in the described re-presentation assay, and so the necrotic cell extracts were first fractionated by centrifugal ultrafiltration to separate ovalbumin and/or ovalbumin fragments from GRP94. This level of activation approximated that observed upon addition of 100 nM ovalbumin. Immunoffinity depletion of GRP94 from the retentate fraction reduced re-presentation to nearly background levels, defined as RAW309 and B3Z cells cultured in the presence of normal culture medium. These data indicate that GRP94 contains nearly the entirety of the high molecular weight OVA-specific immunogenic activity present in a freeze/thaw-derived cell extract.

**Apoptosis Does Not Elicit GRP94 Release**—In contrast to pathological cell death, apoptotic cell death is a tightly regulated process (27, 29). Furthermore, endogenous signals derived from necrotic cells stimulate APC activation and maturation, whereas those from apoptotic cells do not (16, 18, 19). To assess the effects of apoptotic cell death on GRP94 release, EL4 cells were serum-deprived, and over a 21-h time course, aliquots of cell-free tissue culture supernatant were assayed for GRP94 content (Fig. 3A). In paired analyses, aliquots of the cell fraction were assayed for cell death (trypan blue, vital dye

**Fig. 2.** Biochemical fractionation of freeze/thaw-derived cell extracts. A, fractionation of purified GRP94 and ovalbumin by centrifugal ultrafiltration. A solution of purified GRP94 and ovalbumin was subjected to two cycles of centrifugal ultrafiltration on a Microcon100 ultrafiltration device. Filtrate and retentate fractions were concentrated by acid precipitation, resolved by SDS-PAGE, and identified by Coomassie Blue staining. B, ultrafiltration-based size exclusion fractionation of GRP94 in freeze/thaw-derived cell extracts. E.G7-OVA cells were killed by freeze/thaw treatment, centrifuged to remove the insoluble fraction, and fractionated as described above. A Coomassie Blue-stained gel and immunoblot analysis of the fractions is presented. C, linearity of OVA re-presentation assay. β-Galactosidase (LacZ) activity produced by reporter B3Z T cells following stimulation by RAW309 Cr.1 macrophages pulsed with increasing concentrations of OVA peptide (SIINFEKL). D, GRP94 is the primary OVA-bearing immunogenic component of the high molecular weight fraction of freeze/thaw-derived lysates. RAW309 macrophage cultures were pulsed with the ultrafiltration retentate of a freeze/thaw-treated lysate derived from E.G7-OVA cultures, and re-presentation was assayed as B3Z cell activation. 100 nM ovalbumin was used as a positive control; LacZ produced by co-culture of RAW309 and B3Z cells alone was subtracted from all readings as background. The GRP94 component of the retentate was depleted by batch adsorption to an affinity-purified anti-GRP94 IgG matrix. Error bars are S.D. of four independently generated killed cell supernatants.
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Fig. 4. GRP94 and Hsp90 are dominant OVA-specific immunogenic components of cells infected with OVA-expressing recombinant vaccinia virus. 1 × 10⁶ P815 cells were infected with rVV ES-OVAMet258–265 at a multiplicity of infection of 5 for 48 h. The cell pellet was extracted in detergent, and the supernatant was subjected to MonoQ chromatography. GRP94-containing fractions were pooled and separated on a Sephacryl S300 column. Results from the S300 fractionation are shown. A, protein elution profile (solid line) and the paired B3Z activity profile (filled circles) are presented. Aliquots of the indicated column fractions were incubated overnight with 10⁶ B3Z cells and then concentrated and fractionated by gel filtration chromatography on Sephacryl S-300 resin (Fig. 5). Consistent with results obtained in analyses of the cell-derived chaperone fraction, the peak of B3Z activation coincided with the peak GRP94-containing fractions (Fig. 5A), as determined by immunoblot analysis (Fig. 5B).

The fractions obtained by gel filtration chromatography of the concentrated tissue culture supernatant contained a complex, diverse array of proteins, reflecting both serum components and those cellular proteins released in response to pathological cell death (data not shown). To address the relative contribution of GRP94 to the total OVA-specific immunogenic activity of the peak activity fractions, a GRP94 immunodepletion experiment was performed. As depicted in Fig. 5C, immunofluorescence depletion of GRP94 from a peak activity fraction yielded a 60% decrease in OVA-specific activity. Under the described immunofluorescence depletion conditions, GRP94 was efficiently removed from the indicated fraction (Fig. 5C, inset). On the basis of the data presented in Fig. 4, it is likely that the remaining OVA-specific activity is derived from cytosolic Hsp90. In summary, these data identify GRP94 as a prominent, soluble immunogenic component released from cells in response to pathological cell death.

DISCUSSION

As a consequence of pathological cell death, which accompanies lethal infection with vaccinia virus, GRP94 is released into the extracellular space and can function as a cross-presentation antigen in the MHC class I pathway. These observations extend recent findings demonstrating the release of GRP94...
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The mechanism of vaccinia-elicited cell death is, as yet, unexplained. In a manner similar to a number of viruses, including Epstein-Barr, adenovirus, and human papilloma virus, infection with vaccinia results in the expression of virally encoded ER chaperones, including calreticulin, into the extracellular space. It remains to be determined whether in vivo pathological cell death yields the release of GRP94 into the extracellular space. The data included herein identify a potential role for GRP94 in the regulation of immune responses to pathological cell death.

The mechanism of vaccinia-induced cell death is, as yet, undefined. In a manner similar to a number of viruses, including Epstein-Barr, adenovirus, and human papilloma virus, infection with vaccinia results in the expression of virally encoded anti-apoptotic signals that block the progression to apoptotic cell death, indicating that lethal vaccinia infection elicits cell death by a nonapoptotic mechanism (30). In contrast, however, vaccinia infection of immature dendritic cells and immature B-lymphocytes has been reported to elicit apoptotic cell death (31, 32). Etoposide-induced apoptotic cell death results in the calpain-mediated proteolysis of GRP94 (28). That GRP94 was recovered in the tissue culture supernatant fraction in a structurally intact form following vaccinia infection (Fig. 1) supports the conclusion that terminal infection with vaccinia elicits non-apoptotic cell death. Most significantly, and regardless of the precise molecular mechanism by which terminal vaccinia infection elicits cell mortality, the end stage of a lethal vaccinia infection is accompanied by a loss of both plasma and ER membrane integrity and the release of cellular components, including ER chaperones, into the extracellular space.

Maztinger (15), in the danger hypothesis, has proposed that necrotic, but not apoptotic, cell death yields the production of inflammatory signals necessary for the activation of cellular immune responses. In support of this hypothesis, recent studies have demonstrated that lysed cell extracts contain components capable of eliciting dendritic cell activation and maturation (16–19). There exists substantial experimental evidence indicating that heat shock and chaperone proteins can themselves perform such functions. For example, Hsp70 has been demonstrated to elicit cytokine release as well as to direct peptide antigens into the MHC class I antigen presentation pathway (17, 20, 26, 33). Similarly, GRP94 can function as an MHC class I cross-presentation antigen, and recent data convincingly demonstrate that GRP94 induces cytokine expression in both macrophages and dendritic cells (3, 5, 8, 11, 19). Interestingly, the profile of cytokines secreted in response to GRP94 is distinct from those released following addition of bacterial lipopolysaccharide (19). Given that Hsp70 and GRP94 appear to fulfill the requirements predicted of a cell death messenger, it becomes important to identify the physiological scenarios in which pathological cell death contributes to heat shock or chaperone protein release. In addition to lethal viral infection, as illustrated herein, it would be of value to determine whether chaperones and heat shock proteins are released into the extracellular space during chronic inflammation, particularly as accompanies the onset of autoimmune disease (34, 35).

Previously, we and others reported that the ER chaperone, calreticulin, can elicit epitope- and tumor-specific CD8+ T cell responses (3, 36). Of relevance to the current study, calreticulin from an ovalbumin-expressing cell line, E.G7-OVA, was reported to elicit an OVA-specific cytotoxic T lymphocyte response (3). As is evident from Fig. 4, however, the calreticulin fraction obtained from cells infected with a recombinant vaccinia virus expressing an OVA minigene did not display activity in an OVA re-presentation assay. If it is assumed that the uptake and processing of calreticulin from virus-infected cells are identical to that occurring with calreticulin from normal and tumor tissue, it would appear, then, that calreticulin is unable to bind the minimum OVA epitope in a stable manner. At present, the sequence and size determinants for peptide binding to calreticulin are not known, although the ability of calreticulin to function as a lectin is well established (37, 38). Asn-292 of ovalbumin is subject to N-linked glycosylation, and so the possibility exists that calreticulin binds an OVA precursor peptide that extends through the OVA epitope (amino acids 257–264) and includes Asn-292, the site of OVA re-presentation activity. If it is assumed that the cardiac cytoskeleton is well suited to the analysis of the immunogenic and regulatory components produced and/or released by cells undergoing pathological cell death.

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