Recent advances in immunotherapy for hepatocellular cancer

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Summary

There is a continuing need for innovative, alternative therapies for hepatocellular carcinoma (HCC). Immunotherapy of cancer is attractive because of the exquisite specificity of the immune response. Activation of an HCC-specific response can be accomplished by strategies targeting tumour-associated antigens (for example: alpha fetoprotein (AFP)) or viral antigens in those patients infected with hepatitis B or C. Uncharacterised and mutated antigens can also be targeted with whole tumour cell or tumour lysate-based immunisation strategies. Viral vectors coding for genes which make the patient's tumour immunogenic can allow the immune system to naturally evolve specificity against immunogenic target antigens. Strategies which have been tested in human clinical trials include adoptive transfer of lymphocytes, cytokine injections, autologous tumour-pulsed dendritic cells (DC) as well as AFP-derived peptides in adjuvant and pulsed onto autologous DC. These trials, testing novel immune-based interventions in HCC subjects, have resulted in immunological responses and some have impacted recurrence and survival of HCC subjects.

Key words: tumour antigen; dendritic cell; adoptive transfer; T cell; cytokine

Introduction

There is a pressing need for novel strategies to impact development and recurrence of hepatocellular carcinoma (HCC). The goal of immune-based therapies is to harness the sensitivity, specificity and self-regulation of the immune system to eradicate any and all tumour cells.

To activate an immune response, the immune system must first detect the target cell. CD8+ effector T cells (the subset of lymphocytes thought to be most critical for antitumour imunity) identify targets by detecting the 8–11 amino acid peptide for which their T cell receptor is specific. Proteins in the cytoplasm of cells are processed into small peptides by the proteosome complex, which are transported by "transporter associated with antigen processing" (TAP) proteins into the endoplasmic reticulum where they can associate with MHC class I molecules and be transported to the cell surface (figure 1). It is there that these peptides, presented in the context of self major histocompatibility complex (MHC), are seen by CD8+ T cells. Exogenous proteins taken up by cells can
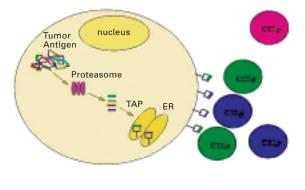
Abbreviations

HCC	hepatocellular carcinoma	PEG	polyethylene glycol
AFP	alpha fetoprotein	TNFα	tumour necrosis factor alpha
MHC	major histocompatibility complex	LAK	lymphocyte activated killer cells
HBV	hepatitis B virus	TIL	tumour infiltrating lymphocytes
HCV	hepatitis C virus	PBL	peripheral blood lymphocytes
APC	antigen presenting cell	IFN γ	interferon gamma
DC	dendritic cells	IL	interleukin
TAP	transporter associated with antigen processing	GM CSF	granulocyte macrophage colony stimulating factor
DTH	delayed type hypersensitivity	KLH	keyhole limpet haemocyanin

also be presented to CD8+ effector cells, by professional antigen presenting cells, like DC.

Many specific proteins have been identified with differential expression in HCC cells, and many strategies are also directed towards uncharacterised tumour-specific proteins. This review will describe potential targets for immunotherapy, the wide variety of strategies being employed in *in vitro* studies to activate effective tumour-specific immune responses, as well as summarise results from clinical trials testing immunotherapy in subjects with HCC.

New HCC targets

Serum alpha fetoprotein (AFP) is a diagnostic marker for the presence of HCC and an obvious choice of a protein target made specifically by HCC cells (table 1). AFP is the major serum protein expressed by the foetus, in which serum levels are up to 3 mg/ml at 10–13 weeks of development, are 30–100 µg/ml at birth, and are 1–3 ng/ml in normal adults [1]. Importantly, 50% to 80% of HCC reactivate AFP expression, secreting up to 1 mg/ml in serum. Targeting AFP is not a new idea, it was intensely studied in the 1970's and 1980's as a target for antibody-based therapies, but these efforts were largely unsuccessful. Even though AFP is secreted, its peptides are processed by the cell and can be presented to both CD8+ and CD4+ T cells. Because AFP is a normal "self" antigen, there is concern that anti-tumour immunity could evolve into autoimmunity. AFP can be transiently reactivated during liver regeneration (from necrosis or hepatitis [2, 3]) therefore AFP-based strategies should track potential toxicity. In our pre-clinical work using murine AFP-based immunisation for murine HCC, pathological examination did not reveal toxicity [4].

Antigens originally characterised by their melanoma tumour-specific expression have also been found to be expressed in HCC, for example, MAGE-A family genes and NY-ESO, members of tumour-specific "cancer-testes" gene families. These genes are on the X chromosome and are up-regulated in tumours by modulation of promoter methylation. Table 1 shows the reported percentage of tumours which express one of the members of this gene family [5–12]. Despite being "self" antigens, the cancer-testes antigens have been demonstrated to be immunogenic in melanoma patients and are being pursued as targets in HCC. Other normal proteins whose expression is increased in a majority of HCC tumours includes Aurora-A [13] and Glypican-3 [14].

Hepatitis viral antigens are other potential targets for T cell responses. They have the additional benefit of being "foreign" proteins, not seen as "self" by the immune system. Viral genes from hepatitis B virus (HBV) and hepatitis C virus (HCV), like the HBx protein (which is believed to modulate p53 expression), are potential targets. When considering viral targets, it is important to consider directing the immune system to infected but non-malignant cells. Importantly, immune responses targeting viral gene products have been implicated in the pathogenesis of chronic liver cell injury that which precedes HCC.

There are also uncharacterised tumour-specific antigens, some of which are "private" or only expressed by a single patient's tumour, compared to "public" or "shared" antigens which are more applicable to generalisable immunisation strategies.

Table 1

Gene Expression Changes in HCC.

Change	Gene Product (% of HCC tested)
Increased	RARa, p53 (22–46%*), tetraspanin CO-029 (26%), cyclin D1, TGFβ, TGFα, IGFII, N-ras, c-myc, c-fos, survivin, MAGE-A family (86%), AFP (50–80%), Aurora-A (61%), Glypican-3 (84%), NY-ESO (24%)
Decreased	CCR5, IFNγ, IGFBPs, tetraspanins CD9 and CD82
Mutated	p53 (30–50%), pRB (20–25%), β-catenin
Viral	HBV and HCV gene expression (HBV X, HBV preS1/2; HCV E2 glycoprotein, NS5B)

Abbreviations: RARa, retinoic acid receptor; TGF, transforming growth factor; IGF, insulin growth factor; CCR, C-C family chemokine receptor; IFN, interferon; IGFBP, insulin growth factor binding protein; pRB, retinoblastoma protein
*when many HCC tumours were tested, the %positive tumours is shown in parentheses

Strategies

There are many strategies commonly employed to stimulate antitumour immunity; making the tumour itself more immunogenic, altering the immunosuppressive tumour microenvironment, generalised immune activation as well as antigen-specific immune activation. Despite the wide vari-

ety of genetic changes and gene regulation changes found in HCC cells, these tumours are not immunogenic. A central issue is that HCC cells (like most tumours) do not appear to activate the immune system – they may express MHC class I, presenting peptides derived from proteins expressed internally, but they do not generally express MHC class II to activate helper cells, nor do they express other adhesion and costimulatory molecules to give strong positive signals to effector cells (figure 2A). Antigen presenting cells (APC), like B cells, macrophages and especially dendritic cells (DC) do express these molecules and are specifically designed to stimulate immunity (figure 2B). Immunotherapy strategies must activate an effective antitumour response by enabling cells of the immune system to detect tumours and respond.

In vitro studies

A simple method of increasing immunogenicity of tumours is to create stress. Heat-shock or hyperthermia treatment of cells enhances the expression of heat shock proteins which have been shown to be chaperones of proteins and peptides and an immunogenic delivery method for tumour-derived antigens. Heat shock creates a stress and/or immunological "danger" environment. Tumour lysate from heat-shocked HepG2 cells has been shown to be more immunogenic when pulsed onto APC than lysate from non-heat shocked HepG2 cells [15].

DC have been the subject of extremely active research for the last decade. These bone-marrow-derived cells have been identified as the most potent immune-stimulatory cells known, specialised for the initiation of and shaping of immune responses of all kinds [16]. Defects in the function of DC have been identified in HCC patients, and those infected with either HBV or HCV. For example, the balance of different types of DC, myeloid and plasmacytoid DC, has been found to be skewed in subjects with HBV infection [17], and generalised impairment in these cells' ability to function has been reported in both HBV and HCV positive subjects in some studies [18] but not others [19]. A recent study found that some functional defects could be reversed by antiviral therapy to go from RNA-positive to HCV RNA-negative cells [20].

T cells specific for HCC have been isolated and analysed *in vitro*. These pre-clinical studies have been directed at identifying tumour antigen-specific cells or non-specific effector cells with antitumour activity [21]. MAGE antigen-specific T cells have been found in TIL from HCC subjects [12], and AFP-specific T cells have been identified in patients with cirrhosis and HCC, with and without immunisation [22–25].

Recent analysis of antiviral T cells specific for HCV antigens have brought to light a population of liver resident, HCV antigen-specific regulatory cells expressing IL-10 which can suppress effective antiviral T cell responses [26]. These suppressive cells in liver could potentially impact antitumour immunity as well.
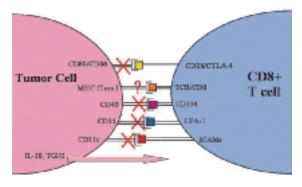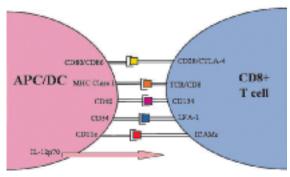

Figure 2A

Tumour cells do not activate CD8 T cells. Tumour cells may or may not express MHC class I/peptide complexes on their surface, enabling recognition by previously activated T cells. They do not express the optimal arrangement of costimulatory and adhesion molecules required for activation of naïve T cells. They may also secrete inhibitory cytokines such as IL-10 and TGFβ. Abbreviations: ICAM, intercellular adhesion molecule; TCR, T cell receptor.

Figure 2B

Antigen Presenting Cells (APC) activate T cells. APC, like B cells, macrophages and DC, express an array of costimulatory molecules in addition to high levels of MHC class I and II. This enables APC to strongly activate T cells, assisted by additional molecules like adhesion molecules and synthesis of cytokines like IL-12 p70.

Human clinical trials

Immunotherapy trials for HCC to date, fall into several categories: cytokine injection to non-specifically activate subsets of immune cells and/or make the tumour more immunogenic; addition of lymphokine-activated killer (LAK) cells to therapy; infusion of TIL, or activated peripheral blood lymphocytes (PBL); infusion of antigen presenting cells, and one recent report of an autologous tumour-based vaccine. The results of these different trials are mixed, but many have shown biological activity and a subset have demonstrated clinical effects (table 2).

Table 2

Immunotherapy
clinical trials.

Strategy[1]	Year/Author	Patients[2]	Setting[3]	Responses[4]	Comments[5]
Chemotherapy +IFNγ+IL-2	'95 Lygidakis et al.	20 stage III–IV	Trans-arterial infusion	14/20 tumour size decrease	14/20 reduced serum AFP
IFNγ+GM-CSF	'02 Reinisch et al.	15 inoperable stage III–IV	cytokines s.c.	no survival benefit vs historical controls	
IL-12 Adenovirus	'04 Sangro et al.	8 HCC of 21	AdVIL-12 dose escalation	6 SD and 1 PR	
Chemotherapy ± LAK	'91 Une et al.	randomised 12 pt./arm	adriamycin ± IL-2-activated spleen LAK i.a.	improved recurrence rate with LAK	
Chemotherapy ± LAK	'95 Kawata et al.	randomised 12 pt./arm	adriamycin ± IL-2-activated spleen LAK i.a	no differences in outcomes	
TIL	'91 Takayama et al.	3 (1 HCC, 2 colorectal mets)	In[111] labelled TIL (IL-2/αCD3) + chemotherapy	2/3 PR	cells trafficked to liver
TIL	'97 Wang et al.	10 pt	IL-2/LAK supernatant activated TIL	improved recurrence rates rates vs historical controls	
PBL	'00 Takayama et al.	150 randomised post curative resection	PBL+ IL-2/αCD3, i.v.	Significant improvements in risk of recurr., time to recurr., recurr.-free survival	Overall survival p = 0.09, ns.
PBL	'04 Shi et al.	13 pt.	PBL+ IL-2/αCD3, i.v.	Increases in lymphocytes and DC subsets	
Dendritic Cells	'02 Ladhams et al.	2 metastatic	GM/IL-4 DC+tumour	1 pt. slowed tumour growth	
Dendritic Cells	'03 Iwashita et al.	10 unresectable.	GM/IL-4 DC+tumour lysate+TNFα+KLH, l.n.	1/10 MR DTH+	7/10 KLH
Dendritic Cells	'03 Stift et al.	2 HCC of 20 total	GM/IL-4 DC+tumour lysate+TNFα+IL-2, l.n.	no PR or CR	
Dendritic Cells	'05 Mazzolini et al.	8 HCC of 17 total	GM/IL-4 DC +adenovirus IL-12	2 SD (of HCC pt.)	Increased serum IFNγ, some increased NK activity
Dendritic Cells	'05 Lee et al.	31 stage IV HCC	DC+tumour lysate	Improved survival	Boosting improved outcome
Dendritic Cells	'05 Chi et al.	14 advanced HCC	Radiotherapy the 1-2 DC i.t.	8 pt. + immune response	3 pt. serum AFP decrease
Autologous Tumour + Cytokines	'04 Kuang et al.	41 pt. randomised, 19 received vaccine	Formalin-fixed autologous tumour+GM-CSF +IL-2+BCG, i.d.	Statistically significant improvements in risk of recurr., time to recurr., recurr.-free survival	Improved overall surviva p = 0.01
Autologous Tumour + Cytokines	'05 Peng et al.	60 pt., post curative resection, randomised to vaccine	Formalin-fixed autologous tumour+GM-CSF +IL-2+BCG, i.d.	Statistically significant improvements in risk of recurr., time to recurr., recurr.-free survival	
AFP Peptides	'03 Butterfield et al.	6 stage IVa and IVb pt.	AFP peptides in Montanide adjuvant i.d.	No PR or CR	Increased frequency of AFP T cells in blood
AFP Peptide-pulsed DC	'06 Butterfield et al.	10 stage III and IV pt.	AFP peptides on DC i.d.	No PR or CR	Increased frequency of AFP T cells in blood

[1] IFN, interferon; IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; LAK, lymphokine-activated killers; TIL, tumour infiltrating lymphocytes; PBL, peripheral blood lymphocytes

[2] pt., patient

[3] Routes of administration: s.c. subcutaneous; i.a. intra-arterial; i.v. intra-venous; l.n. intra-lymphatically; i.d. intradermal, i.t. intratumoral

[4] PR, partial response; MR, mixed response; CR, complete response

[5] ns, not significant; KLH, keyhole limpet hemocyanin; DTH, delayed-type hypersensitivity; AACR, American Association for Cancer Research

Cytokines

In a trial with 20 stage III–IV patients from 1995, transarterial chemotherapy was combined with interferon gamma (IFNγ, which would increase MHC expression for improved antigen presentation and possibly skew the immune response towards activation of cytotoxic T cells) and interleukin (IL)-2 (which activates and acts as a growth factor for T cells) [27]. Fourteen subjects had some tumour size decrease and reduced serum AFP levels, indicating a clear positive biological

effect of this treatment combination. In a trial of 15 patients with advanced, inoperable HCC, subcutaneous injection of IFNγ and granulocyte-macrophage colony stimulating factor (GM-CSF: which acts as a growth factor for antigen presenting cells like DC which reside in the skin) was tested [28]. However, no clinical responses were observed.

More recently, Sangro et al. [29] tested an adenovirus (AdV) encoding IL-12 in intratumoral injections for a variety of digestive tumours (including 8 HCC patients). IL-12 is a potent cytokine able to activate and skew T and NK cells towards cytotoxic responses and have anti-angiogenic effects [4]. Of the 8 HCC patients, there were 6 disease stabilisations (SD), 1 partial response (PR) and 1 progressive disease (PD) post therapy, indicating the antitumour effects of this strategy. Members of this group performed an imaging study of intra-tumoral (i.t.) injected AdV and found no transduction of normal or cirrhotic tissue and gene expression in tumour at 2 days but not at 9 days [30]. The same group also tested i.t. injections of autologous DC transduced with an adenovirus encoding IL-12 [31]. This trial of 17 patients (8 with HCC) demonstrated safety of this approach and many interesting immunological effects of the vaccine. Importantly, 2 of the HCC patients had disease stabilisations.

Effector cells

Several trials have been reported testing infusion of different types of cytotoxic lymphocytes. Two separate but similar randomised trials were performed in 1991 [32] and 1995 [33] combining chemotherapy (adriamycin) with lymphocyte activated killer (LAK) cells generated from splenocytes, post-resection. The first study showed a decrease in rate of recurrence, the second showed no benefit overall. The second study did show a benefit for the subset of subjects with a negative margin of ≥1 cm post resection. The reason for this discrepancy is unknown. One concern with LAK cells is their lack of tumour antigen-specificity. In contrast, TIL, because they are derived from T cells infiltrating the tumour, have been shown to contain tumour antigen-specific T cells.

A pilot trafficking study of Indium[111] labelled TIL was performed which found that these cells (activated by IL-2 and CD3 receptor triggering), delivered via intra-hepatic artery infusion, trafficked to sites of disease preferentially in all of 3 subjects [34]. One subject had HCC, the other 2 had liver metastases from colorectal cancer. In addition, 2 of the 3 subjects (1 HCC) had partial tumour responses of 5–10 months duration.

In a study from 1997, 10 subjects received TIL (activated by IL-2 and LAK cell supernatant) [35]. Treated subjects had improved recurrence rates compared to historical controls.

In the largest trial of its kind, 150 patients were randomised to receive either IL-2 and anti-CD3-activated PBL or observation post curative resection [36]. This trial found statistically significant improvements in risk of recurrence, time to recurrence as well as recurrence-free survival. Overall survival fell short of statistical significance at p = 0.09. Given the randomised nature of this large trial, its results offer more objective support for the potential for immunotherapy, especially in the adjuvant setting.

In a recent study, cytokine-activated PBL, which were cultured in IFNg, anti-CD3 and IL-2, were infused into 13 HCC patients to determine immunological effects [37]. Results showed by several measures that immunity (including peripheral blood DC percentages) improved.

Antigen presenting cells

Many strategies utilising the immune activating ability of professional APC, are being pursued. There are many ways to utilise the potent DC to present tumour-derived antigens to the immune system. Adding tumour lysate or purified proteins to DC takes advantage of the active uptake mechanisms of the immature DC to load them with protein. This is subsequently processed into MHC class I and II to activate both CD8+ and CD4+ T cells. One can coat DC with either acid-eluted tumour-derived peptides or high levels of synthetic peptides. Antigen engineering of DC with plasmids (which is inefficient), or with viral vectors like adenovirus [38] allows the DC to express entire antigens. Fusion of DC and tumour cells via polyethylene glycol (PEG) or more recently, electro-fusion, transfers any and all tumour proteins to a cell which also expresses necessary immune activating molecules.

Several studies have been performed testing infusion of APC. In each case, the APC were mixed with antigen in the form of tumour, tumour lysate or peptides, to prepare a patient-specific vaccine to activate an immune response to antigens in the tumour.

Four publications have used DC generated *ex vivo* from peripheral blood, pulsed with tumour or tumour lysate. In one report, two patients with

metastatic HCC were treated with immature DC [39], grown in GM-CSF (growth factor) and IL-4 (to block macrophage development from monocyte precursors), the most common and straight forward method for generation of DC. Of the two patients, one had slowed tumour growth compared their pre-treatment status. In general, immature DC are more effective at antigen acquisition than for T cell stimulation. Immature DC are, therefore, optimal for mixing with tumour or tumour lysate, to allow uptake and processing of large amounts of tumour antigens. However, more recent data indicates that more potent T cell activation can result from a subsequent maturation step (post antigen uptake), which reduces phagocytosis activity and upregulates T cell activation cell surface molecules, improves trafficking to lymph nodes and increases production of T cell activating cytokines like IL-12p70. The degree of maturation required for optimal T cell activation and the extent to which maturation may occur *in vivo* after injection of DC are unknown.

The next trial used DC loaded with tumour lysate, stimulated with TNFα, and mixed with keyhole limpet hemocyanin (KLH) before injection [40]. The KLH was added as a foreign protein which activates non-antigen-specific helper T cells and serves as a marker of successful immunisation by subsequent DTH testing. In this trial, in which 10 subjects with unresectable HCC were treated, 7 developed positive DTH responses to the KLH (indicating successful vaccination), and 1 subject had a mixed tumour response.

A similar trial with tumour lysate loaded DC which were matured with TNFα, but also mixed with IL-2, was performed in a mixed population of subjects, 2 of whom had HCC [41]. There were no tumour responses in any treated subjects. Recently, the largest trial to date was reported in which 31 HCC patients received DC pulsed with autologous tumour lysate [42]. Fourteen patients received 5 weekly injections i.v., the other 17 also received monthly boosts. Overall, there were 14 PR and 17 SD, and boosted patients had improved 1 year survival rates (63% *vs* 10%). Treated patients had significantly improved 1 year survival (40%) *vs* historical controls (20%, p = 0.038), indicating clinical activity.

DC have also been tested in combination with radiotherapy [43]. Fourteen patients received 8 Gy radiotherapy followed by 1–2 doses of immature DC i.t.. Toxicity was mild (as with most immunotherapy studies) and 3 patients had reduced serum AFP. Eight of 10 patients tested had AFP-specific immunity improvements, suggesting biological activity of this approach.

Autologous tumour

Recently, a randomised phase II trial was published in which formalin-fixed autologous tumour (as a source of tumour antigens) was mixed with GM-CSF (as an APC growth factor), IL-2 (to activate T cells) and BCG as a foreign immune stimulus [44]. Forty-one stage I-IIIa subjects, post curative resection, were enrolled and randomised; 19 received vaccine. Overall, treated patients had statistically significant improvements in risk of recurrence, time to recurrence as well as recurrence-free survival. In this trial, overall survival was also improved at p = 0.01. In a follow up trial [45], 60 patients were randomised to observation or fixed autologous HCC vaccine + GM-CSF+IL-2+tuberculin. Stage I/II patients were immunised i.d., and 3 year recurrence rates were 33% for immunised and 61% for controls, again supporting clinical activity for this approach.

AFP-based immunotherapy

In order to generate tumour antigen-specific immune responses to HCC, AFP has been targeted by our group and others [46]. After studies in murine models [4, 47] and in vitro T cell cultures [48–50], two clinical trials have been conducted.

The Economou group identified several AFP-derived peptides restricted by HLA-A2.1 and recently others have been identified restricted by HLA-A24 [51]. A pilot clinical trial was initiated, in which stage IV patients received the 4 immunodominant peptides (100–500 μg) emulsified in Montanide ISA-51 adjuvant [23]. In these advanced stage HCC patients, no clinical responses were detected, nor did serum AFP levels decrease.

The overall survival post-treatment ranged from 2 to 17 months. The immunological responses in 5 of 6 patients allowed us to demonstrate that AFP peptide epitopes were immunogenic *in vivo* and were able to stimulate antigen-specific T cells in HCC patients with very high serum levels of AFP. Because murine models and *in vitro* T cell cultures suggest that peptides on DC have superior immunogenicity, the follow-up trial tested AFP peptide-pulsed autologous DC. In that trial, 10 patients (stage III–IV) were immunised and 6/10 showed MHC tetramer AFP specific T cell increases and 6/10 had increased frequency of IFNγ-producing AFP-specific T cells [25], demonstrating immunological activity of the AFP-based vaccine.

Conclusions

The promise of immunotherapy is the specific and systemic elimination of tumour, based on the expression of specific proteins by the tumour. To date, several different strategies of immune activation have shown biological activity in HCC subjects and a subset of those have demonstrated clinical efficacy. The published data demonstrates that adoptive transfer of activated effector cells and complex tumour-derived vaccines can impact the recurrence and survival of HCC subjects. Novel vaccines and combinations of vaccines with more standard therapies are just beginning and hold promise for future improvement in impact of immunotherapy for HCC.

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