Determinants of efficacy of immunotherapy with tumor-derived heat shock protein gp96

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Abstract

Immunotherapy with gp96 was highly effective in mice bearing methylcholanthrene-induced fibrosarcomas (Meth A tumors) when treatment began 7 days or less after tumor challenge, but significantly less effective if the treatment began 9 days after challenge. Immunotherapy of pre-existing tumors showed all the hallmarks of specificity of gp96 and dose-restriction observed previously with prophylactic studies. When mice with large primary Meth A tumors were treated with surgery alone, or with surgery followed by therapy with Meth A-derived gp96, the mice that received surgery and immunotherapy did significantly better than those receiving surgery alone. The relationship between the time of initiation of immunotherapy with gp96 and its efficacy was also tested in a metastatic model of the Lewis lung carcinoma. In this model, immunotherapy with gp96 was very effective if treatment began up to 31 days after tumor challenge, but significantly less so if therapy was initiated day 33 post-tumor challenge. These observations suggest that the regulatory phenomena that interfere with immunotherapy gather momentum with surprising speed.

Introduction

Heat shock proteins (HSPs) isolated from cancer cells have been shown be immunogenic specifically against the cancers from which the HSPs were isolated (see 1,2 for reviews). HSPs isolated from normal tissues or from non-autologous tumors are not immunogenic. The specificity of immunogenicity has been shown to derive from the antigenic peptides associated with cancer-derived HSPs but not with normal tissue-derived HSPs (3), as demonstrated in murine (4) and now in human cancers (5). The mechanism of immunogenicity involves interaction of the HSPs with an HSP-receptor CD91, on the antigen presenting cells (APCs), followed by re-presentation of selected chaperoned peptides by the MHC I molecules of the APCs (6,7). The MHC I-peptide complexes then stimulate the cognate CD8+ T lymphocytes. It appears that the HSP-chaperoned peptides are also routed to presentation by the MHC II molecules and the consequent stimulation of cognate CD4+ T cells (8). Most of these aspects have been demonstrated in both the murine and human systems. The principles developed from these studies have also been extended to immunotherapy of viral infections (9,10,11).

Autologous tumor-derived preparations of the HSP gp96 are now being tested in a number of clinical trials (12,13). While tumor-derived HSPs have been shown to be effective in prophylaxis against and in therapy of a number of murine (14,15) and rat tumors (16,17), the optimum parameters have not been defined systematically. These include the optimal dosage, regimen, the stage of tumor growth, primary versus metastatic tumor, and synergy of immunotherapy with surgical excision of the tumor mass, to name a few. As an example, two immunizations have been shown to be effective in immunizing rats (16), mice (14), and frogs (18).
prophylactically against a subsequent cancer challenge, but smaller and larger numbers of immunization have not been tested. Therapy has been carried out with four or more treatments (15,17) but the effects of shorter or prolonged treatments have not been measured. A dose-restriction of immunogenicity of gp96 has been observed in the prophylactic setting (14,19) but its applicability has not been tested in therapy.

An examination of these and other parameters is of crucial significance for translation of this method of immunotherapy from animal experimentation to clinical reality. This study examines some of these parameters using the murine models of the methylcholanthrene-induced BALB/c mouse fibrosarcoma Meth A as a non-metastatic primary tumor, and the spontaneous Lewis lung carcinoma of C57BL/6 mice, as a metastatic tumor.

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**Results**

**Immunotherapy of early and late primary Meth A tumors**

BALB/c mice were inoculated with 100,000 Meth A cells intradermally. The tumors were allowed to grow for 5, 7 or 9 days at which time the tumors were typically 2.0, 5.1 and 8.4 mm in diameter respectively. Mice bearing these tumors were then treated either with saline, Meth A-derived gp96, or as a negative control, BALB/c liver-derived gp96. Gp96 was administered intradermally and 0.5, 1 or 5 µg gp96 per injection were administered. Each mouse received four immunizations given on alternate days. As an example, mice that began treatment on day 5 were immunized on days 5, 7, 9 and 11 (Fig. 1).

![Figure 1. Immunotherapy of early and late primary Meth A tumors with gp96.](http://www.cancerimmunity.org/v1p7/010307.htm)

It was observed that mice that began treatment with Meth A gp96 5 or 7 days after tumor cell implantation responded vigorously to immunotherapy (Figs. 1 and 2). While tumors continued to grow for up to 10 days after
tumor cell implantation in all groups of mice, the tumors of Meth A gp96-treated mice began to regress at about
day 10-12 and the regression continued for the next several weeks. Most mice in these groups were substantially
or completely cured of visible disease. Immunization with gp96 derived from normal liver had no influence on the
kinetics of tumor growth (Figs. 1 and 2) as reported previously in models of prophylactic immunity to Meth A (3).
Further, immunization was effective in mice that received 1 µg per immunization, but not in those that received
less (0.5 µg per immunization) or more (5 µg per immunization) gp96 (Figs. 1 and 2). Thus, the activity of gp96
was dose-restricted as has also been reported previously in models of prophylactic immunity to Meth A (14,19).

Figure 2. Immunotherapy of early, intermediate and late primary Meth A tumors
demonstrating antigen specificity of gp96, timing of treatment and dosage - Summary of the
data in Figure 1. (A) Tumor-bearing mice were treated with tumor-derived gp96, liver-derived gp96
or buffer alone. Tumor diameters were monitored. (B) Tumor-bearing mice received gp96-adjuvant
therapy starting on day 5 (early), day 7 (intermediate) or day 9 (late). Tumor diameters were
monitored. (C) Tumor-bearing mice were treated with varying doses of Meth A-derived gp96.
Amounts indicated in the legend represent the dose given per injection 4 times on alternate days.
Tumor diameters were monitored. Each line is an average of the data from 5 animals.

In contrast to mice that were treated beginning 5-7 days after tumor cell implantation and that responded well to
immunotherapy, mice that began treatment 9 days after tumor cell implantation responded significantly but
relatively poorly to immunotherapy with Meth A-derived gp96. Tumors of these mice began to undergo shrinkage
with kinetics initially similar to that of mice which began treatment early; however, by day 12-15, the tumors of
late-treated mice began to grow again but remained significantly smaller than the tumors of saline-treated mice
(Figs. 1 and 2).

Influence of immunotherapy as an adjunct to partial surgical excision of established Meth A tumors

As mice bearing 9 day old Meth A tumors were relatively refractory to immunotherapy with gp96, further
experimental treatments were attempted with them. They were treated by surgery alone or by surgery followed
by immunization with 5 injections of Meth A-derived gp96 on alternate days (see Fig. 3 for experimental design).
Immunization was carried out with the 1 µg per injection dose determined to be optimal in the experiments shown
in Figure 2, as well as in previous studies (19). It was observed that while tumors resumed growth in mice which
had undergone surgery alone (Fig. 4 B), tumors of mice that had received immunotherapy with gp96 as an
adjunct to surgery remained relatively stable for nearly 20 days after surgery (Fig. 4 C, D). At that point, tumors in
some of the gp96-treated mice resumed growth. However, there remained a significant difference in the size of
tumors in mice that were treated with surgery alone versus those that received immunotherapy as an adjunct to surgery.

**Figure 3. Study design for tumor-gp96-adjuvant therapy of post-surgical residual disease.** Mice bearing late tumors underwent partial resection on day 9, with 60% residual tumor volume, and were treated with tumor-derived gp96 thereafter, as indicated.

**Figure 4. Results of tumor-derived gp96 adjuvant therapy.** Tumor volumes from mice that underwent surgical excision followed by treatment with buffer (A) or 5 doses of tumor-gp96 (1 µg/dose starting on day 9) after surgical incision (B). Data from (A) and (B) are summarized in (C).

**Immunotherapy of metastatic Lewis lung carcinoma**

Highly metastatic lines of the spontaneous Lewis lung carcinoma have been used as models of aggressive and metastatic spontaneous human cancers. This model has been used in several types of immunotherapy including immunotherapy with autologous tumor-derived gp96 (15). Syngeneic C57BL/6 mice were inoculated with 100,000 cells of the D122 line of Lewis lung carcinoma and the tumor was allowed to grow until day 24 post-tumor cell implantation. At this time, the tumor was typically 3-4 mm in diameter and was surgically excised by amputation of the foot. Untreated, these mice succumb to metastatic tumor growth in the lungs. Therapy of these mice was begun on day 29, 31 or 33 after tumor cell implantation (or day 5, 7 or 9 after surgery) (see Fig. 5...
for experimental design). Mice were treated with 4 immunizations of 1 µg D122-gp96 per intradermal injection given on alternate days. Survival of mice was monitored on day 33 post-tumor cell implantation. Two significant patterns were observed (Table 1). While nearly all mice treated with buffer succumbed to tumor growth (27/28), 100% of the 20 mice that began treatment with D122-gp96 on day 29 and 31 post-tumor cell implantation (day 5 or 7 post-surgery) remained tumor-free. In contrast, of the mice that began treatment only 2 days later, i.e. on day 33 after tumor cell implantation (or day 9 post-surgery), 40% (4/10) succumbed to metastatic tumor growth. Figure 6 is a set of representative photographs that demonstrate the tumor burden within the thoraces of mice treated with gp96 at various time points.

Figure 5. Study design of post-operative adjuvant therapy of metastatic disease using gp96 derived from the completely resected primary tumor. Primary tumors, inoculated in the foot, were removed from mice by amputation of the foot, and tumors were used as a source of gp96. Mice with subsequent tumor metastasis were treated early, intermediate or late depending on the day of commencement of post-operative treatment. Control animals received either buffer or gp96 derived from liver.

Table 1. Gp96-therapy of metastatic disease.

<table>
<thead>
<tr>
<th>Start of treatment post-tumor challenge (day)</th>
<th>Disease-incidence in mice treated with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>29</td>
<td>89% (8/9)</td>
</tr>
<tr>
<td>31</td>
<td>100% (10/10)</td>
</tr>
<tr>
<td>33</td>
<td>100% (9/9)</td>
</tr>
</tbody>
</table>

The second notable pattern concerns the immunological activity of liver-gp96. Significant protection from tumor growth was observed in mice that began treatment with liver-gp96 on days 29 or 31 post-tumor cell implantation. Exactly 50% of such mice (9/18) remained tumor-free. While this is significantly lower than 100% of the D122-gp96-treated mice that remained tumor-free, it is also significantly higher than the absence of protection in the buffer-treated mice. However, liver-gp96 afforded no protection among mice that began to receive treatment on day 33 post-tumor transplant.
Figure 6. Representative photographs of thoracic cavities of D122-bearing mice treated with buffer or tumor-derived gp96. Refer to the legend to Figure 5 and in Table 1. Thoracotomies were performed via a midline sternal incision, subsequently exposing the heart and the lungs.

Discussion

Our observations confirm and extend the previous studies on the immunological activity of tumor-derived heat shock protein gp96. In addition, they uncover novel aspects of the activity of gp96 in immunotherapy of pre-existing cancers and these have a significant bearing on the use of gp96 for immunotherapy of human cancer. Previous observations confirmed by our results include the ability of tumor-derived gp96 to mediate anti-tumor activity in primary and metastatic tumor models (15). Two other major facets of immunogenicity of gp96 are also reproduced here: tumor-derived but not normal tissue-derived gp96 preparations elicit anti-tumor activity (3,4) and the dose-restricted immunogenicity of gp96 (14,19).

The novelty of our observations lies in two major areas. We demonstrate a dramatic correlation between the time of initiation of treatment and the consequence of immunotherapy with gp96. Immunotherapy is very effective when begun 7 days post-tumor cell implantation (Meth A) or 31 days post-tumor cell implantation (D122). A delay of two additional days in either model makes the tumors refractory to immunotherapy. In the case of Meth A, the timing of relative resistance to immunotherapy (approximately day 9 post-tumor cell implantation) correlates well with the previously reported timing of appearance of suppressor T lymphocytes (20). It is particularly noteworthy that even at 9 days post-tumor cell implantation, the mice respond to immunotherapy with gp96 until day 15 at which time tumor growth takes over sharply and uniformly in all mice. Surgical intervention on day 9, coupled with immunotherapy with gp96, stabilizes tumor growth further. These observations indicate that although down-regulatory events begin to take the lead somewhere between days 9 and 15, these events are still not irreversible. Surgical excision of the tumor tilts the balance in favor of the host such that immunotherapy coupled with surgery still enables immunological activity against the tumor, resulting in prolonged stabilization of tumor size. These observations highlight the dynamic and fragile interplay between immunogenic and down-regulatory events between days 9-15 in Meth A and between days 31-33 in D122. The sharpness with which the mice become relatively refractory to immunotherapy suggests that the down-regulatory immunological activities are active, dominant and gain-of-function events rather than passive, co-dominant and loss-of-function events.

The second novel aspect of our results lies in the observed efficacy of liver-derived gp96, in the D122 model (Table 1). In this model, liver-gp96 is clearly more active than PBS and clearly less active than D122-gp96. The immunological activity of gp96 has been repeatedly shown to be present solely in the autologous tumor-derived
gp96 preparations (3,4,15). However, if one examines the data carefully, one notes that although the tumor-derived gp96 preparations are far more efficacious than control gp96 preparations, the latter have some activity above the saline controls (4). Indeed, in the D122 model, Tamura et al. (15) noticed and commented upon this fact during a statistical analysis of the anti-tumor activity in mice immunized with saline versus normal tissue-derived gp96. The immunological basis of this activity may lie in the activation of the innate components of the immune response stimulated by interaction of gp96 with APCs as demonstrated recently (21,22). Such activation may provide a more favorable microenvironment for amplification of the adaptive anti-tumor immune response elicited by the tumor itself.

Our observations highlight the need for the examination of a number of issues, including new opportunities, for the translation of the HSP approach to human cancer immunotherapy. These include the identification of a mechanistic basis for the resistance to immunotherapy that develops in hosts with tumors growing for longer periods, the detailed and systematic analysis of the effective dosage and regimen in a number of animal models of cancer, and the use of autologous (i.e. individual tumor-derived) and generic gp96 preparations and possibly their combinations in the therapy of experimental cancers.

Abbreviations

HSP, heat shock protein; Meth A, methylcholanthrene-induced fibrosarcoma

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References


Materials and methods

Purification of gp96

Gp96 was purified from normal liver, Meth A ascitic cells and D122 Lewis lung carcinoma as described (23).

Immunization and tumor challenge studies

Mice were challenged with Meth A or D122 cells and were immunized with gp96 as described (15,19).

Surgical techniques

BALB/c mice were inoculated with 100,000 live Meth A cells intradermally on their backs. On day 9 following inoculation, these tumors were measured in three axes, including the diameters in two planes and the thickness. After deeply anesthetizing them, surgery was performed under sterile conditions approved by the Animal Care Committee at the Akron General Medical Center. Tumors were excised tangentially in a plane parallel to the underlying skin, taking care to leave at least 60% residual tumor volume. The tangential excision was preferred to create a new tumor surface that would be equidistant from the tumor bed, ensuring an equal nutrient supply to the outer peripheral surface. Hemostasis was achieved with pressure alone, thus preventing any confounding impact of thermocoagulation on tumors. Mice were then housed in separate cages for 2-3 hours and their recovery closely monitored. Residual tumor volumes as well as those of the tumors excised were recorded.
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