Antiangiogenic Tumor Therapy by DNA Vaccine Inducing Aquaporin-1–Specific CTL Based on Ubiquitin–Proteasome System in Mice

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Aquaporin-1 (AQP-1) is a water channel protein widely expressed in the vascular endothelial cells of proliferating tissues including malignant cancers. Given that in APC ubiquitinated peptides are effectively introduced into proteasomes from which CD8 epipopes are excised, we fused ubiquitin with AQP-1 (pUB–AQP-1) to produce a DNA vaccine. In C57BL/6J mice immunized with pUB–AQP-1, the growth of B16F10 melanoma was profoundly inhibited. The antitumor effect of the pUB–AQP-1 DNA vaccine was largely mediated by CD8 T cells, which secrete IFN-γ, perforin, and granzyme-B in the presence of APCs transfected with pUB–AQP-1. AQP-1–specific CD8 T cells possessed cytotoxic activity both in vivo and in vitro. After tumor challenge, the microvesSEL density decreased and the ratio of total blood vessel area to tumor area was significantly reduced as compared with control mice, resulting in a dramatic suppression of tumor growth. The immunization effect was completely abrogated in immunoproteasome-deficient mice. Strikingly, this pUB–AQP-1 DNA vaccine was also effective against Colon 26 colon tumors (BALB/c) and MBT/2 bladder tumors (C3H/HeN). Thus, this ubiquitin-conjugated DNA immunization-targeting tumor vasculature is a valid and promising antituor therapy. This vaccine works across the barriers of tumor species and MHC class I differences in host mice. The Journal of Immunology, 2012, 189: 1618–1626.

Quoaporin-1 (AQP-1) is a water channel protein highly expressed in the vascular endothelial cells of proliferating tissues including malignant cancers. Given that in APC ubiquitinated peptides are effectively introduced into proteasomes from which CD8 epipopes are excised, we fused ubiquitin with AQP-1 (pUB–AQP-1) to produce a DNA vaccine. In C57BL/6J mice immunized with pUB–AQP-1, the growth of B16F10 melanoma was profoundly inhibited. The antitumor effect of the pUB–AQP-1 DNA vaccine was largely mediated by CD8 T cells, which secrete IFN-γ, perforin, and granzyme-B in the presence of APCs transfected with pUB–AQP-1. AQP-1–specific CD8 T cells possessed cytotoxic activity both in vivo and in vitro. After tumor challenge, the microvesSEL density decreased and the ratio of total blood vessel area to tumor area was significantly reduced as compared with control mice, resulting in a dramatic suppression of tumor growth. The immunization effect was completely abrogated in immunoproteasome-deficient mice. Strikingly, this pUB–AQP-1 DNA vaccine was also effective against Colon 26 colon tumors (BALB/c) and MBT/2 bladder tumors (C3H/HeN). Thus, this ubiquitin-conjugated DNA immunization-targeting tumor vasculature is a valid and promising antituor therapy. This vaccine works across the barriers of tumor species and MHC class I differences in host mice. The Journal of Immunology, 2012, 189: 1618–1626.
system and the peptides are presented to the TCRs of CD8+ T cells. Vaccination with naked DNA encoding endogenous Ags fused to mono ubiquitin (UB) will direct proteins to be degraded by the ubiquitin–fusion degradation pathway (UFD) via the UPS. This pathway is an efficient means for the induction of functionally activated CD8+ T cells (22).

In the current study, a DNA encoding AQP-1 fused to ubiquitin (pUB–AQP-1) was constructed and C57BL/6 mice were vaccinated with the DNA by gene gun, either before or after transplantation of B16F10 (H-2b)—that is, prophylactically or therapeutically, respectively. The vaccination was clearly effective both therapeutically and prophylactically, as evaluated by inhibition of tumor growth. The angiogenesis of tumor tissues was profoundly suppressed in the vaccinated mice. Such an effect was not seen in mice vaccinated with plasmid encoding AQP-1 (pAQP-1). It is noteworthy that this pUB–AQP-1 DNA vaccine was extremely effective against B16F10 melanoma cells despite the defective or downregulated expression of MHC class I molecules on their cell surface. Strikingly, this pUB–AQP-1 DNA vaccine was also effective against Colon 26 colon tumors in BALB/c mice (H-2b) and against MBT2/2 bladder tumors in C3H/HeN mice (H-2b) in addition to B16F10 (C57BL/6, H-2b), strongly suggesting that the antitumor effect conferred by pUB–AQP-1 vaccination is valid across both tumor-species and MHC barriers.

Materials and Methods

Animals and cell lines

Eight week-old female C57BL/6, BALB/c and C3H/HeN mice were purchased from Seac Yoshitomi (Fukuoka, Japan) and kept in accordance with the institutional guidelines of Kyushu University and Fukuoka University. Proteasome activator PA28 knockout (PA28α/β−/−) mice were established by our group (23), and immunoproteasome subunit LMP2 or LMP7 knockout (LMP2−/− or LMP7−/−) mice were provided by Dr. Hans Jorg Feiling (Institute of Immunology, University Clinics Ulm, Germany). B16F10 cells (H-2b), Colon 26 (H-2b), MBT2/p (H-2k), and C57BL/6 mice (H-2b) were established by our group (23), and immunoproteasome subunit LMP2 or LMP7 knockout (LMP2−/− or LMP7−/−) mice were provided by Dr. Hans Jorg Feiling (Institute of Immunology, University Clinics Ulm, Germany). B16F10 cells (H-2b), Colon 26 (H-2b), MBT2/p (H-2b), and C57BL/6 mice (H-2b) were provided by Dr. Hans Jorg Feiling (Institute of Immunology, University Clinics Ulm, Germany).

Plasmid construction

A plasmid encoding His-tagged AQP-1 was constructed as follows: total RNA was derived from clinical melanoma sections isolated from mice and reverse-transcribed to produce cDNA. AQP-1 cDNA was then amplified by PCR using sense 5’-GCATCTGTCATACACTCAGTCATTCCCACTGGAGAGAGAAAC-3’ and antisense 5’-GTCACTCTCTGATTTGTGATGTTTGGCTCATTCCACGCTGGAGG-3’ primers. The AQP-1 cDNA PCR product was ligated to the pGem-T Easy vector (Promega), and this plasmid was transformed to and amplified in DH5α (Invitrogen). The vector encoding UB (pUB), which we constructed previously (24), was used to construct pUB–AQP-1 tagged with His tag. The AQP-1 gene was amplified again using sense 5’-GCATCTGTCATACACTCAGTCATTCCCACTGGAGAGAGAAAC-3’ and the same antisense primers as above. The AQP-1 PCR product was treated with SacII and XhoI, and then was inserted into the pUB vector.

In vitro cell transfection, selection, and Western blotting

Two million COS-7 cells were transfected at a dose of 2 μg of plasmid DNA using Lipofectamine (Invitrogen). Twenty-four hours after transfection, cell lysates were prepared, and 15-μg proteins were used for Western blotting as described previously (22). Anti-His Ab (Sigma) was used as the first Ab, and peroxidase-conjugated anti-mouse IgG (H + L; Zymed Laboratories) was used as the second Ab. The proteasome inhibitor MG-132 (Bostonchem, Boston, MA) was added 12 h after transfection at a concentration of 10 μM, and cell lysates were prepared 12 h after the addition of MG-132. Two million DC2.4 cells were transfected with 2 μg pUB–AQP-1 using Lipofectamine. Six hours after transfection, G418 was added to the medium at a concentration of 500 μg/ml for selection. After 12–18 d, G418-resistant cell clones were isolated and transferred to separate culture dishes for expansion and analysis.

In vivo gene transfer and implantation of melanoma cells

For gene transfer, we used a Helios Gene Gun (Bio-Rad, New York, NY) as described previously (24). C57BL/6 mice were immunized four times at 2-week intervals with 6 μg of each plasmid. Ten days after the last vaccination, 100,000 B16F10 cells in 200 μl PBS were injected s.c. into each B6 mouse. Tumors were measured twice a week using a caliper, and tumor volume was calculated as m/6 × [(a × b)3], where a and b are two perpendicular major diameters. For the therapeutic experiments, 5000 B16F10 cells in 200 μl PBS were implanted s.c. in the abdominal area of C57BL/6 mice. One day after the implantation, mice were treated with 6 μg of each plasmid and then subsequently treated with 6 μg of each plasmid twice a week for 2 weeks (total of five times).

Flow cytometry, ELISA, and cell proliferation

Mice were sacrificed at 10 d after the last vaccination. One million splenocytes were cultured in RPMI 1640 complete medium containing FCS (50 mg/ml), calcium ionophore (1 μg/ml), and brefeldin A (1 μg/ml) for 4 h using a 12-well plate. After centrifugation, cells were stained with allophycocyanin-labeled anti-CD4 Ab (clone GK1.5; BD Pharmingen), PE-labeled anti-CD8 Ab (clone 53-6.72; BD Pharmingen) and FITC-labeled anti-IFN-γ Ab (clone XMG1.2; BD Pharmingen) at 4°C for 30 min. After washing twice, cells were collected on a FACSscan flow cytometer (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences).

CD8+ T cells were isolated from spleen cells using MACS and 1 million CD8+ T cells cultured with one hundred thousand pUB–AQP-1–transfected DC2.4 cells irradiated with 5.5 Gy gamma-irradiation in a 12-well plate. Three days later, the supernatants were collected and IFN-γ production was measured by ELISA as described previously.

Twenty thousand CD8+ T cells were cultured with 2000 pUB–AQP-1–transfected DC2.4 cells irradiated with 5.5 Gy gamma-irradiation in a 96-well plate. Three days later, [3H]thymidine was added to the medium. After 10 h of incubation, the cells and their media were harvested onto glass-fiber filters, the radioactivity level was assessed using a β scintillation counter, and proliferation was calculated.

In vivo and in vitro CTL assays

Splenocytes (6 × 107) isolated from C57BL/6J mice were cultured in 35-mm plates and transfected with or without 20 μg pUB–AQP-1 plasmid DNA using Lipofectamine. Finally, AQP-1–transfected splenocytes were labeled with 0.2 μM CFSE, and nontransfected splenocytes were labeled with 2 μM CFSE. Equal numbers of these two types of CFSE-labeled splenocytes were mixed, and then 2 × 106 splenocytes of these 1:1 mixtures were transferred to pUB–AQP-1–immunized mice or control mice via i.v. injection. Mouse spleens were isolated 18 h after the cell transfer. Specific lysis was analyzed by flow cytometry. Cell A is pUB–AQP-1–transfected splenocytes labeled with 0.2 μM CFSE, and cell B is nontransfected splenocytes labeled with 2 μM CFSE:

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\% Lysis = \left(\frac{No. of cell B - No. of cell A}{No. of cell B}\right) \times 100
\]

CD8+ T cells (1 × 105) were isolated from pUB–AQP-1–immunized mice or control mice and then cultured with 1 × 106 DC2.4 cells transfected with pUB–AQP-1 or pCDNA3.1 (control) in 1 ml RPMI 1640 medium for 36 h. FITC−CD107a Ab was added to the medium, and the cells were cultured for an additional 2 h and then stained with PE-CD8 and APC–TCR-β Ab. Cells were analyzed by flow cytometry (BD FACS-Canto). The percentage of CD8+CD107a+ on the CD8+ cell gate was calculated, and the difference of the percentage of CD8+CD107a− after CD8+ T cells were isolated from spleen cells using MACS and 1 million CD8+ T cells cultured with one hundred thousand pUB–AQP-1–transfected DC2.4 cells irradiated with 5.5 Gy gamma-irradiation in a 12-well plate. Three days later, the supernatants were collected and IFN-γ production was measured by ELISA as described previously.

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Histology and immunohistochemistry

Fresh melanoma tissues were isolated from B6 mice sacrificed 2 wk after challenge with B16F10 cells. The samples were immediately frozen in liquid nitrogen-cooled OCT compound and stored at −20°C. Cryosections (5 μm thick) were cut and mounted on glass slides. Slides were washed...
in PBS three times for 5 min each, incubated in 0.2% Triton-X100 in PBS for 15 min, and washed again in PBS. Samples were incubated with 1% BSA in PBS (PBS-BSA) for 30 min at room temperature for blocking nonspecific binding. FITC–anti-aquaporin 1 Ab (Biorbyt) was used for the fluorescence immunostain of aquaporin-1 expressed in tumor or skin tissue. The sections were stained with a combination of FITC–anti-Aquaporin 1 Ab (1:1000), PE–anti-CD31 mAb (1:1000) and anti-DAPI (1:1000) overnight at 4°C. After rinsing in PBS three times for 5 min each, slides were mounted in Prolong Gold (Invitrogen) with DAPI. Specimens were examined with a Biozero fluorescence microscope BZ-8100 (Keyence, Osaka, Japan). Pictures of immunofluorescence were analyzed with Image J software (National Institutes of Health).

Statistical analysis
Data are expressed as means ± SD. Differences between experimental groups within each experiment were analyzed using the unpaired Student t test and were considered significant at p ≤ 0.05. Survival was analyzed using the log-rank test, and differences were considered significant at p ≤ 0.05.

Results
Enhancement of proteasome-mediated degradation of aquaporin-1 fused with ubiquitin
AQP-1 and UB–AQP-1 expression plasmids were constructed as described (Fig. 1A), and the constructs were confirmed by DNA sequencing. The expression of AQP-1 or the fusion protein UB–AQP-1 was confirmed by Western blotting after transfection of COS7 cells with these plasmids (Fig. 1B).

Inhibition of B16F10 melanoma tumor growth induced by pUB–AQP-1 vaccination is dependent on the ubiquitin–proteasome system
To survey the immune effect of these plasmids, we delivered pUB, pAQP-1, or pUB–AQP-1 into the abdominal skin of B6 mice using a gene gun, three times, at 1-wk intervals. Ten days after the last immunization, mice were challenged with 100,000 B16F10 cells by s.c. injection at a site on the abdominal skin. Strikingly, the growth of melanoma in mice immunized with pUB–AQP-1 was severely retarded compared with control mice or mice immunized with pUB or pAQP-1 (Fig. 2A). Fifty percent of mice immunized with pUB–AQP-1 survived more than 45 d after the implantation of B16F10 cells, whereas all mice of the other three groups were dead within 35 d (Fig. 2B). These data show that inhibition of the growth of B16F10 melanoma cells occurs only when mice are vaccinated with pUB–AQP-1.

To determine whether growth inhibition of melanoma by vaccination with monoubiquitinated AQP-1 in vivo was dependent on the proteasomal degradation pathway, we used mice lacking the proteasome regulator PA28 complex (α/β) or the immunoproteasome subunits LMP2 or LMP7 (Fig. 2C, 2D). The vaccination effect was completely abrogated in PA28α/β knockout (KO) mice or in LMP7 KO mice, and partially impaired in LMP2 KO mice. Therefore, the efficacy of vaccination with pUB–AQP-1 on growth inhibition of B16F10 melanoma cells is dependent on the UPS and, in particular, on PA28α/β and LMP7.

To investigate whether immunization with pUB–AQP-1 is effective in inhibiting the metastasis of B16F10 melanoma, we used a mouse experimental metastasis model. Mice were challenged i.v. with 2 × 10^5 B16F10 cells after being immunized three times with pUB–AQP-1 and sacrificed 18 d after tumor challenge. As shown in Fig. 2E, the metastatic spots of B16F10 in the lung were significantly decreased in mice immunized with pUB–AQP-1 compared with those of the control group.

Critical role of CD8+ T cells in the antitumor immunity induced by vaccination with pUB–AQP-1
To accurately define the effector mechanisms involved, mice were depleted of CD4+ or CD8+ T cells by treatment with the corresponding specific Abs 1 d before the tumor challenge. Tumor growth (Fig. 3A) and survival rate (Fig. 3B) were similar in pUB–AQP-1-immunized mice, with or without depletion of CD4+ T cells. By contrast, depletion of CD8+ T cells almost completely abolished the protective immunity induced by vaccination with pUB–AQP-1. Therefore, CD8+ T cells are the effector cells required for the protective immunity raised by vaccination with...
pUB–AQP-1. Of note, CD4⁺ T cells were unnecessary in the effector phase of raising immunity to the growth of melanoma cells.

Functional analyses of AQP-1–specific CD8⁺ T cells induced by vaccination with pUB–AQP-1

It is well known that Th1 type immunity and cytotoxic CD8⁺ T cells are important for the resistance and clearance of melanoma. Therefore, we examined whether Th1 type immunity and activated CD8⁺ T cells were induced by vaccination with pUB–AQP-1. Spleen cells, isolated 10 d after the last immunization, were stimulated with PMA and calcium ionophore. The production of intracellular IFN-γ, perforin, and granzyme-B (GZM-B) by CD8⁺ T cells was analyzed by flow cytometry (Fig. 4A). Production of IFN-γ, perforin, and GZM-B was strongly induced in CD8⁺ T cells of mice vaccinated with pUB–AQP-1 (Fig. 4A, right panel). The number of those CD8⁺ T cells was significantly higher in pUB–AQP-1–immunized mice than in control mice or mice vaccinated with pUB or pAQP-1 (Fig. 4B). Thus, activation of Th-1 type CD8⁺ T cells is strongly induced upon vaccination with pUB–AQP-1.

Next, CD8⁺ T cells were isolated from the spleens of control mice, pAQP-1–vaccinated mice, or pUB–AQP-1–vaccinated mice and cultured alone or with either nontransfected DC2.4 cells or DC2.4 cells transfected with pUB-AQP-1. CD8⁺ T cells obtained from spleens of pUB–AQP-1–vaccinated mice secreted significantly higher levels of IFN-γ in response to stimulation with DC2.4 transfected with pUB–AQP-1 (Fig. 4C). Furthermore, the proliferation of CD8⁺ T cells obtained from spleens of pUB–AQP-1–vaccinated mice was significantly enhanced by this vaccination (Fig. 4D). However, we could not detect any significant IFN-γ secretion or proliferation of CD8⁺ T cells obtained from control mice and mice vaccinated with pAQP-1. These results suggest that CD8⁺ T cells specific for AQP-1 protein were greatly activated by vaccination with pUB–AQP-1. CTL assays

FIGURE 2. Inhibition of B16F10 melanoma tumor growth induced by pUB–AQP-1 vaccination is dependent on the ubiquitin–proteasome system. C57BL/6 mice were immunized with pcDNA (open triangles), pUB (open squares), pAQP-1 (closed squares), pUB–AQP-1 (closed triangles), and then challenged with 100,000 B16F10 cells. Mice were surveyed on the basis of tumor size (A) and survival (B). C57BL/6 mice and immunoproteasome subunit-deficient mice, (PA28αβ⁻/⁻, LMP2⁻/⁻ and LMP7⁻/⁻ mice), were immunized with pUB–AQP-1. Mice were challenged with melanoma cells, and tumor size (C) and survival rate (D) were assessed. (E) C57BL/6 mice immunized with pUB–AQP-1 were challenged with B16F10 melanoma cells i.v. 14 d after the challenge, mice were sacrificed and metastases in lung were analyzed (n = 7). In (A), (C), and (E), each value represents the mean ± SD from seven or eight mice in each group. Every experiment had been done at least three times, and one representative experiment is shown. *p < 0.05, **p < 0.02 compared with other groups by the Student t test. In (B) and (D), results are expressed as the percentages of surviving mice in groups of seven or eight mice. *p < 0.05, **p < 0.02 compared with other groups by the log-rank test.

FIGURE 3. Critical roles of CD8⁺ T cells in the antitumor immunity raised by vaccination with pUB–AQP-1. (A) Mice vaccinated with pUB–AQP-1 were treated with anti-CD4 or anti-CD8Abs and then challenged with B16F10 cells. Each value is the mean ± SD from eight mice in each group. *p < 0.05, **p < 0.02 compared with control group by the Student t test. (B) Results are expressed as percentages of surviving mice in groups of eight mice. Every experiment had done three times, and one representative experiment is shown. *p < 0.05, **p < 0.02 compared with control group by the log-rank test.
were processed to verify the specific CTL activity of CD8+ T cells in vivo (Fig. 4E) and in vitro (Fig. 4F). As showed in Fig. 4E, AQP-1–specific CTLs were significantly increased in mice immunized with pUB–AQP-1 compared with mice in the control group. pUB–AQP-1 immunization inhibits angiogenesis and enlarges necrotic areas of B16F10 melanoma tissues in vivo

AQP-1 protein is expressed widely in the vascular endothelia of proliferating microvessels and is reportedly responsible for angiogenesis in B16F10 melanomas (6). To clarify the mechanisms of resistance to challenge with B16F10 in mice immunized with pUB–AQP-1, we examined the angiogenesis of B16F10 melanoma in control mice and UB–AQP-1–vaccinated mice. As show in Fig. 5A, in which tumor tissues were stained with fluorescently labeled anti-CD31 Ab, the density of microvessels in B16F10 melanoma tissues was significantly decreased in mice immunized with pUB–AQP-1 compared with control mice (Fig. 5B). To obtain more information for tumor angiogenesis, we examined the ratio of blood vessel area to total tumor area using the IMAGE J software. In this study, this ratio in mice of the control group was ∼7.44 ± 6.15%. This ratio was drastically decreased to 3.58 ± 0.2% in mice immunized with pUB–AQP-1 compared with those of control mice. However, AQP-1 expression on blood vessels in normal skin was not significantly affected in mice immunized with pUB–AQP-1 compared with control mice (Fig. 6B). These results suggest that the antitumor effects of pUB–AQP-1 vaccination were exerted by AQP-1–specific CD8+ T cells and that these cells may inhibit the angiogenesis of melanoma tumors and induce necrosis of melanoma tumor tissues by CTL activity against AQP-1–positive endothelial cells of newly formed microvessels. However, the AQP-1–specific CD8+ T cells do not react against the tumor cells themselves.

Therapeutic effects of pUB–AQP-1 vaccination on B16F10 melanoma and two additional tumor models

From the point of view of clinical application, it is important to examine the therapeutic effects of this vaccination strategy against melanoma transplanted before vaccination. As shown in Fig. 7 immunization of tumor-transplanted mice with pUB–AQP-1 vaccination resulted in marked suppression of tumor growth compared with control mice or mice immunized with pAQP-1 (Fig. 7B). These results suggest that the antitumor effects of pUB–AQP-1 vaccination were exerted by AQP-1–specific CD8+ T cells and that these cells may inhibit the angiogenesis of melanoma tumors and induce necrosis of melanoma tumor tissues by CTL activity against AQP-1–positive endothelial cells of newly formed microvessels. However, the AQP-1–specific CD8+ T cells do not react against the tumor cells themselves.
DNA plasmid is effective even after initiation of the malignant B16F10 melanoma, thus confirming its potential clinical effectiveness against tumors. Furthermore, we also tested the therapeutic effect of pUB–AQP-1 in different tumors and strains of mice. In C3H/HeN mice, the rate of growth of MBT2/p, a murine bladder transitional cell carcinoma cell line, was inhibited by immunization with pUB–AQP-1, and the survival of mice carrying this tumor was also prolonged (Fig. 7B). In BALB/c mice, the rate of growth of colon26, a colorectal carcinoma cell line, was inhibited by immunization with pUB–AQP-1, and survival of mice with this tumor was also prolonged (Fig. 7C). These results clearly suggest that immunization with pUB–AQP-1 is widely effective against tumors.

**FIGURE 5.** pUB–AQP-1 immunization inhibited angiogenesis and enlarged necrotic areas of B16F10 melanoma tissues in vivo. (A) Tumor tissues from the control or pUB–AQP-1–immunized groups were stained with PE–anti-CD31 and DAPI. Immunofluorescence images were analyzed with Image J software and blood vessels areas were shown as filled black. The images were merged from 100–120 pictures of original magnification ×200. (B and C) The density of blood vessels in melanoma tissues and the ratio of the total blood vessel area to the total tumor area were analyzed by Image J software. (D) H&E staining images of whole tumor tissues and processed images using Image J software. Four whole tumor tissues of control mice and pUB-AQP-1 immunized mice were analyzed and the representatives were shown. The images were merged from 30–40 pictures of original magnification ×40. (E) Percentage of necrosis area of tumor tissues from control mice or pUB–AQP-1–immunized mice was calculated (n = 4). *p < 0.05 compared with control group by the Student t test.
applicable as an antitumor vaccination in different types of tumors and different strains of mice.

Because AQP-1 is expressed in kidneys, we examined whether there is any physiologic or pathologic disturbance in kidneys of mice immunized with pUB–AQP-1. As shown in Fig. 7D, we found no significant differences in the morphologic structure of kidneys comparing control mice and pUB–AQP-1–immunized mice (Fig. 7D). To examine the physiologic functions of the kidney, the RBC, WBC, sugar, and protein content in the urine of mice were measured. There were no abnormal findings in the urine of pUB–AQP-1–immunized mice (data not shown). There was also no disturbance in the body weights of mice immunized with pUB–AQP-1 (Fig. 7E). These results indicate that pUB–AQP-1 vaccination can be used safely in mice to induce effective antitumor immunity.

**Discussion**

In this study, we demonstrated that DNA immunization with pUB–AQP-1 rendered mice significantly resistant to challenge with malignant tumors. Despite the negligible expression of AQP-1 in B16F10 cells themselves (data not shown), AQP-1–specific CD8 T cells effectively inhibited the angiogenesis of melanoma in vivo, conferring striking resistance to challenge with B16F10 in mice. AQP-1–specific CD8 cytotoxic T cells killed AQP-1–expressing endothelial cells in tumor neovasculature, causing impairment of angiogenesis in tumors. The density of blood vessels in tumor tissue was reduced in mice immunized with pUB–AQP-1, and more necrosis was found in tumor tissues of immunized mice compared with control mice. It is noteworthy that no significant side effects or clinical symptoms were detected in mice immunized with pUB–AQP-1 in the current study.

CD8 killer T cells have long been proposed to be the main effectors of immunologic cancer therapy targeting tumor specific Ags (27). Unfortunately, however, no satisfactory results have been obtained. One can postulate several reasons for these failures. First, it is essential that tumor cells express MHC class I molecules on their surface to be targeted by Ag-specific CD8 T cells. However, malignant tumor cells often lack or show decreased levels of MHC class I molecules on their surface and thus successfully escape CD8 killer T cell attack. Second, for many tumors, tumor-specific Ags have not been identified. Third, MHC class I-restricted peptides must be identified to use a tumor-specific
Ag for the induction of CD8 T cells. Furthermore, MHC class I alleles are different in each individual tumor-bearing host. Thus, exact peptides from tumor-specific Ags, which are suitable for binding the patient’s own MHC class I, must be identified for the successful application of tumor-specific, Ag-driven antitumor therapy.

AQP-1 is widely expressed in vascular endothelial cells, especially in those of proliferating microvessels. The intimate relationship between AQP-1 function and the development of tumors has been noted recently (6). AQP-1 facilitates water permeability in cell protrusions and thus enhances the migration of endothelial cells for tumor angiogenesis, although the details of the mechanism are still under investigation. Angiogenesis is crucial for the growth of malignant tumors as these cells require tremendous nutrition to be transported via blood vessels. Thus, a protein such as AQP-1 that is highly expressed in the proliferating blood vessels of tumor tissues may be an ideal immune system target for the inhibition of tumor growth. These findings prompted us to test whether inducing AQP-1–specific CD8 killer T cells by DNA vaccination would lead to impairment of angiogenesis in tumor tissues and thus provide an effective strategy against malignant tumors.

In this study, we successfully established CD8 CTLs specific to AQP-1 by vaccination with plasmids encoding ubiquitin-conjugated AQP-1. AQP-1–specific CD8 T cells killed AQP-1–expressing endothelial cells in tumor vasculature, rendered tumor tissue necrotic and thus abolished tumor growth. It is important to note that the CD8 T cells induced by this UB–AQP-1 DNA vaccine are specific for tumor neovasculature but not for the tumor cell itself. Although malignant tumor cells decrease or lose surface MHC class I expression, MHC class I expression on endothelial cells in tumor neovasculature remains intact. Therefore, AQP-1–specific CD8 killer T cells can successfully inhibit the growth of class I-deficient malignant tumor cells by cytolysis and the destruction of neovasculature. This unique characteristic is a solid foundation for the effectiveness of this vaccination against malignant tumors, which lose MHC class I expression.

Furthermore, we provide evidence that UB–AQP-1 DNA vaccination is effective not only against B16F10 melanoma cells but also against different types of tumors such as Colon 26 in BALB/c (H-2d) mice and MBT2/p in C3H/HeN mice (H-2k). We demonstrate that the same DNA vaccine is effective for all three strains of mice, which have different MHC class I alleles (H-2b,d,k). These results strongly suggest that this UB–AQP-1 DNA vaccine is a universal vaccine, which works efficiently across the barriers of different MHC class I haplotypes and different tumor-specific Ags.

Specific immunity in mice has been achieved using multiple approaches, including DNA genetic immunization and recombinant viral vectors (17, 22). Clinical application of viral vectors is still limited by the potential toxicity of viral infection, the transient nature of transgene expression, and host antivirus immunity (28, 29). On the other hand, immunization with plasmid DNA has some advantages compared with other approaches. DNA plasmids are safe, easy to store, ready to deliver, and molecularly defined and can be easily constructed to enhance antigenicity by various means, such as enhancement of Ag processing or adjuvant activity through a CpG motif included in the plasmid.

AQP-1 fused to ubiquitin is expressed within cytosol in APCs, and the UPS has a central role in degrading this protein and activating AQP-1–specific CD8+ T cells just as endogenous Ags are.
processed by the proteasome after being ubiquitinated. The chimeric protein, AQP-1 fused to monoubiquitin, is likely to be poly-ubiquitinated by E4 ubiquitination factor and processed in the proteasome to antigenic peptides. The immunoproteasome contains the proteasome regulator PA28 (23) and immune subunits LMP2 and LMP7, which are induced by IFN-γ stimulation (21, 30). In this study, we used mice carrying deletions of these three genes to investigate the role of the ubiquitin–proteasome system in the induction of immunity. The resistance to tumors induced by immunization with pUB–AQP-1 was abrogated in mice deficient in the proteasome activator PA28α/β or the immunoproteasome subunit LMP7 and partially cancelled in mice deficient in LMP2. Therefore, we hypothesize that in this study, the dominant AQP-1 epitopes responsible for the induction of the immune response were mainly dependent on LMP7 rather than LMP2.

Several approaches have been used to attempt to inhibit tumor growth by suppressing angiogenesis. For example, immunization against FLK1 (murine VEGFR2) elicited significant antitumor activity (31). The immune response to FLK1 was achieved by immunization against FLK1 (murine VEGFR2) elicited significant antitumor growth. For example, immunization with chemotactic-prostate tumor associated antigen gene-modified tumor cell and blockade of B7-H1. Clin. Immunol. 27: 117–130.


