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Immunoprevention of Basal Cell Carcinomas with Recombinant Hedgehog-interacting Protein

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Abstract

Basal cell carcinomas (BCCs) are driven by abnormal hedgehog signaling and highly overexpress several hedgehog target genes. We report here our use of one of these target genes, hedgehog-interacting protein (Hip1), as a tumor-associated antigen for immunoprevention of BCCs in Ptch1$^{+/-}$ mice treated with ionizing radiation. Hip1 mRNA is expressed in adult mouse tissues at levels considerably lower than those in BCCs. Immunization with either of two large recombinant Hip1 polypeptides was well tolerated in Ptch1$^{+/-}$ mice, induced B and T cell responses detectable by enzyme-linked immunosorbent assay, Western blot, delayed type hypersensitivity, and enzyme-linked immunospot assay, and reduced the number of BCCs by 42% ($P < 0.001$) and 32% ($P < 0.01$), respectively. We conclude that immunization with proteins specifically up-regulated by hedgehog signaling may hold promise as a preventive option for patients such as those with the basal cell nevus syndrome who are destined to develop large numbers of BCCs.

Key words: basal cell nevus syndrome • patched • skin cancer • immunization • mouse

Introduction

In the rare autosomal dominant basal cell nevus syndrome (BCNS; Gorlin syndrome; MIM 109400) patients develop dozens to hundreds of basal cell carcinomas (BCCs) starting early in childhood or adolescence (1). Local growth and treatment of the multiple tumors in these patients inevitably causes significant scarring and disfigurement. A noninvasive, ideally preventive therapy against BCCs would substantially improve life quality and the management of individuals such as those with BCNS at high risk of developing BCCs. Since BCNS patients develop such large numbers of BCCs, even partial prevention of tumors would provide great clinical benefit for these patients.

Mutational activation of hedgehog signaling with overexpression of hedgehog target genes is the pivotal step in the development of BCCs. This activation most commonly is effected by mutations in PTCH1, which encodes a protein which normally inhibits hedgehog signaling, and BCNS patients inherit one defective copy of this tumor suppressor gene (2, 3). Among the hedgehog target genes uniformly overexpressed in BCCs is the recently identified putative transmembrane protein hedgehog-interacting protein (Hip1; Hhip) (4). This consistent overexpression potentially provides a target for immunization. As reviewed recently by Finn and Forni, the rationale for preventive immunization against malignancies is strong (5). When challenged before cancer has developed, the host's immune system has not been impaired by tumor-induced suppression, and the immune system may eliminate subclinical tumors much more effectively than it can eliminate macroscopic tumors. Indeed, recent animal studies have shown that cancer vaccines not only are capable of protecting against tumor challenge but also can reduce the occurrence of tumors in genetically predisposed animals. For example, vaccination with dendritic–tumor fusion cells prevented the development of tumors in up to 43% of transgenic mice predisposed to develop spontaneous develop mammary carcinomas (6).

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Abbreviations used in this paper: BCC, basal cell carcinoma; BCNS, basal cell nevus syndrome; DTH, delayed-type hypersensitivity; Hip1, hedgehog-interacting protein; IR, ionizing radiation; ODN, oligodeoxynucleotides; TAA, tumor-associated antigen.

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$Ptch1^{+/-}$ mice, unlike previously tested mouse models of skin carcinogenesis, develop BCCs and medulloblastomas, rhabdomyosarcomas, and other phenotypic abnormalities similar to those of BCNS patients (3, 7). We have studied the Goodrich et al. model in which the lacZ and neo genes were substituted for part of exons 1 and 2 (8). These $Ptch1^{+/-}$ mice are treated with 5 Gy ionizing radiation (IR) at the age of 2 mo, all develop microscopic BCC-like tumors by age 7 mo, and 50% of surviving individuals develop visible BCCs by age 15 mo, making this a reliable and practical tumor model that closely mimics human BCC development (8).

Because Ptch1 is itself a target of hedgehog signaling, activation of the lacZ reporter gene inserted at the Ptch1 locus allows convenient detection of Ptch1 promotor activity and hence of hedgehog target gene up-regulation. Consistent with the finding of activated hedgehog signaling in human BCCs, high levels of β-galactosidase can be found in all BCCs of $Ptch1^{+/-}$ mice, whereas normal epidermis in tumor-bearing mice remains negative for β-galactosidase (8).

There are some hints that the immune system may help control human BCCs. Thus, Curson and Weedon first described the infiltration of BCCs by immune cells together with possible signs of regression in BCCs—disruption of the palisaded architecture of the tumor cells at the periphery, occurrence of apoptotic cells, and dermal deposition of collagen (9). Using these criteria, they found that 81 out of 400 tumors examined had evidence of previous or continuing regression (9). Several groups have found significantly increased numbers of $CD3^+$ and $CD4^+$ cells in the tumors, and the finding of increased levels of IFNγ mRNA in tumors suggests a possible role for $CD4^+$ cells and Th1 cytokines in the control of BCCs (10–12). Furthermore, nonspecific stimulation of the immune system by local injection of IFNα or by application directly to BCC tumors of the immune response modifier imiquimod can reduce tumor size or even eliminate BCCs (13–17). Postulated mechanisms for the efficacy of IFNα include the stimulation of the CD95 (Fas) receptor in the BCCs, which in untreated patients constitutively express CD95 ligand but not the receptor. The peritumoral infiltrate could support the resulting apoptotic suicide by the secretion of IFNγ or IL-2, which may trigger further up-regulation of CD95 on BCC cells. Imiquimod activates the immune system through localized induction of cytokines such as IFNα, IFNγ, and IL12, and thus its mechanism of action may be similar to that of direct cytokine treatment with IFNα. Although cytokine therapy of BCCs is still experimental and its long-term efficacy has been questioned (15), the clinical success of local immunostimulation with IFNα and imiquimod and the putative role of infiltrating T cells in regressing BCCs have encouraged us to assess the possibility of BCC immunoprevention using a tumor-associated antigen.

In this study, we first investigated the expression of Hip1 mRNA in extracutaneous tissues of adult mice. Based on our finding of the limitation of strong expression of Hip1 mRNA to BCCs, we immunized mice with recombinant Hip1 fragments. We found that these peptides are immunogenic and that immunization can reduce the formation of endogenous BCCs in $Ptch1^{+/-}$ mice.

Materials and Methods

Constructs and Peptides. The coding sequence of pPT27, a 45-kD His-tagged mouse Hip1 fragment (aa 147–483), was cloned into a pRSETB (Amp) vector (Invitrogen) using EcoRI and expressed in BL21 (DE3) Gold-competent cells (Stratagene) using 1 mM IPTG (Sigma-Aldrich) for 2.5 h. Cells were lysed, and the peptide was purified on Talon metal affinity chromatography resin (CLONTECH Laboratories, Inc.).

pPT209, encoding a 148-aa fragment of mouse Hip1 (aa 482–678) fused to pseudomonas exotoxin A in a pVCH6 (Amp) vector was expressed in BL21 (DE3) pLysS Gold-competent cells (Stratagene). The resulting fusion protein was highly insoluble. Therefore, crude bacterial lysate containing PT209 was electrophoresed directly on a 15% SDS page gel; the band at 85 kD was excised, minced, homogenized in 0.85% sterile saline, and used for injections.

Mice. $Ptch1^{+/-}$ mice were maintained at ~50:50 C57BL/6.DBA/2 (B6.D2) background by continued breeding to C57BL/6.DBA/2 F1 breeders (Jackson Labs) (8). They were fed Purina 5008 laboratory chow and water ad libitum and were housed in plastic cages with metal lids at 50% humidity and 70–74°F with 12 h/d of white light from 34-W fluorescent bulbs. To induce BCC formation, mice were irradiated at 8 wk (56 ± 3 d) with 5 Gy with a cesium-137 radiation device (half-value layer, 0.60 cm Pb, dose rate 0.94 Gy/min; Best Industries) (8).

β-Gal Expression in Extracutaneous Tissues. Tissues harvested from 10 adult B6.D2 $Ptch1^{+/-}$ mice were analyzed microscopically for lacZ expression as an indicator of hedgehog signaling pathway activation (8).

Quantitative RT-PCR. Hip1 expression was quantitated in (a) commercially obtained RNA (Ambion) and (b) extracts of tissues from 5 adult $Ptch1^{+/-}$ B6.D2 mice. TaqMan RT-PCR (Applied Biosystems) was performed as described, comparing the differences in fluorescence (threshold) between tubes with GAPDH amplification and those with the specific Hip mRNA amplification (18).

Immunization. Injections were started at age 11 wk, 3 wk after IR treatment: 30 mice (a) received 10 μg PT27 in 100 μl sterile saline and ImmunEasy adjuvant (QIAGEN), a commercially available mix of CpG-oligodeoxynucleotides (ODNs), prepared freshly before each injection according to the manufacturer's recommendation. Control groups of 30 mice each were injected with (b) 0.85% sterile saline or (c) ImmunEasy adjuvant in 0.85% sterile saline, respectively. A fourth study group of 17 animals was injected s.c. with 2 aliquots of 50 μl of a suspension of SDS-gel slices containing (d) 10 μg PT209 homogenized in sterile saline. Groups a–c mice received injections at 2-wk intervals until age 32 wk; group d mice received injections every 4 wk until age 32 wk. At age 28 wk, blood from the tail vein was assessed for anti-PT27 B cell responses. A 1-cm^2 skin biopsy was taken from the upper back at the age of 32 wk and assessed for BCCs with the viewer "blinded" to the treatment of the mouse from which the skin was taken (19). After the skin biopsies, delayed type hypersensitivity tests (DTH) were performed on all mice, and spleens from 10 mice per group were harvested to assess in vitro T cell responses

ELISA. Costar 96-well plates (Corning Inc.) were coated with 100 μg/ml PT 27 in PBS, pH 7.4. Blocking was performed with 0.1% gelatin in PBS and 0.1% Tween 20 (PBS-T), and 50 μl serum in PBS-T was added starting at a dilution of 1/50. Plates were developed using a phosphatase-coupled anti–mouse antibody (Sigma-Aldrich) and p-Nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read with a 96-well plate reader (Fisher Scientific Thermo Labsystems Multiskan MCC/340) at 405 nm.

Western Blot. Samples of 400 ng PT27 were run on a 15% SDS gel and transferred onto a PVDP membrane (E&K Scientific). The membrane was cut into strips, which then were blocked separately with 5% nonfat dry milk and incubated with serum in blocking buffer at 1:40 dilutions. Blots were developed using a peroxidase-coupled anti–mouse antibody (Sigma-Aldrich) and ECL Chemiluminescence kit (Amersham Biosciences). Control and test sample strips were exposed on the same film.

In Vivo DTH. 10 μl of 0.85% sterile saline were injected into the left ear pinna of each mouse, and 10 μl saline containing PT27, 2 mg/ml, were injected in the pinna of the right ear using 32 G needles. The ear thickness was measured after 48 h using a calliper (Mitoyo); DTH reactivity was expressed as the ratio between swelling of the antigen-injected site and the saline-injected site (20).

ELISPOT. Splenocytes (10^7 cells/ml) from immunized mice and from control mice were used for ELISPOT assays (21). The mean number of spots in unstimulated wells was subtracted from the experimental values of the stimulated cells. Data are means of 10 mice per group. Error bars represent SDs.

Cytokine ELISA. Splenocytes (10^7 cells/ml) from immunized or control mice were stimulated in 24 well plates with 10 μg/ml PT 27 or PBS alone, respectively. Supernatants were collected after 24 h, and capture ELISA was performed in duplicates (22). Data are means of 10 mice per group. Error bars represent SDs.

Data Analysis. Mean, SD, minimum, maximum, median, and interquartile range were used to summarize the data. Because of the small sample sizes, the Wilcoxon Rank Sum Test was used to determine the significance of the differences between groups (23). A box plot was used for visual description of median, range, and interquartile range of the data (24). Statistical Software package SAS was used to perform statistical analysis.

Results

Hip1 Expression in Adult Mice. Expression of hedgehog target genes such as PTCH1, GLI1, and HIP1 is abundant in human BCCs and BCC-like tumors of $Ptch1^{+/-}$ mice. As found previously in $Ptch1^{+/-}$ mice (8), all BCCs stain positive for β-galactosidase, whereas unaffected epidermis remains negative. Assuming that β-galactosidase activity would be detectable in all tissues with strong expression of hedgehog target genes, we autopsied 10 adult $Ptch1^{+/-}$ mice, which had received 5 Gy Cs^{137} ionizing radiation at the age of 2 mo and which, at the time of the biopsy, had multiple macroscopic and numerous microscopic BCCs, and we examined tissue from major organs for β-galactosidase staining. These extracutaneous tissues had no β-gal staining except for occasional very small stromal foci in sections of the bladder, the ovary, and the cecum (Fig. 1). To verify that lack of transcriptional activation of the lacZ gene at the Ptch1 locus correlates with minimal to no expression of Hip1 in extracutaneous tissues, we assayed the level of Hip1 mRNA in extracts of adult $Ptch1^{+/-}$ mouse tissues and in commercially available standard RNA from various $Ptch1^{+/-}$ wild-type mouse tissues. We detected expression of the Hip1 transcript in all tissues examined ex-

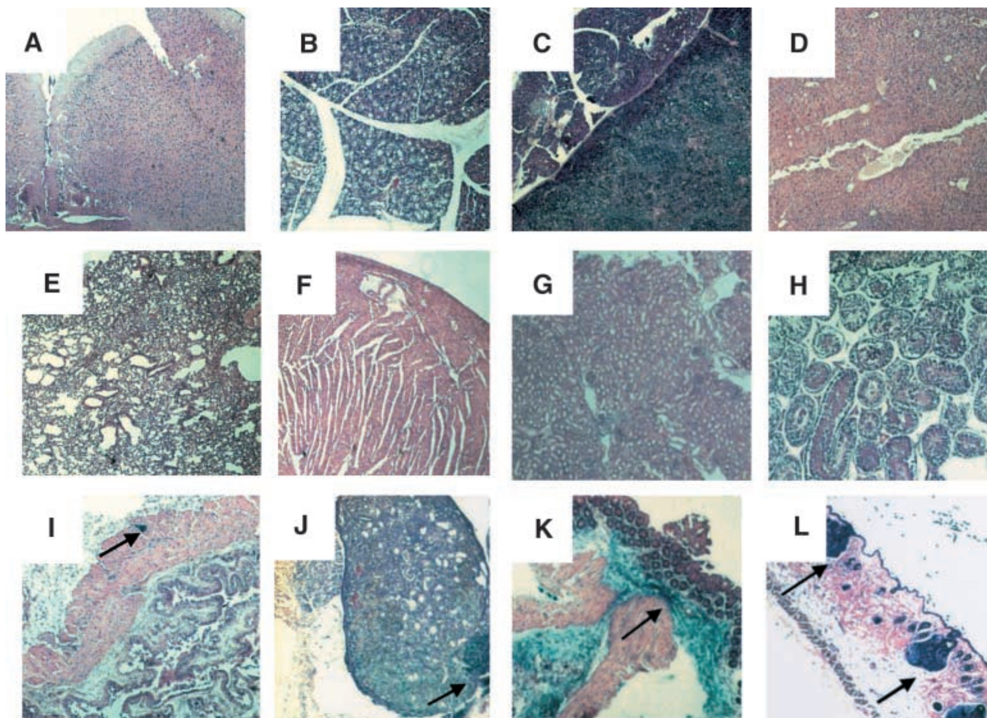

Figure 1. β-Galactosidase staining for hedgehog signaling dysregulation in adult mouse tissues. Histological sections of organs obtained from 10–15-mo-old tumor-bearing $Ptch1^{+/-}$ mice after treatment with 5 Gy ionizing radiation at the age of 2 mo. Sections were stained for β-galactosidase expression. Except for a few stromal foci in cecum, ovary, and bladder of some animals, BCCs were the only adult tissue with strong activation of hedgehog signaling, indicating tumor-specific up-regulation of hedgehog target genes. Arrows indicate positive staining for β-galactosidase. Brain (A), thymus (B), pancreas/spleen (C), liver (D), lung (E), heart (F), kidney (G), bladder (H), testis (I), ovary (J), cecum (K), and skin with microscopic BCCs (L).

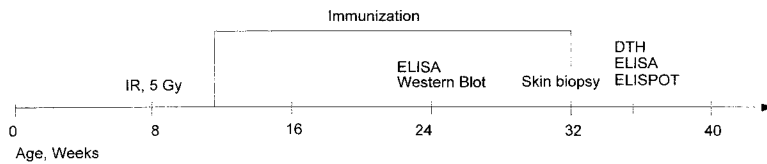

Figure 2. Injection schedule. All mice received 5 Gy ionizing radiation at the age of 8 wk; the injections were started at the age of 11 wk. Mice received 0.85% sterile saline, 0.85% sterile saline, and CpG or the recombinant polypeptide PT27 with CpG in 0.85% sterile saline s.c. every 2 wk. A fourth group of mice received s.c. injections of suspensions of recombinant polypeptide PT209 in SDS-PAGE gel in 0.85% sterile saline every 4 wk. At the age of 24 wk, sera were tested for antibody responses with ELISA and Western blot. 1-cm^2 biopsies were taken at the age of 32 wk. At 36 wk, DTH assays were performed on all study mice. After the DTH assessment, spleens from 10 mice per group were harvested for ELISPOT assays and ELISAs in order to assess peptide-specific T cell responses.

cept heart, but Hip1 mRNA levels were up to 150-fold higher in BCCs than in normal tissue (Table I). Our data suggest that Hip1 is expressed at much higher levels in BCCs than elsewhere in IR-treated adult Ptch1$^{+/-}$ mice. The level of Hip1 mRNA in embryonic tissue was comparable to its levels in some BCCs, which is consistent with the important role of hedgehog signaling in early development (Table I).

Induction of Anti-Hip1 Immune Reactivity. Thus, having identified Hip1 as a tumor-associated antigen in mice and humans we next assessed whether it would be possible to induce immune responses against recombinant fragments of Hip1 and whether the administration of the peptides to Ptch1$^{+/-}$ mice would protect against tumor growth.

Our study included three main groups of mice: those receiving injections with (a) sterile saline, (b) CpG-ODNs, or (c) CpG-ODNs in combination with the recombinant Hip1 fragment PT27 (Fig. 2). A fourth study group received monthly injections of the highly insoluble Hip1-pseudomonas exotoxin A fusion protein PT209 in SDS-PAGE gel without additional adjuvant. Injections were well tolerated by all test animals. We observed no local (at injection sites) or systemic (as judged by changes in behavior or weight gain) toxicity in immunized mice. No change in hair coat was seen. At the age of 32 wk when the skin biopsies were taken, 92% of the saline-injected control group and 89% of the mice receiving CpG-ODNs, PT27, or PT209 were still alive.

Animals immunized with PT27 + CpG developed strong antibody responses to PT27 as detected by ELISA (Fig. 3 A). Consistent with this finding, sera from mice receiving PT27 injections bound strongly to PT27 peptide on Western blots (Fig. 3 C). Mice injected with PT27 + CpG also developed strong cell-mediated immune responses as detected by ELISPOT assay (Fig. 4 A). Spleen cells from immunized mice were assayed for their ability to secrete IFNγ during in vitro stimulation with the polypeptide PT27, which had been used for the immunization.

Table I. *Expression of Hip1 mRNA in Adult Mouse Tissues*

Tissue	ΔCT standard RNA (Ambion)[a]	Adult Ptch1$^{+/-}$ mice (pooled for five adult animals)
Liver	9.6	8.3
Lung	7.1	8.1
Spleen	13.2	10.1
Heart	ND	14.0
Brain	9.3	5.0
Ovary	8.8	8.1
Textix	9.6	8.0
Embryo	5.0	-
Thymus	10.2	9.0
Intestine	-	8.7
BCC-1[b]		1.8
BCC-2		3.4
BCC-3		4.0

[a]mRNA levels were measured by Taqman analysis using primers and probes specific for Hip1 and normalized to the expression level of GAPDH. Values are expressed as ΔCT.
[b]BCC-1, -2, and -3 were individual tumors rather than pooled samples.

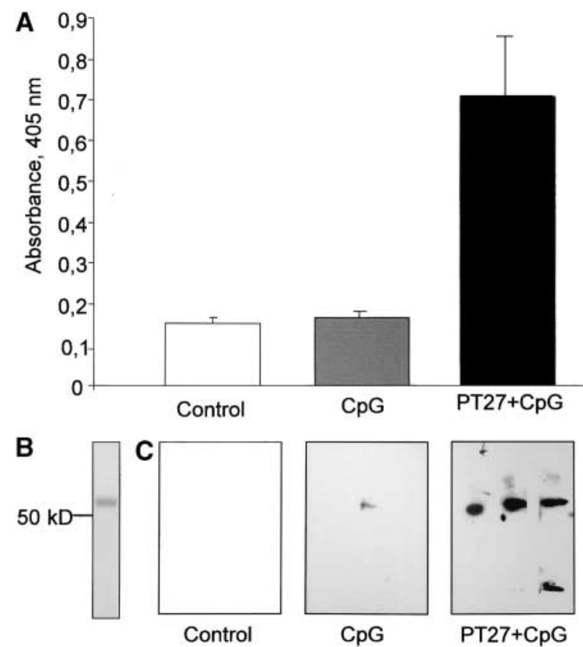

Figure 3. Antibody response to Hip1 immunization. (A) ELISAs were performed on PT27-coated plates using sera from mice receiving sterile saline, CpG in sterile saline, or PT27 + CpG in sterile saline at a dilution of 1:200. (B) Purified PT27 (1 mg/ml) on a 15% SDS polyacrylamide gel. (C) To confirm the specificity of the antibodies, Western blots were performed using sera from mice receiving sterile saline, CpG in sterile saline, or PT27 + CpG in sterile saline at a dilution of 1:40 on purified PT27 (400 ng). Each of the three lanes in the two blots are the assessment of serum from an individual mouse.

Table II. *Descriptive Statistics of the Number, Area, and Burden of Microscopic BCC (1-cm² Skin Biopsies) Per Animal at the Age of 8 Mo*

BCC parameters	Treatments				
	Control	PT209	CpG-ODN	CpG-ODN + PT27	p-value[a] for treatment differences
	(n = 19)	(n = 17)	(n = 22)	(n = 23)	
Lesion number					
Median (range)	11 (5–17)	7 (1–16)	10 (3–18)	5 (1–13)	<0.001
Mean (SD)	10.7 (3.07)	7.29 (4.21)	10.46 (3.53)	6 (3.16)	
Lesion area					
Median (range)	0.0055 (0.003–0.023)	0.0052 (0.0023–0.0112)	0.0066 (0.001–0.0261)	0.0045 (0.0255–0.0263)	0.37
Mean (SD)	0.0087 (0.0056)	0.006 (0.0028)	0.0085 (0.0065)	0.0069 (0.0061)	
Lesion burden					
Median (range)	0.0682 (0.0227–0.02649)	0.0348 (0.0024–0.1788)	0.0572 (0.003–0.3034)	0.0219 (0.0045–0.3423)	0.07
Mean (SD)	0.097 (0.0733)	0.0514 (0.048)	0.0957 (0.0827)	0.0535 (0.0778)	

[a]p-value was calculated by the Wilcoxon Rank Sum Test.

Mice receiving the peptide injections had a threefold increase in spots per well (Fig. 4 A). The findings in the ELISPOT assays were confirmed with capture ELISA of supernatants from splenocyte cultures exposed to PT27 for 24 h. The concentration of IFNγ supernatant in culture media 24 h after restimulation of spleen cells was increased 2.1–2.8-fold compared with the groups that received sterile saline or CpG-ODNs only (Fig. 4 B).

To investigate in vivo cell-based immune responses, we used the DTH skin reaction to detect antigen-specific immunity. 19 of 23 peptide-injected animals developed an erythematous induration at the injection site of the peptide-injected ear pinna. In contrast, no DTH reaction was observed in control mice. The average ear thickness index, the thickness of the peptide-injected ear pinna compared with the water-injected side, was significantly higher in test animals than in the control groups: 1.79 versus 1.07 for saline-injected and 1.04 for CpG-injected.

Inhibition of Tumorigenesis in Immunized Mice (Fig. 5). Table II summarizes the BCC data and the p-values for simultaneous comparisons of all treatment differences. There were significant differences between the four treatment groups in lesion numbers ($P < 0.001$), marginally significant reductions in tumor burden ($P = 0.07$), and no statistically significant differences in BCC area ($P = 0.37$). Table III shows the significance results of pairwise comparisons between groups for BCC number, area, and burden. The Wilcoxon Rank Sum Test confirms that there was no difference in BCCs between mice injected with saline (Table II, Control) and mice injected with the adjuvant (Table II, CpG-ODN) in the BCC number, area, and burden. Neither were there significant differences between BCCs in mice immunized with PT27 versus those immunized with PT209. We further compared the PT27 and PT209 results against those of the controls. Mice injected with CpG-ODN had a median of 10 BCCs and 0.0572 BCC burden. Mice injected with PT27 and CpG + ODN had a median of 5 BCCs and 0.0219 BCC burden. Mice injected with saline control had a median of 11 BCCs and 0.0682 BCC burden. Mice injected with PT209 had a median of 7 BCCs and 0.0348 BCC burden. p-values between CpG-ODN vs. CPG-ODN + PT27 were <0.001 for BCC number, 0.005 for burden, and not sig-

Table III. *Three Pairwise Comparisons for BCC Number, Area, and Burden Using Wilcoxon Rank Sum Test*

Comparisons	BCC	
	Parameters	p-value
Saline vs. CpG-ODN	Number	0.65
	Area	0.99
	Burden	0.87
Saline vs. PT209	Number	0.008
	Area	0.15
	Burden	0.019
CpG-ODN vs. CPG-ODN + PT27	Number	<0.001
	Area	0.24
	Burden	0.005
CPG-ODN + PT27 vs. PT209	Number	0.35
	Area	0.67
	Burden	0.39

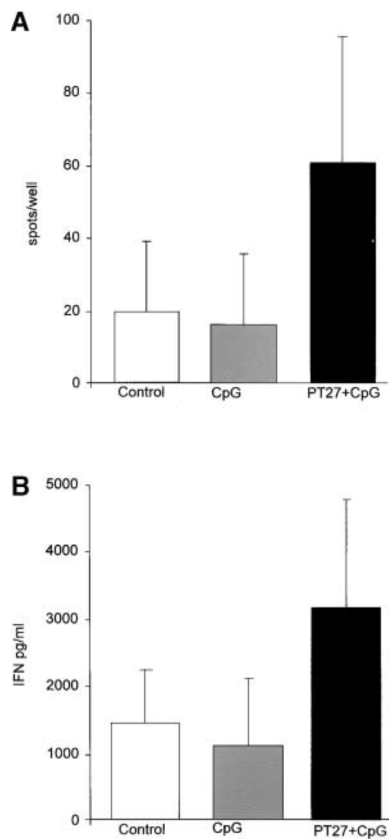

Figure 4. T cell response to Hip1 immunization. (A) T cell responses were assayed in vitro with ELISPOT assays. 5×10^5 isolate spleen cells were pulsed with the recombinant Hip1 fragment PT27. After overnight incubation at 37°C and 5% CO_2, the wells were washed and the number of IFNγ spots revealed as described in Materials and Methods. (B) The data were confirmed by ELISA on culture supernatant of isolate spleen cells stimulated with 10 μg/ml PT27 for 24 h.

nificant for BCC area; between PT209 and saline controls were 0.008 for BCC number, 0.019 for burden, and not significant for BCC area.

Discussion

The identification of the mutations causing BCNS allows the early identification of affected individuals. In a study of 90 Caucasian BCNS patients, 80% had at least one BCC, and the number of BCCs ranged from 1 to >1,000 (25). Their continued accumulation of BCCs makes assessment of preventive agents highly feasible and clinically important. Vaccination with a tumor antigen could be an ideal method to reduce or even prevent tumor formation in these individuals. The recently published reduction of the incidence of HPV-16 infection and of HPV-16–related cervical intraepithelial neoplasia in a study of 2,392 young women receiving HPV-16 virus-like particle vaccine demonstrates the great potential of antitumor vaccines, although the target in this study was an infectious agent which causes tumors rather than a TAA (26). Direct vaccination with tumor-associated antigens, as performed in our study, extends this traditional concept of vaccination to endogenously forming tumors.

BCCs are comparatively homogenous tumors. Genetic instability of BCCs appears to be much less than that of most other tumors, and metastasis is rare. Thus, one study found aneuploidy in 19% of 509 BCCs compared with 75% in other solid tumors (27). Allelic deletions on chromosome 9 at the patched gene locus are characteristic for BCCs, but deletions are uncommon at other chromosomal sites. Accordingly, Quinn et al. found loss of heterozygosity of one or more 9q markers in 33 of 44 BCCs (28). In animal models, mutational activation of hedgehog signaling is sufficient to induce BCC-like lesions, and mutations in PTCH1 and/or SMO, both components of the hedgehog signaling cascade, have been found in >90% of human BCCs (29–31). This characteristic defect of BCCs led us to investigate further the expression of the hedgehog target gene Hip1 as a possible candidate for future immunization studies.

In our study of various tissues of $Ptch1^{+/-}$ mice, quantitative real-time RT-PCR identified basal expression of Hip1 mRNA in all tissues examined except heart tissue. These results are in accordance with reports by Bak et al. who found expression of human Hip1 in fetal and adult human tissues except fetal ovary (32). Nonetheless, with up to 150-fold higher mRNA levels in BCCs than in normal skin and other organs, Hip1 appears to be up-regulated in adult mice specifically in BCCs and therefore may be a suitable target for vaccination strategies. Most identified human tumor-associated antigens (TAAs) are "self-antigens" with some expression in normal adult tissue. Carcinoembryonic antigen, for example, is a TAA which is overexpressed in various carcinomas including gastrointestinal carcinomas but also is expressed in normal colonic mucosa (33). Similarly tyrosinase-related protein 2 (34) and the HER-2/neu (35) antigen are expressed not only on cancerous cells but also on normal tissue, and in some cases successful rejection of established tumors could be achieved only in the presence of severe autoimmune responses (36). However antitumor responses against other normal differentiation antigens can be generated with little evidence for destructive autoimmunity (37–39). Consistent with these latter reports, injections of Hip1 polypeptides were well tolerated in our study and yet induced peptide-specific immune responses. Although expression of hedgehog target genes has been reported in the bulge region of the adult hair follicle in humans and in mice and although involvement of hedgehog signaling in the regulation of the adult hair cycle has been proposed (40–42), no gross change in hair was seen in immunized $Ptch1^{+/-}$ mice. Because hedgehog signaling is important in embryonic development, we assumed that the antigenicity of Hip1 would be low and that immunization against Hip1 would require the coadministration of adjuvants. In rodents and humans, CpG-ODNs stimulate the expression of Th1-like cytokines and of costimulatory molecules and lead to a general increase in antigen-presenting function (43). If used as adjuvant, CpG-ODNs give potent antibody and CTL responses. In a recently published paper,

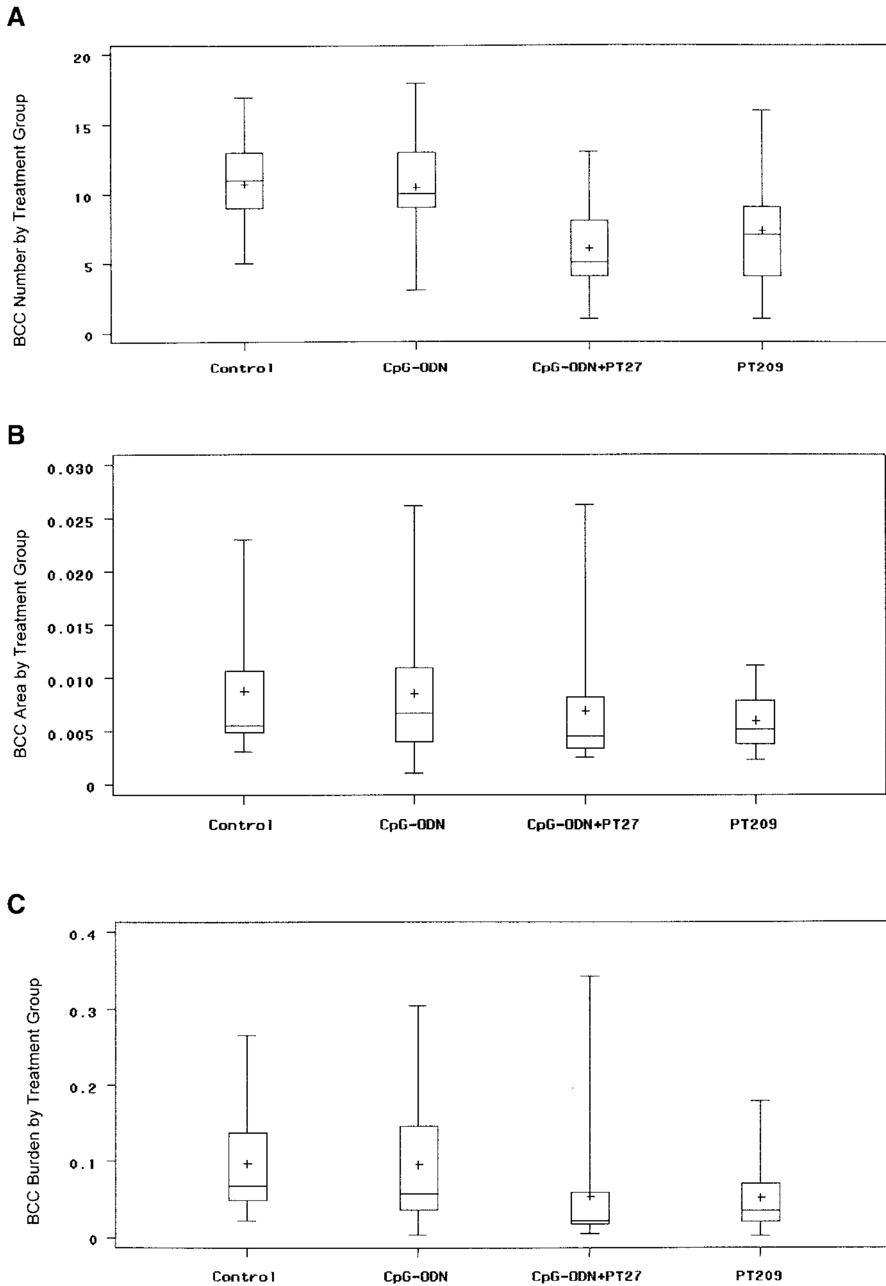

Figure 5. At the age of 32 wk, 1-cm^2 skin biopsies were taken from each animal. The median number of microscopic BCC per animal (A), the median cross-sectional area of tumors in each sample (B), and the median total tumor burden per animal (C) were determined microscopically. Each rectangular box indicates interquartile range (25% and 75% percentiles) with median inside the box and average as shown with +. The highest and lowest bars are the maximum and minimum values. Statistical analysis using the Wilcoxon Rank Sum Test showed a significant reduction of tumor number in mice immunized with CpG-ODN + PT27 and in mice immunized with the fusion protein PT209 against control groups (A); a significant reduction of tumor burden in mice with PT27 + CpG and in mice with PT209 against controls (C). There were no significant differences of BCC number, area, and burden either between Control and CpG-ODN or between CpG-ODN + PT27 and PT209.

Kim et al. demonstrated that immunization of C57BL/6 mice with the HPV oncogenic proteins E6 and E7 in combination with CpG-ODNs significantly reduced growth of HPV-driven tumor cells not only when administered before but even after murine tumors had developed (44). In some animal models, even monotherapy with peritumoral CpG-ODN injections enhanced antitumor immunity with rejection of established tumors at the injection site and at distant sites (45). CpG-ODNs are not immunogenic and unlike Freund's adjuvant do not cause granulomatous reactions. Therefore, they can be injected repetitively without severe side effects (46, 47). In our study, the repetitive administration of PT27 in combination with CpG-ODNs was sufficient to overcome the self-tolerance expected due to high-level expression of Hip1 during development and low-level expression in adult tissues.

We found strong PT27-specific antibody responses in PT27-injected mice, and this peptide also induces anti-Hip1 antibodies in rabbits (unpublished data). The exact localization of Hip1 within the cells remains unclear, and its immunogenic properties so far have not been investigated in detail. Yet, as a putative transmembrane protein, Hip1 might well be accessible to antibody binding. In other cancer models antitumor antibodies have been shown to mediate anti-tumor effects effectively. Antitumor activities of the recently approved therapeutic antibodies Trastuzumab (Herceptin), specific for the protooncogene p185HER-2/neu in breast cancer, and Rituximab (Rituxan), a monoclonal

IgG1 specific for the B cell marker CD20, prove that antitumor antibodies can be a powerful tool in cancer therapy.

Spleen cells from PT27-injected Ptch1$^{+/-}$ mice produced significantly more IFNγ in response to in vitro stimulation with the recombinant Hip1 peptide PT27 than did spleen cells from control mice receiving saline or CpG injections. Moreover, 19 out of 23 peptide-injected mice developed DTH. Detailed immunology-focused studies will help to elucidate further the immunological properties of Hip1 and to optimise future immunization strategies. Central to our study is the more general observation that Hip1 injections were well tolerated and induced detectable B cell and T cell responses and that these findings may be of clinical relevance because Hip1-injected mice developed significantly fewer microscopic tumors than did control mice.

This is to our knowledge the first attempt to immunize against BCCs, and therefore we consider the partial reduction of microscopic tumors with two different peptides to be a very encouraging result. For BCNS patients and others developing many BCCs, even a partial reduction of tumors would substantially improve their quality of life.

In summary, we have defined Hip1 as a tumor-associated protein and presented evidence that immunization against BCCs with recombinant fragments of Hip1 induced immune responses despite the "self-nature" of this protein. Moreover, injection of two different Hip1 polypeptides in two different test groups led to a significant reduction of microscopic tumors in the peptide-injected mice compared with saline- and adjuvant-injected animals. Based on these data, we believe that Hip1 is a suitable molecule for further studies in this field. Ptch1$^{+/-}$ mice present a valuable model for the investigation of therapies against BCCs because the mice develop endogenous BCCs, closely mimicking the course of disease in high-risk individuals such as BCNS patients. Future studies will focus on the immunology of Hip1 and other proteins which are up-regulated by hedgehog signaling and on why some tumors appear to have escaped the immune response.

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