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EDITORIAL

STRATEGIES FOR SUCCESSFUL VACCINATION AGAINST HEPATOCELLULAR CARCINOMA

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Current therapies against hepatocellular carcinoma (HCC) are not curative in the majority of patients. In the past, immunotherapy approaches aimed to non-specifically stimulate immune response were quite ineffective. New treatments based on stimulation of specific anti-tumor immune response are currently proposed and appear more promising. Tumor-specific antigens identified in HCC demonstrated immunogenicity both in preclinical and clinical trials. Effectiveness in animal studies raised interest in the clinical applicability of non-specific adoptive immunotherapy that prevented disease recurrence after tumor resection. Dendritic cell (DC)-based tumor vaccines achieved encouraging results, and cellular vaccines based on DCs have already entered clinical trials. Preventive and therapeutic DNA vaccination have been proposed, all based on tumor-associated antigens (TAAs), either modified or not, an example being alpha-fetoprotein (AFP). The concomitant expression of co-stimulatory molecules and cytokines was used to increase tumor immunogenicity. Syngeneic or nude mice models indicated that immunotherapy for HCC could stimulate an anti-tumor T-cell response leading to clinical benefit devoid of significant toxicity. The use of DNA-based vaccination raises exciting possibilities in preventing HCC in high-risk individuals such as those with cirrhosis. Novel immunotherapy strategies may contribute in the future to prevention and treatment of HCC.

Worldwide, hepatocellular carcinoma (HCC) ranks fifth among solid tumors and third as a cause of cancer-related death (1). Non-surgical therapies, e.g. systemic or regional chemotherapy, failed to cure the disease (2). Published studies of systemic chemotherapy report a response rate of 25% at best, whilst overall survival never improved in any subset of HCC patients (3). Surgical resection helped a minority of subjects, those without advanced disease at diagnosis. Recurrent rates reached 50% at 2 years after surgery (4). Immune mechanisms appear important in the control of HCC, since several cases of spontaneous tumor regression have been reported (5). HCCs offer an attractive target for immunotherapy because the malignancy is densely infiltrated with lymphocytes, and patients with high levels of tumor infiltrating lymphocytes (TIL) fared better after tumor resection (6). TIL isolated and expanded *in vitro* were shown to be cytotoxic to autologous HCC (7). Furthermore, active specific immunotherapy of cytotoxic T-lymphocyte (CTL) vaccine seems suitable for HCC since, as a rule, hepatoma cells strongly express HLA class I antigens; their loss allows human tumors to escape

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immune surveillance (8).

The limitation of treatment modalities for HCC stimulated interest for new ones, such as immunotherapy. Promising strategies developed in the laboratory have been applied in the clinical setting. In the past, immunotherapy based on tumor cells and BCG (Bacillus Calmette Guerin) was investigated and achieved non-specific immune response in the local environment of the tumor. Temporal efficacy of the immune response was however unpredictable, and overall quite ineffective (9). To increase immunogenicity of HCC, immunomodulatory genes or ligands were delivered to tumor cells, but success was dismal (10).

Several works investigated antigen specific immunotherapy against potential tumor-associated antigens (TAAs) in HCC, including alpha-fetoprotein (AFP) (10-11), carcinoembryonic antigen (CEA) (12), glypican-3 (GPC3) (13), Wilm's tumor 1 gene (WT1) (14), cancer testis antigen NY-ESO-1 (15) and melanoma-associated antigens (MAGE) (16). In particular, AFP is an oncofetal protein whose expression level is increased in many primary liver tumors; as a result, elevated serum levels of AFP may serve as a liver tumor marker (11). MAGE is also frequently expressed in HCCs, and associated with expansion of specific CD8+ cells observed in HCC patients (16).

In animal models tolerance to HCC associated antigens can be overcome allowing to prevent growth of implanted tumors, using a number of different techniques. The most promising technique is based on the use of dendritic cells (DCs), which are the most potent professional antigen presenting cells (APCs) and are able to activate naïve T cells, thus driving primary immune response. Immature DCs are poised to capture antigen, then migrate to draining lymphoid organs while undergoing maturation, ultimately driving antigen specific immunity and immune responses. When DCs are loaded with tumor antigens, they can stimulate a specific and durable anti-tumor response.

Targets and immunotherapeutic strategies for HCC

AFP may be used as target for active immunotherapy of HCC (10). In mice, prophylactic DNA vaccination with AFP-expressing plasmid vector induced syngeneic tumor rejection, even though specific CTLs could not be detected *in vitro* (17). Two genetic immunization strategies, i.e. genetically engineered DCs and plasmid DNA, also generated AFP-specific T-cell responses; both modalities induced AFP-specific immunity, as demonstrated by *in vivo* protection against AFP-producing murine tumors and by stimulation of AFP-specific CTLs (10). The murine and human T cell repertoires can recognize AFP-derived peptide epitopes in the context of MHC class I. A complete analysis of approximately 70 AFP-derived peptides was performed by Butterfield et al.; at least four different HLA-A2 restricted immunodominant epitopes were naturally processed and presented in the context of class I, were immunogenic, thereby representing potential targets for HCC immunotherapy (18). Among HLA-matched patients bearing AFP-positive HCCs, 100% developed specific T-cell responses when immunized with three of the four AFP peptides; this finding underscores that tolerance to HCC TAAs can be overcome (19). This was supported by i) the recent identification of AFP-derived CD4+ T-cell epitopes recognized in association with HLA-DR (11) and ii) the unmasking of AFP-specific CD4+ T-cell response in HCC patients undergoing arterial tumor embolization. Taken together these observations provide a rationale for combining conventional cancer treatment with immunotherapy in HCC patients (11). Notably, responses to AFP subdominant or cryptic epitopes might be more clinically relevant in HCC patients due to the reduced effects of central and peripheral tolerance on T cells (20). The advantage of inducing an immune response to AFP is that up to 80% of HCC reactivate AFP expression, and the majority of HCCs overexpress this oncofetal antigen (11). Nevertheless, immunotherapy based on monovalent antigen vaccination may prompt clonal expansion of antigen-negative variant tumor cells, thus limiting the durability of any response.

To overcome this event a number of approaches have been undertaken to generate a polyvalent vaccine (Fig. I). DCs fused with syngeneic hepatoma cells were effective in preventing growth of subcutaneously implanted HCCs and inhibited local recurrences after tumor resection in rats (21). Analogous results were obtained in mice using an hybrid vaccine of DCs and hepatocarcinoma

Table I. *Representative anti-HCC strategies tested in animal models.*

APPROACH	IMMUNE ELEMENTS	RESULTS	REFERENCE
Increasing tumor immunogenicity by targeting to tumor	Cytokines or chemokines	antitumor immunity	(10)
		complete tumor metastases regression	(30)
		median survival period doubled *vs* control group	(31)
	Co-stimulatory molecules	median survival period doubled *vs* control group	(31)
Immunization with tumor cells fused with antigen-presenting cells		specific CTL activity; tumor growth inhibition; regression of pre-established tumor	(21)
Adoptive transfer of cells	NK	growth inhibition of tumor metastases	(32)
Targeting AFP-expressing HCC cells by	Plasmid DNA	effective AFP-specific T cell responses; antitumor effect on AFP-producing tumors	(28)
	Adenovirus	regression of pre-established tumor	(33)
	Peptides	T-cells AFP-specific	(18)
DCs strategies		potent T-cell responses; protective immunity	(10)
		life span prolongation; tumor growth suppression; enhanced NK and $CD8^+$ functions; augmentation of IFN-γ production	(23)
Cell death strategies	Tissue inhibitors of metalloproteinase	Tumor growth delay	(34)
	Prodrug therapy	tumor growth inhibition; life span prolongation	(35)
	TRAIL induced apoptosis	growth inhibition of tumor metastasis	(32)

CTL: cytotoxic T lymphocyte; NK: natural killer cells; AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma; DC: dendritic cell; IFN-γ: interferon-γ; TRAIL: TNF(tumor necrosis factor)-related apoptosis-inducing ligand

cells (22). Other strategies aimed at generating APCs capable of presenting multiple TAAs both via class I and II pathways, e.g. DCs pulsed with tumor lysate (23). The safety and feasibility of this approach were evaluated in HCC patients and shown devoid of toxicity (24). However, clinical efficacy of tumor lysate is hampered by the risk of autoimmunity against 'housekeeping' proteins. Total RNA amplified from microdissected tumor cells was used to load DCs with multiple TAAs (25). TAAs introduced as mRNA by electroporation were processed and presented as peptides on lymphocyte surface, analogously to CEA (12). Induction of AFP-specific CD4+ and CD8+ T cell response has been reported following mRNA transfection of DCs generated from HCC patients (26).

Overview of HCC vaccination strategies

The need for novel effective treatments for HCC makes immunotherapy for HCC attractive; the exquisite specificity of the immune response is indeed an added benefit, and side effects are not expected. An HCC-specific response was activated by strategies using tumor-associated self-antigens. A recent study employed cells from HCC patients, in which DCs transfected with genes coding for AFP and interleukin-18 (IL-18) induced AFP specific CD4+ and CD8+ T cells (27). Gene array and proteomics studies have added to the list of HCC-specific gene products that can be targeted (24). Vectors bearing genes that render HCC immunogenic were employed in animal models. Molecular chaperones, such as heat-shock protein 70 (HSP70),

Table II. *Representative anti-HCC strategies tested in human clinical trials.*

APPROACH	IMMUNE ELEMENTS[1]	RESPONSES[2]	REFERENCE
Enhancing tumor immunogenicity by targeting to tumor	IFN-γ + IL-2	14/20 tumor size decrease	(10,24)
	IFN-γ + GM-CSF	No clinical responses	(10,24)
	IL-12	6/8 SD, 1/8 PR	(24)
Adoptive cell therapy	LAK + chemotherapy	Improved recurrence rate	(10,24)
		No differences in outcome	(10,24)
	CIK / TIL	Lowered recurrence rate	(39)
		Reduced recurrence rate	(10,24)
		Lowered recurrence rate, improved recurrence-free outcomes, time to recurrence	(10,24)
		Improved immunological status in HCC patients	(24)
DC-based immunotherapy	DCs + Ad-IL-12	2/8 disease stabilization; 5/8 augmented TIL percentage	(24,40)
	Tumor lysate-pulsed DCs	1/2 slowed tumor growth	(10,24)
		1/10 MR; 7/10 KLH DTH+	(10,24)
		No PR or CR	(10,24)
		4/31 PR; 17/31 SD; improved 1 year survival	(24)
AFP-based immunotherapy strategies	AFP-derived peptides in Montanide adjuvant	No PR or CR; AFP-specific T cells in HCC patients	(19)
	AFP peptides pulsed-DCs	No PR or CR; increased levels of AFP-specific T cell	(41)
Autologous patient-derived DCs/HCC cells fusion		Induction of antigen-specific CTL and generation of Treg by DCs/HCC cells fusions	(42)
Autologous formalin-fixed tumor vaccine		Reduced risk of recurrence, time to recurrence, improved recurrence-free survival	(24)
Enzyme/prodrug therapy	Prodrug-activating enzymes	Detectable transgene expression in the tumor	(40)

[1]IFN-γ: interferon-gamma; IL: interleukin; GM-CSF: granulocyte macrophage colony stimulating factor; LAK: lymphokine-activated killer cells; CIK: cytokine-induced killer; TIL: tumor infiltrating lymphocytes; DC: dendritic cell; Ad: adenovirus; AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma

[2]PR: partial response; SD: stable disease; CR: complete response; MR: mixed response; KLH: keyhole limpet hemocyanin; DTH: delayed-type hypersensitivity

combined with other antigens can strongly prime immunogenicity presumably through improved processing and presenting of antigens to their APCs. A DNA vaccine encoding AFP and HSP70 could generate antitumor immunity. Such effective AFP-specific T-cells response and definite antitumor effects on AFP-producing tumors prompted clinical testing of this approach as a therapeutic vaccine for HCC (28). Induction of partial tumor regression was observed in mice treated with a AFP-specific DNA-based combinatorial therapy (10). Very recently, Lan and colleagues reported anti-HCC effects in a

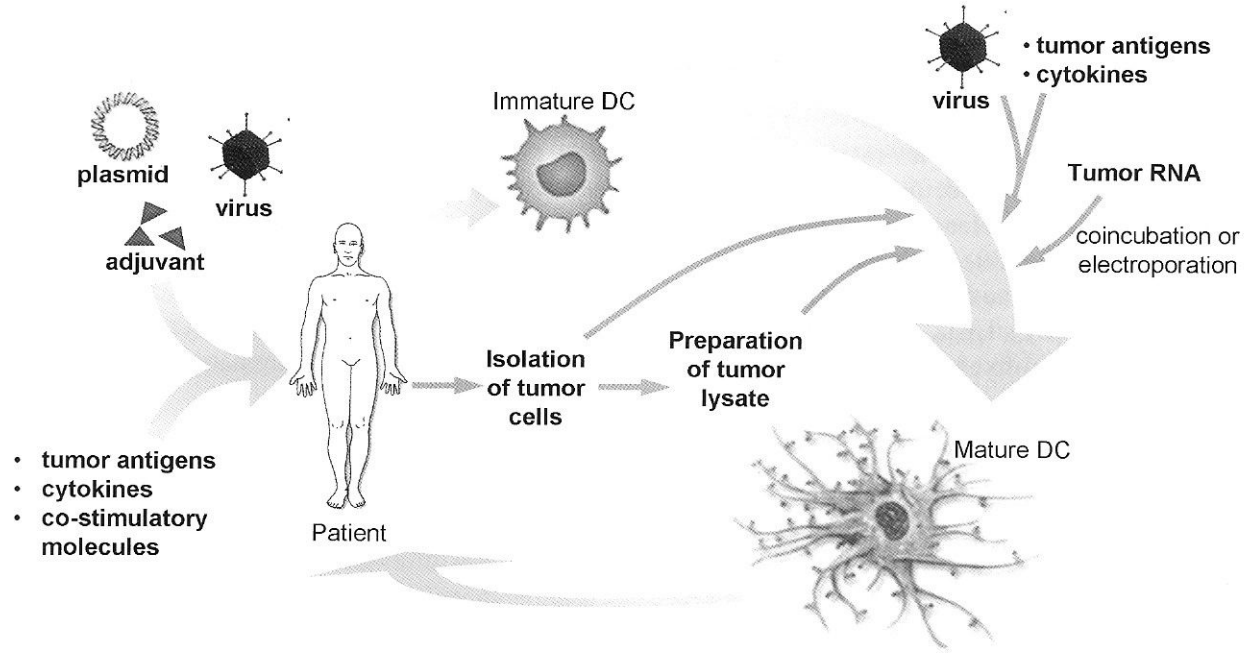

Fig. 1. *Representative anti-HCC immunotherapeutic strategies to generate mono/polyvalent vaccines. Activation of an HCC-specific response can be accomplished by DC-based vaccines using ex vivo loaded DCs (right side) or by peptide/DNA-based vaccines (left side).*

therapeutic setting with a DNA vaccine encoding mouse AFP plus HSP70 genes; this vaccination strategy may therefore contribute to effective treatments for human HCC (28). A combined DNA vaccine encoding a fusion protein of AFP and CTLA-4 antigen (that binds co-stimulatory B7-1 and B7-2 molecules) obtained both potent specific CTL response and antitumor effect on AFP-producing tumor in mice, without impairing hepatic and renal functions (29).

Also uncharacterized and mutated antigens can be targeted with whole tumor cell or tumor lysate-based immunization strategies, as well as using vectors bearing genes making tumor cells immunogenic; the immune system in these instances shall evolve specificity against these new immunogenic target antigens.

Table I summarizes representative strategies investigated in animal models: (a) to increase tumor immunogenicity by targeting cytokines (IL-2, IL-4, IL-6, IL-7, IL-12, IFN-γ, TNF, GM-CSF, CD40L, Flt3L) or co-stimulatory molecules (B7-1) towards HCC (10, 30-31); (b) to immunize with tumor cells fused with antigen-presenting cells (21); (c) to adoptively transfer cells (32); and (d) to target AFP-expressing HCC cells by several different vectors, including plasmid DNA (28), adenovirus (33), peptide (18) or DCs strategies (10, 23). Several strategies aimed at selectively destroying cancer cells by other different mechanisms were also investigated (32, 34-35).

DNA vaccines represent an especially promising methodology against allergens, pathogens and cancer. Indeed, DNA-based immunization induced strong cellular and humoral immune response against a variety of antigens, including tumor-derived ones (melanoma, ovarian carcinoma, breast, neuroblastoma, prostate carcinoma, small-cell lung cancer, etc.). Gene-based vaccines were evaluated either as prophylactic or therapeutic treatment for infectious diseases, allergies and cancer including HCC [reviewed in (36)]. Our contribution documented feasibility and safety of naked DNA vaccination (37-38).

Human clinical trials by HCC vaccines

Success of animal studies led to the clinical application of HCC immunotherapy. Table II shows strategies tested in human clinical trials to enhance anti-tumor immune responses. Worthy of mention are non-specific immunostimulants (10, 24), adoptive transfer of autologous activated lymphocytes (10, 24, 39), trials using DCs (10, 24, 40), AFP-based immunotherapy (19, 41), autologous patient-derived DCs/HCC cells fusion (42), autologous formalin-fixed tumor vaccine (24) and gene-directed enzyme/pro-drug approaches (40). These phase I/II clinical trials, testing novel immune-based interventions in HCC subjects, highlighted effective immunologic responses and positively affected recurrence and survival rates in human patients (24). Even when only mild antitumor effects were achieved, almost all immunotherapy trials for HCC were feasible and well-tolerated. At present, immunotherapeutic approaches hold promise to prolong survival in patients with advanced HCC who respond by enhanced tumor immunogenicity. The boosted immunological function in HCC patients can play an important role in reducing the recurrence rate of HCC, since the efficacy of several treatments appears to be limited by the activation of immunosuppressive mechanisms (43). Further studies are aimed at ameliorating clinical efficacy. Gene therapy approaches of human liver cancer were unsatisfactory, especially in advanced cases, despite favourable immune-gene therapy results in animal models, that appeared to prevent tumor formation and/or reduce distant metastases (40). Furthermore, combination of different targeted therapies might attain success in the control of primary liver malignancy. Whilst DNA-based vaccine might enhance immune response in HCC patients, optimal results are still to be obtained. New, more efficient and specific anti-tumor vaccination strategies require more and thorough investigations. Preclinical studies identified already antigen-specific effector cells with anti-tumor activity (10). AFP-specific T cells have been demonstrated in patients with HCC superimposed on chronic liver diseases (24, 44). Thus, prophylactic AFP-specific vaccination might prevent growth of AFP-positive malignancy in cirrhotic patients. Effector cells against a tumor-associated antigen were activated following DNA vaccination combined with intra-tumoral chemokine and cytokine expression. Such therapy obtained tumor regression in all the animals; complete regression was achieved in 25% of mice that survived long-term. Arguably, combined stimulation of different immunological mechanisms may be crucial for an efficient anti-tumor effect (45). AFP DNA vaccination combined with chemokine/cytokine expression warrants evaluation in clinical trials, particularly in patients at high risk of developing HCC, or to eliminate minimal residual disease after cancer resection.

CONCLUSION

Active immunotherapy is still in preclinical or in early phases of clinical trials. Successful anti-HCC immunotherapy relies on expression of modified or unmodified TAAs that are abnormally expressed or masked in the tumor. Several strategies were assessed to activate immune response, to overcome tolerance, and to avoid anergy. A combination of antigen-specific DNA vaccination with intratumoral pro-inflammatory cytokine/chemokine expression and accessory/co-stimulatory signals holds the promise for improvements of HCC immunotherapy.

The growing knowledge in vector engineering, genomics and proteomics will provide further innovations in immunotherapy. Thus, more efficient tools will be available for both tumor prevention and active immunotherapy of HCC patients. Impact might be especially valuable in countries with high prevalence of viral hepatitis that largely contributes to HCC development.

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