ptosis (*26*). Additional study is needed to delineate the exact mechanisms by which activation of I_K might promote neuronal apoptosis. One possible arena for linkage between these events is in cell cycle control, because K^+ channels and a decrease in intracellular K^+ have been implicated in initiation of mitosis (*27*), and apoptosis has been postulated to reflect an "abortive mitosis" (*28*). Furthermore, agents that reduce intracellular K^+ may activate caspases in macrophages or monocytes (*29*). We suggest that interventions directed at blocking excessive K^+ efflux, in particular by the noninactivating delayed rectifier K^+ channel, be explored as a strategy for attenuating neuronal apoptosis in disease states.

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Immunotherapy of Tumors with Autologous Tumor-Derived Heat Shock Protein Preparations

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Immunotherapy of mice with preexisting cancers with heat shock protein preparations derived from autologous cancer resulted in retarded progression of the primary cancer, a reduced metastatic load, and prolongation of life-span. Treatment with heat shock protein preparations derived from cancers other than the autologous cancer did not provide significant protection. Spontaneous cancers (lung cancer and melanoma), chemically induced cancers (fibrosarcoma and colon carcinoma), and an ultraviolet radiation-induced spindle cell carcinoma were tested, and the results support the efficacy of autologous cancer–derived heat shock protein–peptide complexes in immunotherapy of cancers without the need to identify specific tumor antigenic epitopes.

Heat shock proteins (HSPs) GP96, HSP90, and HSP70, when purified from cells, are associated with a broad range of peptides derived from that particular cell, such that the HSPs chaperone the antigenic repertoire of the cells from which they are purified (*1*). Immunization with HSP-peptide complexes, whether derived endogenously (*2–4*) or reconstituted in vitro (*5, 6*), elicits potent T cell responses against the chaperoned peptides and hence against the cells from which the HSPs are purified, as seen in studies with cancers (*1, 3, 7–9*), viruses (*4, 10–12*), model antigens (*5, 6, 13*), and minor histocompatibility antigens (*13*). We now examine the use of HSP-peptide complexes in the treatment of a variety of established cancers of spontaneous and experimental origin.

The 3LL (Lewis lung) carcinoma of C57BL/6 mice (*14, 15*) is a nonimmunogenic spontaneous cancer. It metastasizes naturally to the lungs if injected subcutaneously or in the footpad. HSP GP96 prepa-

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rations from D122, a highly metastatic clone of 3LL (*16*), were tested for the ability to eradicate a preexisting tumor. In a perisurgical protocol (Fig. 1A) (*11*), mice were injected with 10^5 D122 cells in the footpad, and the tumors were allowed to grow for 11 days, at which time they were visible and palpable. Five weekly treatments with D122-GP96 (20 μg in saline) (Fig. 1B) were begun. Other groups of mice were treated with phosphate-buffered saline (PBS), GP96 derived from normal liver, or the nonautologous but syngeneic melanoma B16.F10. Tumors on each mouse were measured and surgically excised once they reached an average diameter of 8 mm. Lungs were isolated, and the number of metastases was counted 18 days after surgery. Mice treated with D122-GP96 averaged ~20 metastases per lung, whereas mice given PBS had ~200 metastases per lung [Fig. 1, C (left) and D]. Treatment with only 5 μg of D122-GP96 had little effect. Two of the five mice treated with liver-GP96 or B16.F10-GP96 had a reduced metastatic burden. Treatment with D122-GP96 also reduced the rate of growth of the primary footpad tumor compared with PBS-treated mice (Fig. 1E).

To identify the immune cells mediating the efficacy of GP96, we depleted mice of $CD4^+$ or $CD8^+$ T lymphocytes or natural killer (NK) cells by appropriate antibodies, immediately before the first treatment. Depletion of any of the three cell types abrogated the efficacy of immunization with GP96 (Fig. 1C, center). Mock depletion with a control immunoglobulin G (IgG) preparation had no effect. This result is consistent with our previous finding that immunization with GP96 requires $CD4^+$ and $CD8^+$ T lymphocytes in the effector phase of tumor immunity (*8*). The requirement for NK cells is in accord with our previous results, which showed that GP96 preparations stimulate release of interleukin-12 and other cytokines by antigen-presenting cells (*17*). Thus, although antitumor $CD8^+$ T cells were primarily stimulated by immunization with GP96, nonspecific NK effector cells were also recruited for tumor eradication.

D122-derived HSP70 preparations were also tested for efficacy of immunization in this protocol (Fig. 1C, right). HSP70 preparations were obtained by adenosine 5′-diphosphate (ADP), instead of the conventional adenosine 5′-triphosphate (ATP), affinity chromatography, as ADP does not hinder the HSP70-peptide interaction (*3*, *9*) (Fig. 1B). Tumor-bearing mice were treated, four times weekly, with ADP-purified D122-HSP70 or with corresponding ATP-purified, peptide-depleted preparations. ADP-purified liver-HSP70 and D122-GP96 were used as controls. ADP-purified D122-HSP70 was effective in reducing the micrometastatic burden to <10% of that in the untreated group. ADP-purified liver-HSP70 or ATP-purified D122-HSP70 did not protect, indicating that the immunity was elicited by HSP70-chaperoned antigenic peptides (Fig. 1C, right).

The efficacy of HSP immunization was tested in another type of therapy protocol that simulates the treatment of minimal residual disease (Fig. 2). D122 cells were injected into the footpads of naïve mice, and the tumor grew to 5 mm in diameter. The tumor-bearing footpads were removed, and the animals were apparently disease-free. However, all mice died of metastatic lung disease within <45 days of surgery. Thus, the animals were already carrying a micrometastatic burden at the time of the surgical removal of the primary tumor, as is often seen in humans. When mice were treated after surgery four times at weekly intervals with D122-GP96 (20 μg per immunization), 80% of the immunized mice survived to >250 days after surgery and were free of detectable lesions (Fig. 2). In contrast, mice immunized with liver-GP96 showed little protection.

As it may often be difficult or impossible to obtain a tumor sample for preparation of GP96 from a patient's primary tumor because of its inoperability, we investigated whether a metastatic lesion, which may differ substantially from the primary tumor, could be used as a source of GP96. A clone of D122 was selected that differs from the original in being nonmetastatic and in its histological appearance. A GP96 preparation derived from this variant was tested for its ability to treat a micrometastatic burden

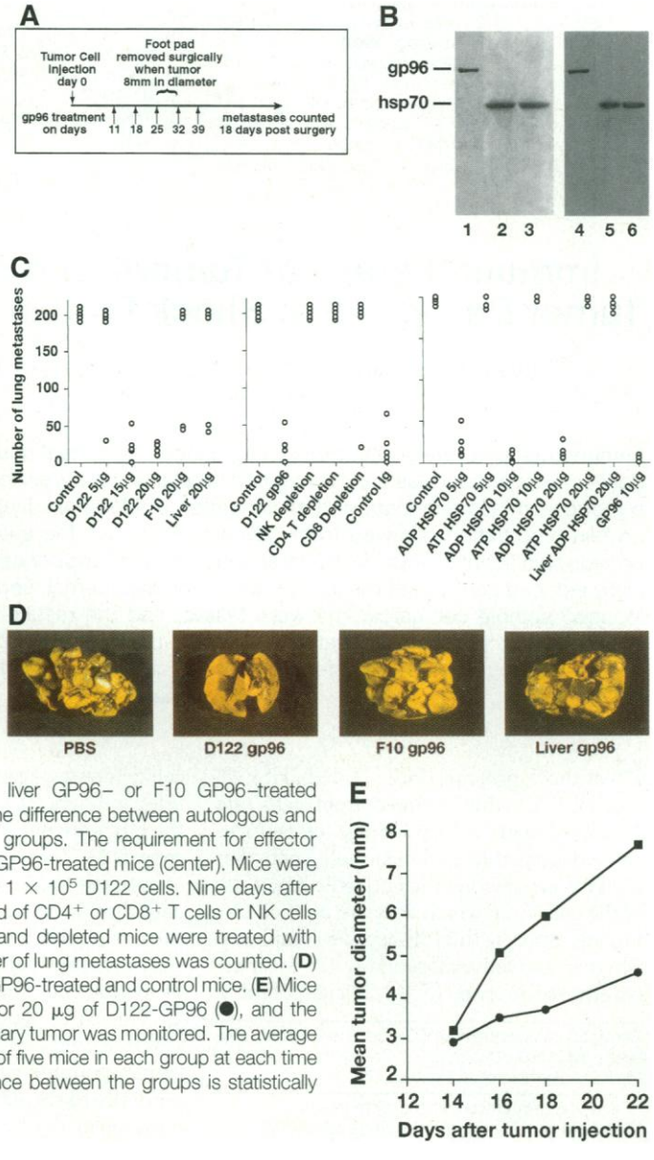

Fig. 1. Immunotherapy with D122-derived HSPs confers immunological protection against the primary tumor and lung metastases of D122 (*26*). (**A**) The protocol used for immunotherapy. (**B**) Silver-stained SDS–polyacrylamide gels of GP96 (lane 1), ATP affinity–purified HSP70 (lane 2), and ADP affinity–purified HSP70 (lane 3). Lanes 4 to 6 show immunoblots of the samples in lanes 1 to 3 with the relevant antibodies (*27*). (**C**) Immunotherapy with D122-GP96 and HSP70 confers protection against the lung metastases. Each circle represents the number of metastases per mouse, treated with 20 μg of GP96 (left) or HSP70 (right) as shown in (C). Among GP96-treated mice, $P < 0.001$ for the difference between PBS-treated and autologous GP96-treated groups, $P < 0.2$ for the difference between PBS-treated and liver GP96– or F10 GP96–treated groups, and $P < 0.02$ for the difference between autologous and heterologous GP96-treated groups. The requirement for effector cells was determined in the GP96-treated mice (center). Mice were injected in the footpad with 1×10^5 D122 cells. Nine days after injection, mice were depleted of $CD4^+$ or $CD8^+$ T cells or NK cells as described (*28*). Control and depleted mice were treated with D122-GP96, and the number of lung metastases was counted. (**D**) Photographs of lungs from GP96-treated and control mice. (**E**) Mice were treated with PBS (■) or 20 μg of D122-GP96 (●), and the kinetics of growth of the primary tumor was monitored. The average diameter of primary tumors of five mice in each group at each time point is shown. The difference between the groups is statistically significant ($P < 0.001$).

derived from the parental D122, and it was effective in conferring long-lived protection against the parental tumor (Fig. 2). Such efficacy derives, presumably, from the ability of HSPs to chaperone the entire antigenic repertoire of the cells from which they are isolated.

The generality of the therapeutic efficacy of HSP vaccination was tested in other tumors of different histological origins (melanoma, spindle cell carcinoma, colon carcinoma, and fibrosarcoma), different methods of induction (spontaneous, ultraviolet (UV) radiation induced, and chemically induced), different haplotypes (C57BL/6, C3H, and BALB/c), different degrees of immunogenicity (very high to undetectable), and different types of behavior (localized intradermally or micrometastatic). Mice bearing the spontaneous, poorly immunogenic melanoma B16.F10 were untreated, treated with B16.F10- GP96, or treated with D122-GP96 as a negative control. Treatment was begun 7 days after injection of the primary tumor, when the tumor was visible and palpable. Treatment with autologous but not heterologous GP96 resulted in a reduced growth rate for the tumor in all mice (Fig. 3A). The tumors in control mice grew to about 20% of their body weight by day 21, at which time the animals were killed. In contrast, the tumors of mice in the group treated with autologous GP96 did not grow to such size until day 45. Similar results were observed in the case of a UV radiation–induced spindle cell carcinoma 6139 (Fig. 3B), in which mice were treated starting at day 3, when the tumor was palpable. The widely used and extremely aggressive fibrosarcoma Meth A was also examined in a therapy model (Fig. 3C). We treated the mice starting at day 6 after tumor injection, when the tumor was visible and palpable. Treatment with Meth A–GP96 slowed the growth of the primary tumor, whereas treatment with irradiated Meth A cells, which are highly effective in prophylaxis (*1*, *3*, *7–9*), had no effect in therapy. The survival of Meth A–bearing mice treated with Meth A–GP96 was also significantly greater (~50%) than that of untreated mice. As a test case for hematogenous metastatic dissemination and a model for another major cancer, therapy of the colon carcinoma CT26 was attempted. Tumor cells (5×10^4) were injected into the retro-orbital sinus and mice were untreated or treated with CT26-GP96 or Meth A–GP96 as a negative control, starting on day 5. Five treatments were done on alternate days. CT26-bearing mice treated with autologous GP96 preparations had significantly longer survival times than the untreated mice or mice treated with heterologous GP96 (Fig. 3D). Whereas the efficacy of immunization with GP96 was consistently significant in all models tested, the extent of the antitumor effect varied. The regimens used were not optimized for dosage, frequency of administration, duration of treatment, site and route of vaccination, and combinations of HSPs and hence represent the minimum rather than optimal efficacy.

The extensive prophylactic immunization experiments with GP96 and HSP70 have uniformly demonstrated specificity in cancer (*1*, *3*, *7–9*), viral (*4*, *10–12*), and other (*5*, *6*, *13*) models. This specificity was also seen in the models for treating established tumors that we now report. However, rare instances of cross-reactivity were observed [Figs. 1C (left) and 2B] and may be derived from the ability of HSPs (*17*, *18*) to stimulate antigen-presenting cells to secrete cytokines, which can exert a nonspecific antitumor effect. Immunization with GP96 elicits an inherently nonspecific NK response [this study and (*19*)].

As the HSPs, like the major histocompatibility complex molecules, chaperone a broad repertoire of peptides, immunization with endogenous HSP-peptide complexes might elicit pathological autoimmune responses. However, in several hundred mice that have been immunized with tumor or

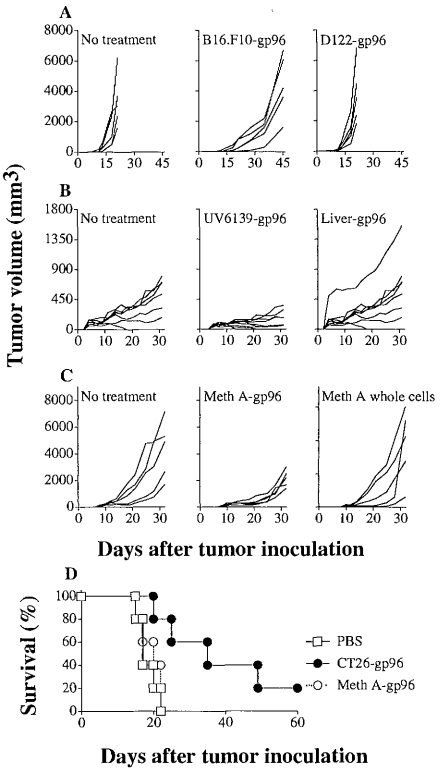

Fig. 3. Immunotherapy with autologous but not heterologous cancer-derived GP96 preparations is effective in therapy of (**A**) B16.F10 melanoma (C57BL/6) of spontaneous origin ($P < 0.01$ for untreated versus autologous GP96-treated groups, $P < 0.9$ for untreated versus heterologous GP96-treated groups, $P < 0.01$ for autologous versus heterologous GP96-treated groups), (**B**) UV radiation–induced 6139 spindle cell carcinoma (C3H) ($P < 0.02$ for untreated versus autologous GP96-treated groups, $P < 0.9$ for heterologous GP96-treated versus untreated groups, $P < 0.02$ for autologous versus heterologous GP96-treated groups), (**C**) methylcholanthrene-induced Meth A fibrosarcoma (BALB/c) ($P < 0.1$ for untreated versus autologous GP96-treated groups, $P < 0.9$ for autologous irradiated cells versus untreated mice), and (**D**) CT26 colon carcinoma (BALB/c). For the difference between untreated and autologous GP96-treated groups, $P < 0.01$ on days 22 to 25, $P < 0.05$ on days 25 to 35, and $P < 0.2$ on day 50. (A to C) Kinetics of growth of the primary tumor. (D) Survival analysis. Treated mice in all groups received 20 μg of GP96 per subcutaneous immunization five times on alternate days. Each line represents a single mouse in (A) to (C), whereas in (D), five mice were treated in each group.

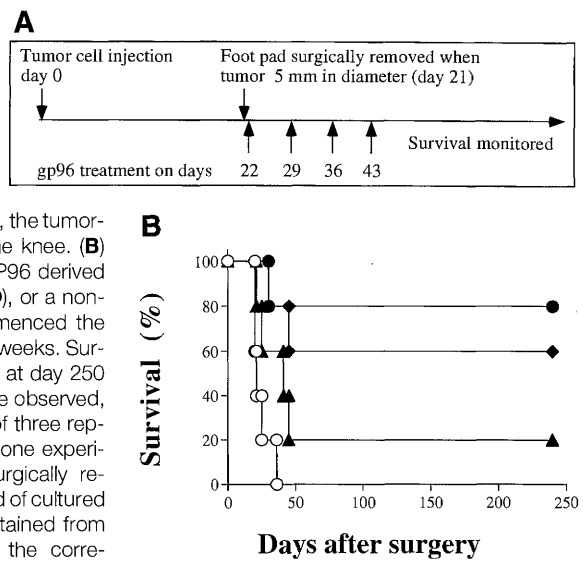

Fig 2. Treatment with D122-GP96 is effective in curing minimal residual disease. (**A**) Protocol used for postsurgical immunotherapy and treatment with D122-GP96. Mice (10 mice per group) were injected with 1×10^5 D122 cells in the footpad. When the primary tumor reached an average diameter of 5 mm, the tumor-bearing leg was amputated below the knee. (**B**) Treatment with PBS (○), 20 μg of GP96 derived from liver (▲), D122 metastatic line (●), or a nonmetastatic variant of D122 (◆) commenced the same day and continued weekly for 4 weeks. Survival was monitored. Mice were killed at day 250 after surgery because of age and were observed, at autopsy, to be disease-free. One of three representative experiments is shown. In one experiment, GP96 was prepared from surgically resected solid D122 tumor tissue instead of cultured cell lines. The GP96 preparations obtained from such preparation were identical to the corresponding preparations from cultured cells in therapeutic activity.

normal liver-derived GP96 and monitored for long periods for gross measures such as weight loss, ruffling of fur, life-span, and more specific parameters such as hepatotoxicity, no adverse consequences indicative of autoimmune phenomena have been detected (*20*). Further, in a recent phase I study, in which patients with progressive malignancies were immunized with autologous cancer-derived GP96 preparations, patients were followed for over a year specifically for signs of autoimmunity. No such signs were detected (*19*).

Regardless of the method of their induction or their lack of intentional induction, cancers are antigenically individually distinct (*21, 22*). This antigenic repertoire may consist of peptides that are rendered antigenic by the mutations within cancers; because the mutations are random and many, a repertoire becomes a fingerprint, which has little probability of repeating itself (*1, 22*). Immunization with cancer-derived HSP preparations permits access to the antigenic fingerprint of a cancer without a need for identification of this repertoire for each patient's cancer. Although immunization with defined cytolytic T lymphocyte epitopes that are shared between different cancers is widely perceived as a potential method of generating common cancer vaccines (*23, 24*), immunization with such epitopes may not elicit protective tumor immunity (*25*). Thus, therapies based on the complex, dynamic, and individually unique repertoire of tumor antigens may have advantages for treatment of cancer (*1, 22*).

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27. Antibodies against GP96 (9G10.F8.2) and HSP70 (BRM22) were obtained from NeoMarkers (Fremont, CA).
28. Mice were depleted of $CD4^+$ or $CD8^+$ T cells by intraperitoneal (ip) injection of 250 μl of 1:8 diluted GK1.5 ascites or YTS-169.4 ascites, respectively. Injections were continued biweekly. NK cells were depleted by ip injection of 200 μg of purified monoclonal antibody NK1.1 every week. Mice in the control group were injected with 200 μg of rat or mouse Ig once per week. The efficiency of depletion was confirmed by flow cytometry, and the specific depletion was 90 to 100%.

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Rapid Colorectal Adenoma Formation Initiated by Conditional Targeting of the *Apc* Gene

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Familial adenomatous polyposis coli (FAP) is a disease characterized by the development of multiple colorectal adenomas, and affected individuals carry germline mutations in the *APC* gene. With the use of a conditional gene targeting system, a mouse model of FAP was created that circumvents the embryonic lethality of Apc deficiency and directs *Apc* inactivation specifically to the colorectal epithelium. *loxP* sites were inserted into the introns around *Apc* exon 14, and the resultant mutant allele (Apc^{580S}) was introduced into the mouse germline. Mice homozygous for Apc^{580S} were normal; however, upon infection of the colorectal region with an adenovirus encoding the Cre recombinase, the mice developed adenomas within 4 weeks. The adenomas showed deletion of *Apc* exon 14, indicating that the loss of Apc function was caused by Cre-*loxP*–mediated recombination.

Mutations in the *APC* gene are responsible for FAP (*1*), a disease characterized by the development of thousands of colorectal adenomas (*1*). FAP patients have germline *APC* mutations, and their tumors show inactivation of the wild-type allele (*2*). Inactivation of both *APC* alleles also occurs frequently in sporadic colorectal adenomas (*3*). These results suggest that *APC* is a tumor suppressor gene, although the mechanism by which *APC* mutation leads to transformation of colorectal epithelial cells is largely unknown.

Several mouse lines carrying *Apc* mutations in their germline have been established as experimental models for FAP (*4*). For example, the Min mouse, which has a germline mutation in *Apc*, develops more than 100 intestinal adenomas, a subset of which show inactivation of the second *Apc* allele (*4, 5*). However, most of the tumors in the Min mouse develop in the small intestine rather than the colon (*5*), and it is difficult to study the early events in tumorigenesis in this model.

To study the initiation stage of colon

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