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Alpha-Fetoprotein as an Immunoregulator and Immune Response Modifier: Historical Background and Current Update

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SUMMARY

The various immunological roles of human alpha-fetoprotein (HAFP) and its correlation with hepatomas, i.e., hepatocellular carcinomas (HCCs), are not always addressed together in the biomedical literature even though HAFP has long been utilized as a biomarker for hepatomas. Although the well-being of the newborn, infant, and juvenile can be monitored by the measurement of age-dependent HAFP in biological fluid levels throughout these stages, the majority of clinical reports in these age groups do not include hepatomas (except hepatoblastomas) because of their later onset. However, reports concerning the interaction of HAFP and immune-associated proteins and cells in HCC patients in both applied clinical research and investigational settings has gradually increased over the years; thus ,it has expanded our base knowledge of mounting an immunotherapeutic response against AFP in hepatomas. The different immune-associated proteins (cytokines, chemokines, interleukin, kinins) interacting with HAFP has remained less reported due to limitations of appropriate in vitro and in vivo models. Concomitantly, the advances in elucidating the various immunological activities of AFP are opening new vistas of knowledge regarding the physiological roles of AFP in the growth of HCC. The present review surveys HAFP as an immunologic response modifier and regulator for HCC and its use in the generation of AFP – sensitized lymphocytes. An attempt is also made to relate the AFP activities to HCC progression following immunotherapies. Hence, the present review was divided into two major sections; I) AFP structure and homologies in the immune response, and II) the therapeutic use of AFP in HCC patients in adult stages in both in vivo and in vitro models.

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Abbreviations: MHC = major histocompatibility complex; Mus = mouse, Ig = immunoglobulin TNF = tumor necrosis factor; Hum = human leucocyte antigen; HT-Rec = human T-cell receptor; 14 = identity; SIM = similarity.

Introduction

Human Alpha-fetoprotein (HAFP) is a tumor-associated fetal glycoprotein that functions in the regulation of both ontogenic and oncogenic growth, HAFP has been classified as a member of a three-domain cysteine-rich translated protein of the albuminoid gene family which currently consists of four members: albumin (ALB), vitamin-D binding protein (DBP), AFP, and alpha-albumin (α-ALB), HAFP is a 69-kDa single-chain polypeptide, synthesized in the yolk sac, fetal liver, and gastrointestinal tract during pregnancy and is reexpressed in multiple adult tumors of mixed mesodermal/endodermal origin [1-6]. Hence, HAFP has been employed in the clinical laboratory as a fetal defect and serum tumor marker for hepatomas and germ cell tumors including teratomas, ovarian yolk sac, and gastrointestinal tumors [7-9] (Table-1). However, the main biological roles of AFP during infancy and adult stages remain controversial to this day.

A vast biomedical literature has been amassed concerning the use of HAFP during adulthood as a biomarker in human hepatomas, i.e., hepatocellular carcinomas (HCC). These studies have largely addressed the measurement of elevated AFP levels in the sera of adult patients which display both hepatic disorders and cancer. The first hepatic tumors to be associated with abnormal AFP levels were hepatoblastomas and hepatomas [7, 9]. Later, other types of liver dysfunction disorders were found to reflect discordant AFP levels which included abnormalities coincident with cirrhosis and various forms of hepatitis [8,10]. Similar to the elevated fetal and maternal serum AFP levels associated with neural tube fetal defects, adult patients with liver tumors also demonstrate high serum levels. Concurrent with the association of elevated AFP with hepatic distress disorders, additional neurological and wasting disorders have also been reported denoted [5,11]. However, there exists a scarcity of reports that have attempted to correlate AFP serum levels with the various immunoregulatory activities attributed to AFP.

Table 1: Alpha-Fetoprotein Is a Tumor-Associated Fetal Protein That Serves As a Biomarker for both Pregnancy and Adult Disorders

I. Pregnancy Disorder (A). Structural Defects 1. Neural tube defects (spina bifida; anencephaliy). 2. Renal Agenesis 3. Omphalocele 4. Gastroschisis 5. Fetal growth restriction 6. Down Syndrome	II. Adult Disorder (A). Liver disorders: 1. Alcoholic cirrhosis 2. Viral hepatitis 3. Subacute hepatic necrosis 4. General liver distress 5. Hepatic biliary obstruction 6. Choledocholithiasis
(B) Fetal Fluid Disorders 1. Oligohydramnios's 2. Fetal Maternal Bleed 3. Severe Pre-eclampsia 4. Placental Previa 5. Placental Abruption 6. Placental Obstruction	(B). Cancer types 1. Primary hepatomas 2. Secondary hepatomas 3. Malignant teratomas 4. Extra-embryonic tumors 5. Yolk sac tumors 6. Squamous cell carcinomas 7. Various germ cell tumors
(C) Miscellaneous Pregnancy Disorders 1. Premature Labor 2. Prematurity 3. Cystic Hygroma 4. Fetal Demise	(C) Miscellaneous Disorders 1. Ataxia Telangiectasia 2. Oculo-motor Aprasia 3. Combined Immunodeficiencies 4. Hepatoblastoma

Objectives

One aspect of the present review will address the increase of reports of AFP in conjunction with its therapeutic utility against hepatomas (HCCs). Thus, the present review has attempted to link the activities of AFP in the immune response to the presence of HCC in adult patients. Moreover, publication of the biological roles attributed to AFP in the regulation of the immune system during growth and progression of HCCs have nearly matched the flow of clinical reports. Correspondingly, the objectives of the present report are two-fold. First, the immunobiological activities of AFP in adults will be reviewed since many previous reports have focused on AFP as a pregnancy and cancer biomarker. Second, the multiple immune disorders associated with histopathologic and/or serum alterations using AFP as a biomarker will be surveyed. Third, the various properties of AFP as a biomarker probe of the immune response will be described. For more detailed accounts on the physiochemistry and genetics of AFP, the reader is directed to earlier reviews on AFP [12-16].

In view of the above, the present review has been divided into two sections. These will include the following: (I); homologies and immune-functional aspects of AFP; and (II) the relevance of AFP immunotherapy in hepatomas relative to patient outcome. AFP and the Immunoglobulin Superfamily: Genebank Homologies (Note: In the following 3 sections; please refer to Tables – 2 and 3 and the references cited thereof).

The Major Histocompatibility Proteins

The major histocompatibility complex (MHC) genes encode three major sets of molecules; the class I, II, and III proteins. The Class-I proteins address the innate immune system while Class II molecules are involved in the adaptive immunological response with the major histocompatibility complex (MHC) proteins; Class III molecules are concerned with the complement cleavage (lysis) cascade in the inflammatory response. The class I molecules comprise single transmembrane glycoprotein heavy chain proteins complexed with a β2-microglobulin molecule. HAFP displays a class I amino acid (AA) identity homology site on its second domain [17, 18] (Table-2). This amino acid sequence stretches are 23 amino acids (AA) long with an AA identity (Iden) of 39% and a similarity (Sim) of 56%. Class-II proteins consist of two noncovalently joined peptide segments that transverse the plasma membrane at their COOH terminus. HAFP displays a Class-II identity site on domain I [18]. This latter site demonstrated a 33% Iden stretch and Sim = 52% over 25 AAs. Finally, the class III molecules (complement-associated) displayed AA identity sites on HAFP within domain 1, 2 and 3 [18]. The Class-III AA Iden sequences ranged from 50-60% and were 62% sim over amino acids in lengths from 10-12 AA residues [17-19]. Based on these sequence homologies, it is tempting to speculate that HAFP might somehow be involved in NK, MHC, and complement interactions at the onset of pregnancy and during the three terms of gestation.

Immunoglobulin Heavy Chains

The immunoglobulins (Igs), are a group of lymphoid-derived glycoproteins present in the serum and tissue fluids of all mammals. They are produced in large amounts by plasma cells, which developed from precursor β-lymphocytes. Such lymphocytes carry membrane-bound immunoglobulins with antigen-specificities of plasma cell-secreted lgs. These Igs are bound to lymphocytes through receptors present on the cell surface that bind various Ig classes (i.e., the IgG β-Fc γ receptor). Data from the Genbank have identified putative matched sequences on AFP largely with Ig heavy chains and with the IgG receptor (Table-2). HAFP domain 1 appeared to have a matched murine Ig heavy-chain amino acid stretch of 30 AA (Iden = 30%, Sim = 43%); no such sequence was localized on domain 2, but two sites were reported on domain 3 [17]. The Ig heavy-chain sequence on HAFP domain 3 was localized near the carboxyl terminus of the molecule, representing a identity of 29% (Sim = 51%) over 31 AAA in length. Finally, the remaining IgG receptor matched site was detected on HAFP domain 3 which displayed an Iden of 39% and a Sim of 39% over a 16 AA stretch [17-19].

The T-Cell Receptors

The MHC proteins accomplish antigen presentation via a three-way interaction involving the T-cell receptor (TCR), MHC molecules, and lymphocyte-processed antigens. The T-cell receptors fall into two main types, the T-cell receptor-1 (TCR1) and the T-cell receptor-2 (TCR-2), both composed of heterodimer combinations of α-, γ-, or δ-chains, constituting of constant and/or variable chain segment regions. Both rodent and human AFP have been previously implicated in the regulation of immune responses in both the humoral and cell-mediated types [18]. Thus, it was not surprising that HAFP amino acid identity segments involving TCRs were detected on all three AFP domains (Table-2). HAFP domain I bears a β-chain TCR site that displayed a 19-AA stretch exhibiting a 39% identity, (Sim = 33%) while the domain 2 site displayed a 19-AA sequence with a 47% identity (Sim=21%). HAFP domain 2 revealed a further site stretching over a 12-AA length with a 41% identity (Sim=25%). Finally, the third domain of HAFP demonstrated the presence of an identifier site composed of shorter AA lengths representing the δ-chains of the TCR, which displayed a 40% identity over a 15-AA segment [17, 19].

AFP Domain-I

1) HAFP$_{7}$	Y	G	I	A	S	I	L	D	S	Y	Q	C	T	A	E	I	S	L	A	D	L	A	T	I	F					
MHC-II$_{218}$	Y	G	X	G	N	K	Y	K	A	Y	S	C	S	S	N	C	I	V	I	L	I	T	L	I	F					
2) HAFP$_{80}$	E	L	C	H	E	K	Q	I	L	E	K	Y																		
MHC-III$_{784}$	N	L	C	W	E	K	S	I	L	G	L	I																		
3) HAFP$_{78}$	E	L	C	H	E	K	Q	I	L	E	K	Y	G	H	S															
TNF$_{486}$	Q	L	C	K	S	K	Q	S	R	Q	K	Y	P	I	S															
4) HAFP$_{99}$	Q	S	E	E	G	R	H	N	C	F	L	A	H	K	K	P	T	P	A	S	I	P	L	F	Q	V	P	E	P	V
Mus Ig$_{3325}$	K	P	Q	S	Q	P	S	D	H	F	L	Q	K	A	T	P	T	P	T	R	P	H	L	L	I	V	P	L	P	I
5) HAFP$_{137}$	E	T	F	N	N	K	F	I	Y	E	I	A	R	R	H	P	F	L	Y											
HT-Rec$_{1248}$	N	T	F	I	Y	K	Y	L	Y	I	L	I	X	K	M	Y	F	L	Y											

Domain-II:

1) HAFP$_{264}$	A	C	A	V	M	K	N	F	G	T	R	T	F	Q	A	I	T	V											
HumC$_{62233}$	S	C	X	L	F	K	H	F	S	A	K	N	L	Q	A	G	E	V											
2) HAFP$_{233}$	L	S	Q	K	F	T	K	V	N	F	T	E	I	Q															
HumCd1c$_{125}$	L	S	S	S	F	Q	R	I	R	F	T	E	F	P															
3) HAFP$_{241}$	L	D	V	A	H	V	H	E	H	C	C	R																	
HT-Rec$_{22150}$	L	D	L	X	S	L	R	P	H	C	C	S																	
4) HAFP$_{252}$	C	C	R	G	D	V	L	D	C	L	E	D	G	E	K	I	M	S	Y										
HT-Recβ$_{83036}$	C	L	C	G	D	T	G	D	C	L	E	G	S	Y	K	I	L	S	Y										
5) HAFP$_{291}$	H	A	E	N	D	G	K	P	E	Q	L	S	P	N	L	N	R	F	L	G	D	R	D						
Mus MHC$_{743}$	H	X	X	P	A	G	R	A	G	P	F	S	T	E	L	H	I	F	L	G	D	S	E						

Domain-III:

1) HAFP$_{419}$	A	N	R	R	P	C	F	S	S	L	V	V	D	E	T	Y													
HumIg1$_{215}$	N	D	S	L	P	C	I	I	P	L	S	V	G	G	T	V													
2) HAFP$_{511}$	K	F	I	F	H	L	D	L	C	Q																			
HLA-III1$_{865}$	S	F	I	F	E	M	E	L	C	Y																			
3) HAFP$_{538}$	V	K	Q	K	P	Q	I	T	E	E	Q	L	E	A	V	I	A	D	F	S	G	L							
HL60$_{1}$	I	R	V	K	P	Q	V	S	E	S	K	L	X	P	E	V	O	A	Y	L	G	L							
4) HAFP$_{521}$	A	Q	G	V	A	L	Q	T	M	L																			
MHC-III$_{324}$	S	Q	G	Q	E	L	K	T	M	L																			
5) HAFP$_{56}$	L	E	K	C	C	Q	G	Q	E	Q	E	V	S	F	A	E	Q	G	Q	K	L	I	S	K	T	R	A		
HumIg$_{83}$	L	T	C	C	C	C	H	Q	E	E	D	A	P	G	P	V	H	G	E	E	L	X	S	R	D	S	P		

*The amino acid sequences on the three domains of HAFP were compared with protein sequences derived from the GenBank databases using the GCG (Wisconsin Program) FASTA sequence comparison software as described in Ref. Dauphinee & GJM Med Hypo 58: 433, 2002.

Table 3: Selected amino acid sequences derived from human alpha-fetoprotein (HAFP) as Major Histocompatibility Complex (Class-I) antigens were obtained through a Genebank "find patterns" software program (see Ref.17.). The HAFP sequences were then subjected to "FASTA" protein sequence identity matches to T-cell related proteins. Note that each HAFP-MHC site can be matched to either an IgG receptor or a T-cell receptor

Protein	Domain – 1	Domain – 2	Domain – 3
1). Hum Alpha-fetoprotein	$_{137}$ P L F Q V P E P V * - * *- -		
Mus IgG Receptor	$_{3345}$H L L I V P L P T		
2) Hum Alpha-fetoprotein	$_{158}$ F M N K F [IY] E - * * - *		
Hum T-cell Receptor	$_{1248}$ F I Y K L [YI] L		
3) Hum alpha-fetoprotein		^{306} T [T L] E R [GQ] [C I] I * -	
Rat T-Cell Receptor-β		^{7438} T [V P] W I [QG] [F C] I	
4) Hum alpha-fetoprotein			^{485} C I R H E M T P V - - - * - -
Mus T-cell Receptor β			^{1045} C I R D N K T P S
5) Hum alpha-fetoprotein			^{492} P V N P G [V G] Q C - * - - * *

Hum T-cell Receptor-β			1280 P I W P G [A L] P T
6) H alpha-fetoprotein			507 N R R P C F S X [S L] V * - - * - -
Hum IgG Receptor			D S L P C I I P [L S] V
7) H Alpha-fetoprotein			542 G V A L Q T M K Q - - - * -
Mus T-cell Receptor α			35 G E A L R G M L C

Mus = mouse; Hum = human; MHC = major histocompatibility complex
*Ref. 37 stated that HLA-A2 restricted peptides derived from human alpha-fetoprotein elicited T-cell responses.
- Numbers prior to sequence represent amino acid number on the chain of the protein
- dash indicates amino acid identity; *asterisk indicates amino acid similarity; brackets indicate juxtaposed sequences.

AFP-Lymphocyte Interaction

The immunoregulatory functions of HAFP have long been known and studied. Rodent AFP has been shown to exert a significant immunosuppressive activity on the in vivo generation of murine cytotoxic T lymphocytes (CTLs) [20]. This suppression was shown to be independent of the susceptibility to the mixed leukocyte culture activation phase, using various murine strain combinations. The murine strains, whose proliferative responses were refractive to AFP-induced suppression, failed to develop demonstrable cytotoxic lymphocyte (CTLs) activity when incubated with AFP [21]. Data from several species strain combinations revealed that the normal generation of CTLs occurred in cell cultures that contained AFP. This refractive nature of AFP correlated with the presence of nonsuppressible lymphocyte-stimulating alloantigenic systems on the stimulated cell population. Such data provided the basis for proposing possible mechanisms for AFP-induced suppression of T-cell-mediated cytotoxicity. Moreover, the primary target of this suppression appeared to be the proliferating helper T-cells precommited to respond to MHC-associated lymphocyte-activating determinants [21].

Using a direct immunofluorescence assay, researchers have demonstrated that alpha-fetoprotein (AFP), both in purified form and in hepatocellular carcinoma (HCC) sera, was capable of binding 10-20% of T lymphocytes and 5-10% of B lymphocytes in human peripheral blood when preincubated in AFP-containing amniotic fluids at 4 degrees C [22]. After raising the temperature to 37 degrees C, most of the membrane-bound AFP was internalized or shed, and consequently, less than 3% of the cells exhibited positive cell membrane fluorescence. Furthermore, binding of AFP to the lymphocyte surface membrane and the continuous presence of large amounts of AFP in these lymphocyte cell cultures did not interfere with the action of cytotoxic antibodies directed against HLA determinants on the lymphocyte surface [22].

Nonspecific suppressor cell (NPC) activity was induced in vitro by preculturing splenocytes from normal mice in the presence of both mouse amniotic fluid and purified alpha-fetoprotein for 5 days or more. In adoptive transfer experiments in vivo, the AFP-precultured NPCs were shown to reduce the humoral response in mice to sheep red blood cells and to the cell-mediated cytotoxic response to allogeneic tumor cells [23]. Using mixed experiments (in vitro) containing freshly explanted splenocytes, the AFP-precultured splenocytes abrogated the generation of specific cytotoxic T-lymphocytes in primary mixed lymphocyte-tumor cell cultures. However, supernatants of such precultured cells demonstrated only a marginal response effect. The suppressor cells were found to be nylon-wool non-adherent, and their effect could be completely abolished by treatment with anti-Thy-1, 2 serum enriched with complement. NPC precursors were found to be sensitive to cyclophosphamide (in vivo) and to hydrocortisone (both in vivo and in vitro) and were resistant to various doses of radiation [23].

The phylogenetic conservatism of AFP demonstrated by extensive immunological cross reaction between human AFP and other mammalian AFPs, suggest that AFP plays a general role in ensuring the successful pregnancies of most if not many of the mammalian species studied. Investigators had previously demonstrated the antiproliferative effects of human AFP on lymphocytes harvested from normal human donors [24]. The inhibitory effect of human AFP was also found during blast transformations of lymphocytes. In a later study, peripheral blood mononuclear cells were induced to blast transformations with phytohaemagglutin (PHA-M) and the effect of AFP was quantified by the incorporation of [3H]-thymidine into newly synthesized DNA during a 24 hr pulse. Human AFP showed immunosuppressive effects similar to various species of lymphocytes (mouse, rat and hamster) studies, and a parallelism was noted in the respective percentage of thymidine incorporation values at comparable doses. These data served to establish a cross species inhibitory effect of human AFP which the investigators attributed the effect to T-helper cells, while no observable interactions with interleukin-2 (IL-2) were noted [24, 25].

Recombinant AFP as an Adenovirus Co-Vector

AFP has also been employed in a recombinant form as a co-vector for an adenovirus carrying a cargo gene such as melittin (Ad-rAFP-Melittin (Mel)). The Ad-rAFP-Mel construct, used in a Bel-7402 hepatoma cell line, had an inhibitory effect on the proliferation of the BEL-7402 cells [26]. The morphological changes of apoptosis were confirmed by both light microscopy and DNA electrophoresis. The ultrastructural characteristics exhibited by apoptotic cells, such as chromatin condensation and nuclear fragmentation, were further observed by electron microscopy in the Ad-r-AFP-Mel-infected cells. The Ad-rAFP-Mel infection markedly induced cell apoptosis, and FAS expression of Bel-7402 cells infected by the Ad-rAFP-Mel construct was up-regulated. These investigators concluded that the melittin construct could induce apoptosis of the BEL-7402 cells and suggested that adenovirus-mediated delivery of melittin plus AFP as a plausible approach for the treatment of HCC [26].

AFP and NK Cell Interactions

In a earlier study, Spleen cells taken from quails Treated with chicken alpha-fetoprotein (Ch-AFP) had demonstrated reduced natural killer (NK) activity as did spleen cells obtained from quails treated with chicken amniotic fluid (derived AFP ChAmF) [27]. The Ch-AFP-induced reduction of NK cell activity was subsequently shown to be mediated by suppressor T-cells. Later, Ch-AFP-treated quails developed tumors with shorter latent periods than the tumors that grew in untreated quails after inoculation with Rous sarcoma virus; the same was true for ChAMF-treated quails. These data

suggested that reduction of NK-cells by avian AFP induced a susceptibility to subsequent tumor formation [27].

Natural killer (NK) cells are "spontaneous cytotoxic cells" thought to be involved in surveillance against tumor cells, rejection of virally infected cells, regulation of hematopoietic stem cell differentiation, and antibody synthesis. AFP has also been demonstrated to regulate certain T-cell-mediated immune reactions both in vitro and in vivo [28]. The lack of NK activity in newborn mice with high endogenous levels of AFP, together with the presence of cells expressing NK cell surface markers, suggests that AFP could play a role in the regulation of NK activity. In a previous study, the effects of AFP on spontaneous versus activated murine NK function were studied [24]. The lytic ability of AFP on freshly-prepared splenic NK cells and those on AFP arising after incubation for 24 h with interferon, Poly I:C,l, or T-cell growth factor (TCGF) was compared. Only when AFP was in combination with TCGF was the killing phase affected. In contrast, if AFP was added at the beginning of the 24 hrs. lymphokine stimulations, the subsequent NK activity induced by both interferon Poly I:C, and TCGF were significantly suppressed in a dose-and time-dependent fashion by AFP [24]. Delayed addition experiments revealed that AFP present during the first 6 h of in vitro stimulation inhibited interferon and TCGF-induced NK activity by 50-80%. The AFP-mediated inhibitory effect on lymphokine-stimulated NK activity was not the result of increased death of effector cells or non-specific binding of AFP to the enhancing agents.

Furthermore, in vivo injections of Poly I:C or TCGF did not increase neonatal NK function, while administration of interferon caused slightly higher levels of NK activity. Spleen cells from newborn animals cultured for 24 h in the presence of lymphokines markedly elevated NK function and could be suppressed by addition of purified fetal-derived AFP. The authors concluded that the in vivo pattern of NK activation in newborns with high endogenous levels of AFP was very similar to that of adult NK stimulation in vitro when exogenous AFP was added to the mixture [24].

AFP and Lymphocyte/Cytokine Activity

Other researchers further examined the effect of HAFP, at concentrations ranging from the physiological to pathological circulating levels, on the native alpha-IFN-and IL2-induced NK activity of normal peripheral blood lymphocytes (PBL). The cytotoxic function of unstimulated PBL was unchanged after a 16 hr incubation with 200 to 8000 ng/ml HAFP. In contrast, this treatment significantly reduced the responsiveness of PBL to the NK-enhancer factor interferon (IFN) and Interleukin 2. Optimal inhibition was observed when cells were pre-incubated with HAFP prior to being stimulated with IFN. Overall, the authors concluded that the sensitivity of PBL to HAFP mediated inhibition was restricted to very early times (30 Min) of incubation with the Lymphokine stimulating agents [29].

AFP and the Immune State of Pregnancy

The proposed concept that normal human pregnancy is actually a controlled state of inflammation has been validated in the biomedical literature [30]. The human conceptus has classically been viewed as a foreign (non-self) object in the mother's body and has long been considered a tissue allograft residing in the maternal uterus. Investigators have described that the conceptus as residing in an immunologically privileged site situated in juxtaposition to the placental cells barrier which is in direct contact with uterine tissue containing NK (natural killer) cells of the maternal innate immune system [31]. In turn, the maternal NK cells secrete cytokines that attract maternal lymphocytes to the placental boundary causing the maternal cells to view the foreign cell clusters as tissue inflammatory sites, rather than foreign cells/tissues. Such lymphocytes then wall off intruder cells from the maternal tissues at the placental/uterine interface [31]. Thus, the cells of the conceptus are viewed by maternal cells only as sites of tissue inflammation and not foreign (non-self) material. Obviously, any fetal protein that mimics the many lymphokines/cytokines of the immune system in structure and function would have a definitive advantage in sustaining and maintaining the foreign tissue as a site of inflammation. AFP is one such protein consisting of a series of successive modular cassette-like peptides mimicking the immune system cytokine substitutes and back-up immunoregulatory peptides [1, 3].

AFP and Immunoregulation

Since the first reports by Murgita et al in the 1970s, AFP has been recognized as both a B-and T-cell immunoregulatory agent. Full-length HAFP has been found immunosuppressive in both B-and T-cell lectin blast transformation assays [32, 33]. HAFP has further been shown to functionally impair dendritic cells inducing immune dysfunction and apoptosis of antigen processing cells (APCs) [34]. In a latter report, the authors suggested a mechanism by which hepatoma cells could escape immunological surveillance as a result of cells bearing AFP molecules on their cell surfaces [33]. More recent studies have further indicated that not all AFP-specific T-cell clones are deleted during ontogeny, and that potential AFP antigenic sites persist and are recognized by both murine and human T-cells. During the last decade, many research groups have succeeded in mapping T-cell immunodominant epitope sites on HAFP [35-40]. These research groups have reported that at least four major HLA-A epitotopic sites and several minor epitopic determinants can be localized throughout the three domain structure of HAFP [40].

Computer-generated HAFP AA sequences (9-10 AA in length) comprising multiple peptides were screened; such screens produced peptides which represented major histocompatibility complex (MHC) sites of the HLA-A type [37, 41, 42]. Several peptide segments of the latter positive group corresponded to known major T-cell epitopes that had been described in other reports (see above). Of the remaining antigenic determinants, several were classified as minor epitopic sites [37, 40, 43]. Each peptide segment proved capable of inducing specific T-cell lymphocyte responses in vitro from normal human HLA-A *0201 donor lymphocytes; the peptides also recognized HLA-A *0201/AFP primed tumor cells in both cytotoxicity assays and in interferon (IFN-α) induction studies. AFP peptide-bearing specific T-cells were further identified by the researchers in the spleens of mice immunized with dendritic cells transduced with an AFP-expressing adenovirus. These studies and others concluded that the human T-cell repertoire is capable of recognizing AFP in the context of MHC Class-I immune responses even in environments of high circulating HAFP levels in both hepatoma and cirrhotic patients [42-44].

Several of the peptides of HAFP-origin have been subjected to Phase I and II clinical trials for the treatment of hepatocellular carcinoma [44, 45]. Although the AFP epitopes provided a rational for T-cell based immunotherapy against AFP-producing cells, hepatomas, the eventual side effects of circulating antibodies of HAFP epitopes (aside from the T-cell responses described above), have yet to be determined. One such study suggested that the induction of liver autoimmune disease may yet be a factor with which to contend in sensitizing T-cells against HAFP [46]. Nonetheless, later reports have continued to identify subdominant

epitopes from AFP capable of activating high-avidity T-cells; such cells should be able to be identified, expanded, and studied in hepatoma patients [43].

AFP, Dendritic Cells, and Cytotoxic Lymphocytes

The immune response of lymphocytes has been studied after activation by dendritic cells (DCs) sensitized with a cytotoxic T lymphocyte (CTL)-based peptide from the 2nd domain of human alpha-fetoprotein (hAFP, #218-226 LLNQHACAV) [37, 45]. High purity DCs were obtained from plastic-adherent monocyte isolates cultured from healthy human donors of HLA-A2(+) of peripheral blood; these have been co-incubated with granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 7 days [34, 47]. Self-lymphocytes derived from donors were stimulated with DCs treated with the AFP #218-226 peptide segment in culture medium containing interleukin-2 (IL-2) for 7 days. IL-12 and TNF levels were then measured in the culture medium as well as employing specific lysis activity assays of lymphocytes against four strains of primary hepatocellular carcinoma cells.

After stimulation by DCs activated with the hAFP (#218-226) epitope peptide, lymphocytes appeared in good condition and the culture medium of the activated lymphocytes contained a high level of the Th1 type cytokines IL-12 and TNF. The AFP-activated lymphocytes not only specifically lysed HLA-A2(+) HepG2 tumor cell line, but also showed cytotoxicity against three other primary hepatocellular carcinoma cell lines and T2 hepatoma target cells that had been activated with the hAFP peptide. The results of these experiments provided the basis for developing a DC-based vaccine with a HLA-A2 restricted peptide epitopes derived from the hAFP (#218-226 peptide epitope) directed against AFP positive primary hepatocellular carcinoma cells [47]. The recent use of de ndritic cell-derived exosomes from AFP expressing DCs have also been employed to perform HCC therapy [48].

Alpha fetoprotein-derived peptide epitopes, as discussed above can also be recognized by heterologous human T cells in the context of MHC class I antigens. Investigators have determined the identity and AA sequence of AFP-derived peptides, presented as HLA-A*0201 antigens which could be recognized by the human (H) T cell repertoire. Researchers screened 74 peptides and identified 3 new AFP epitopes, hAFP (#137-145), hAFP (#158-166), and hAFP (#325-334), in addition to the previously reported hAFP (542-550) epitope [37]. Each epitope possessed two anchor AA residues and each stabilized HLA-A*0201 on T-cells in a concentration-dependent MHC class I binding assay. The peptide epitopes also showed stability for 2-4 h in a dissociation-kinetics assay. Each peptide induced peptide-specific T-cells in vitro from several normal HLA-A*0201 human donors.

Interestingly, the HAFP peptide-specific T cells also were capable of recognizing HLA-A*0201(+)/AFP(+) bearing tumor cells in both cytotoxicity assays and in IFN-gamma enzyme-linked immunospot assays. The immunogenicity of each peptide was assayed in vivo using HLA-A*0201/Kb- bearing transgenic mice following immunization with each peptide emulsified in adjuvant; in addition, the draining of mouse lymph node cells detected IFN-gamma cytokines following recognition of cells which had been stably transfected in HAFP assays. Furthermore, AFP peptide-specific T-cells could also be identified in the spleens of mice immunized with dendritic cells transduced with an AFP-expressing adenovirus (AdVhAFP) [35, 37, 41]. Three of four such AFP peptide epitopes could be identified by mass spectrometric analysis of surface peptides from an HLA-A*0201 human HCC cell line. The authors presented both immunological and physiochemical evidence which showed that at least four hAFP-derived peptide epitopes were immunogenic, naturally processed, and presented in the context of MHC class I antigens [34, 41]. These investigators proposed that such AFP sites could represent potential targets for hepatocellular carcinoma immunotherapy [37]. Although AFP is a weak immunogen, the immune response against AFP can be enhanced using AFP modified cell or peptide vaccines [49].

Peptide fragments of AFP presented to lymphocytes as major histocompatibility molecules can potentially serve as recognition targets by CD8 T-cells provided that the lymphocytes were not clonally deleted during ontogeny. In one such study, researchers sought to determine whether the human T-cell repertoire could recognize AFP-derived peptide epitopes of a common class I allele, HLA-A2.1. Dendritic cells genetically constructed to express AFP were able to induce AFP-specific T-cell responses in autologous human lymphocytes cultures and in HLA-A2.1/Kb transgenic mice. Such T cells could recognize a 9-mer peptide epitope derived from hAFP (#542-550) (GVALQTMKQ). The HAFP (#542-555) fragment was identified as a potential A2.1-restricted peptide epitope using a computer analysis of the AFP sequence, and was shown to have a low binding affinity to A2.1, with slow off-dissociation-kinetics.

AFP-specific CTL-and IFN-gamma-producing cells were also shown to recognize HAFP (#542-550)-pulsed targets. Conversely, the HAFP (#542-550) peptide-epitope generated T cells from both human lymphocyte cultures and A2.1/Kb transgenic mice which recognized AFP-transfected targets in cytotoxicity assays and in cytokine release assays. These data suggested that not all AFP-reactive clones had been deleted from the human T-cell repertoire since researchers detected at least one immunodominant A2.1-restricted epitope. Such findings have served to establish AFP as a potential target for T-cell-based immunotherapy in hepatomas [50].

Therapeutic use of AFP in Liver Tumors

A previous existing immunological belief was that high concentrations of soluble proteins contribute to the maintenance of peripheral tolerance to self-proteins, such as in pregnancy. This concept was tested in a clinical immunotherapy trial using MHC class I-restricted peptide epitopes derived from HAFP. AFP was previously thought to be a self-protein expressed by the fetal liver at high concentrations, which were transcriptionally repressed at birth. AFP is genetically unrepressed in many hepatocellular carcinomas (HCCs) and such patients display AFP serum levels in the high ug/ml range. One investigative team previously identified four immunodominant HLA-A*0201-restricted peptides derived from human AFP, that could stimulate specific T-cell responses in peripheral blood lymphocytes obtained from normal volunteers [37, 45, 51]. These researchers found that AFP peptide-activated T cells could be expanded in vivo in HCC patients immunized with four different AFP peptide epitopes. A pilot Phase I clinical trial was undertaken in which HLA-A*0201 patients bearing AFP-positive HCC cells were immunized with AFP epitopes. The intradermal vaccinations of four AFP peptides (100 ugm or 500 ugm each) emulsified in incomplete Freund's adjuvant were undertaken [52]. All of the patients (n=6) generated T-cell responses to most or all AFP peptides as measured by direct IFN gamma enzyme-linked immunospot (ELISPOT) and MHC class I tetramer assays, Thus the investigators concluded that the human T-cell repertoire is capable of recognizing AFP presented as MHC class I antigens even in the face of high circulating levels of AFP [45].

AFP and Immune Tolerance in Hepatomas
Even though breaking immunologic tolerance towards the HCC-associated alpha-fetoprotein (AFP) antigens now appears feasible, the use of this concept for the treatment of immunocompromised HCC patients has been limited. In one such study, investigators analyzed whether dendritic cells (DCS) from HCC patients transduced with a human AFP (hAFP)-expressing adenovirus and co-cultured with cytokine-induced killer (CIK) cells, could induce a strong specific immune response against HCC derived cells. An HAFP-encoding adenovirus (Ad-HAFP) was generated and DCs from healthy donors or patients were transduced at high efficacies. DCs were further co-cultured with autologous CIK-cells and studied for their ability to lyse tumor cells. AFP-transduced DCs strongly stimulated CIK cells to lyse 70% of AFP-expressing HCC cells, and cytotoxicity was significantly higher when lymphocytes were co-cultured with Ad-HAFP-transduced DCs rather than with Ad-mock-transduced DCs. This result indicated the presence of an AFP-specific immune response against the HCC cells. Interestingly, CIK cells from patients with AFP-positive HCC could be stimulated to lyse AFP-expressing HCC cells effectively as CIK cells from healthy individuals and stronger than CIK cells obtained from patients without AFP-expressing HCC. These data indicated that patient-derived DCs induced with an AFP-expressing adenovirus and co-cultured with autologous CIK cells could induce a strong AFP-specific immune response against HCC cells. It was proposed by these investigators that this approach may have a potential for use as an adoptive and/or DC based immunotherapy for HC patients [53].

Transfected AFP and Dendritic Cells
Although Alpha-fetoprotein (AFP) could serve as a possible target for a hepatocellular carcinoma (HCC)-specific vaccinations as previously shown, some studies have demonstrated that dendritic cells (DCs) treated with AFP can become dysfunctional. In a previously reported study, researchers were able to transfect AFP mRNA into DCs and observe the ability of DCs to induce AFP-specific CD4(+) and CD8(+) T cells [49]. It was hoped that AFP could be processed and presented by DCs directly, rather than released into culture media, such that there would not be an AFP negative effect on the function of DCs. These investigators employed immature DCs generated from peripheral blood mononuclear cells (PBMCs) of HLA-A2 positive HCC patients which were transfected with AFP mRNA. The transfected, matured DCs were then employed to stimulate autologous T cells.

Their results indicated that the expressions of membrane molecules from DCs following transfection were dramatically increased, and that interleukin-12 (IL-12) p70 release in the supernatant was significantly elevated; moreover, only a minority of AFP cell release was observed in the transfected DC supernatants. CTLs induced by the transfected DCs specifically recognized an HLA-matched AFP positive HepG2 cell line; thus, AFP-specific proliferative T-cell responses could be induced. The researchers' findings indicated that an AFP mRNA transfection strategy could generate fully functional DCs and induce specific T- cells to recognize AFP (+) HCC cells [54]. Additional studies with lentivirus engineered DCs disclosed that such DCs could activate AFP-expressing T-cells to inhibit HCC cells growth [40].

AFP Cytotoxicity against Hepatomas
In order to further assess AFP cytotoxicity against liver tumor cells, cytotoxic T lymphocytes (CTLs) were induced by dendritic cells phagocytosing HLA-A2+ restricted epitope peptides encapsulated in polylactic acid (PLA) microspheres (PLA-AFP218-226 epitope). The studies utilized HepG2 cell lines and T2-cells incubated with HLA-A2+ restricted epitope peptides derived from alpha fetoprotein (AFP#218-226, LLNQHACAV). Mature dendritic ells (DCs) obtained by inducing monocytes isolated from peripheral blood cells of HLA-A2+ healthy donors with GM-CSF and IL-4, were also employed. On 3rd day from onset of cell culture, PLA-AFP #218-226 peptide was added to the culture medium and on day 6 lipopolysaccharide (LPS) was added to induce the maturation of immature DCs. Afterward, there was observed a high avidity between AFP #218-226 and the HLA-A2 cell surface proteins. The DCs phagocytosing PLA-AFP218-226 highly expressed CD83, CD86, and CD40, while the CTLs induced by the DCs destroyed the HepG2 and T2 cells when incubated with AFP #218-226. The strong cytotoxicity against HepG2 cell lines could be induced in vitro by DCs phagocytosing PLA-AFPM #218-226 peptide microspheres, suggesting that such microspheres could serve as a new type of CTL epitope vaccine for prophylaxis and treatment of hepatocellular carcinoma [55].

AFP and T-Cell Immunotherapy
Alpha-fetoprotein (AFP) may be proposed as a potential target for T-cell-based immunotherapy for hepatocellular carcinoma (HCC); however, the number of its identified epitopes is limited and the status of AFP-specific immunological responses in hepatoma patients has not been well-characterized. To address these issues, investigators have examined the feasibility of inducing AFP-specific cytotoxic T cells (CTLs) using novel HLA-A*2402 restricted T-cell (HLA, human leukocyte antigen) derived from AFP. The relationship between its frequency of occurrence and clinical features associated with patients having HCC tumors was then analyzed. Five AFP-derived peptides, containing HLA-A*2402 binding motifs were studied; these showed high binding affinity to HLA-A*2402 which induced CTLs to secrete IFN-gamma that then cytotoxically destroyed an AFP-secreting hepatoma cell line.

The frequency of AFP-specific CTLs produced was 30-190 per 1 x 10 6 peripheral blood mononuclear cells, similar to other immunogenic cancer associated antigen-derived epitopes. Analyses of the relationships between AFP-specific CTL responses and clinical features of patients with liver cancer revealed that AFP epitopes were often recognized by CTLs in patients with advanced HCC and such data correlated to their stage of tumor progression. The analyses of CTL responses before and after therapy showed that the AFP associated treatments changed the frequency of occurrence of AFP-specific CTLs. In summary, the researchers identified five HLA-A*2402- restricted T-cell epitopes derived from AFP. These newly identified AFP epitopes seemingly provide further support for the rationale for future HCC immunotherapy when combined together with analysis of host immune responses to the HCC tumor cells [39].

Cytokine Effects on AFP Expression in Hepatomas
Other researchers investigated the effects of pegylated (PEG)-interferon (IFN)-alpha2b on alpha-fetoprotein (AFP) expression as demonstrated by protein and mRNA levels in multiple hepatoma cell lines. The number of KIM-1 hepatoma cells in culture with PEG-IFN-alpha2b decreased between 24 and 240 hours, whereas the levels of intracellular and secreted AFP increased with levels 1.9-fold and 2.9-fold higher at maximum than control cells. The AFP mRNA levels increased between 72 and 192 h, in which the levels were 3-fold higher than that of the control. In the 72-h culture with 40-5000 IU/mL PEG-IFN-alpha2b, dose-dependent increases occurred in AFP protein and mRNA expression, while a dose-dependent decrease in cell number resulted from both apoptosis and blockage of the cell cycle at the S-phase. The rate of fucosylated

AFP in the cell lysate decreased in both a dose-dependent and time-dependent manner. In the PEG-IFN-alpha2b culture of other hepatoma cell lines, cell proliferation was suppressed, while the expressions of AFP protein and mRNA increased in only two cell lines with suppression of cell proliferation unrelated to the increase of AFP expressions. In summary, these findings indicateded that PEG-IFN-alpha2b induced an increase in AFP expression at both the protein and mRNA levels [56].

Cytokines as Tumor Markers in HCC and Embolism
Serum alpha-fetoprotein (AFP) has long been established as an important tumor marker for hepatocellular carcinoma (HCC). Previous reports have indicated that HCCs were associated with increased levels of interleukin IL-6, IL-10 and hepatocyte growth factor (HGF). One study investigated the role of these cytokines as tumor markers for HCC. The expression of IL-6 or IL-10 (at 3.0 pg/ml), or high levels of HGF (>1000 pg/ml) and AFP (>20 ng/ml) were observed in only 0.3% of normal subjects. Patients with HCC more frequently had higher IL-6 and IL-10 levels ($p<0.05$), whereas HGF levels in HCC patients and were not significantly elevated compared to patients with chronic hepatitis or non-HCC tumors. Among patients with low (<20 ng/ml) AFP levels IL-6 or IL-10 expression was significantly associated with the presence of HCC ($p<0.05$). Patients with large (>5 cm) HCC tumors more often had increased IL-6, IL-10 and AFP levels (p values all<0.05). Serum levels of IL-6 and IL-10 are frequently elevated in patients with HCC but not in benign liver disease or non-HCC tumors. Thus, IL-6 and IL-10 may help identify a subset of HCC patients with low AFP level and may serve as complementary tumor markers in these patients [57].

AFP and CD4 Lymphocyte Responses
The study of AFP-specific T-cell (CD4) lymphocyte responses have been applied to patients bearing hepatocellular carcinomas (hepatomas). Investigators have shown that AFP specific CD4 (+) T cell responses to three immunodominant epitopes in HCC patients were significantly expanded during ($p <0.0001$) and after embolization ($p <0.002$) [58]. The development of higher frequencies of AFP-specific CD4 (+) T cells after treatment were significantly associated with the induction of > 50% necrosis of tumor and an improved clinical outcome. In addition, the authors identified two novel HLA-DR-restricted AFP-derived CD4 (+) T cell epitopes (AFP #137-145) and AFP (#249-258) and showed that the CD4 (+) T cells recognizing these epitopes produced Th1 (IFN-gamma and TNF-alpha) but not Th2 (IL-5)-type cytokines. AFP peptide (#137-145), AFP (#249-258), and AFP (#364-373) epitopes on specific CD4 (+) T cells were detected in HCC patients with no induction of tumor necrosis factor. Furthermore, a conventional cancer treatment was found to unmask tumor rejection Ag-cell-mediated immunity providing a rationale for combining embolization with immunotherapy in AFP secreting HCC patients [47].

Targeting the AFP-MHC complex with the CART-T cell therapy (chimeric antigen receptor t-cell therapy) for liver cancer has also been reported [59]. These results demonstrated that such therapy can elicit a potent anti-tumor response in HCC cancers. Thus, AFP is not just a tumor biomarker for diagnosing HCC, but can also play a very complicated and important role in modifying and regulating proliferation, apoptosis, autophagy, and anti-tumor immune responses in immune-associated cells [60].

Tumor-Derived AFP, Dendritic Cells, and T-Cells
Studies by Butterfield et al, have further shown that blood-derived monocytes (from health donors) cultured in the presence of cord blood derived AFP (NAFP) versus HCC derived AFP (TAFP) significantly inhibited DC differentiation and T-cell proliferation responses [61]. However, the tumor suppressive activity was solely dependent on the presence of TAFP, but not NAFP. Thus, TAFP was found to co-purify (chaperoned) with a low-molecular weight (LMW) entity transported as a ligand bound to AFP. Thus, a LMW hydrophilic ligand contributed to the impairment of dendritic cell differentiation and function. It was later determined that TAFP could directly drive human killer cell activities and tumor cell death [62]. The LMW molecule has yet to be identified, but a fatty acid or Lysophosolipid entity are being considered; however, these have yet to be positively identified. In addition, these same investigators have more recently demonstrated that TAFP decreases fatty acid metabolism and oxidative phosphorylation in the dendritic cells [63]. Finally, it was determined that the route of antigen (AFP) delivery to the DCs impacted the immune-stimulatory function of DCs towards activating both CD4+ and CD8+ T-cells [64].

Concluding Statements
This review has focused on the immunobiological roles of AFP and its utility as a biomarker to predict liver distress and adverse outcomes in liver cancer. Since the discovery that AFP was tumor associated in the mid-1960s, the functional roles of AFP have slowly emerged concomitant with its ever-growing use as a biomarker in the clinical laboratory. Even though the quantitative serum levels of AFP do not always correlate with increasing size of the hepatoma and other tumors, the use of AFP as a tumor marker for hepatomas has not abated even to the present day. Its popularity as a fetal-associated tumor marker increased dramatically in the 1970s and 1980s and achieved prominence in the postoperative monitoring of hepatocellular carcinoma and germ cell tumors (Table-1). Since AFP has been employed as a biomarker to screen and predict the presence of liver cancer, its relevant association with HCCs have prompted the underpinnings for advances of other serum markers for liver cancer [65].

The discoveries of discordant AFP serum/fluid levels correlated with liver distress and adverse hepatic outcomes gradually emerged in reports emanating from the late 1980-1990s. With each passing decade, the physiological roles of AFP has gradually increased, but only few attempts were made to merge those functions with the multitude of immunological-associated hepatoma therapies that have been reported. The research findings that small AFP-derived peptides could mount an immune response in the context of MHC class-I antigens was a milestone discovery. In the future, we can expect AFP peptide-based vaccines to become more fully utilized including those employing DNA primed methodologies [62]. These vaccines together with adenoviral-and lentivirally engineered dendritic cell interactions with cytokines, interleukins, CD1 molecules and NK receptors need to be more fully explored [66-69].

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