Drugs of the Future 1999, 24(1): 55-65

CCC: 0377-8282/99

Review Article

Significance ϧ α-fetoprotein in the development of novel therapeutic agents

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Introduction

During the past 10 years, progress in protein research has been impressive, including investigation in isolation, purification, structure determination and synthesis. Many of these advances have been made possible due to new technologies that have emerged: gene cloning and expression in foreign organisms and automated evaluation of amino acid sequences. Consequently, renewed interest has focused on functional proteins, one of which is α-fetoprotein (AFP).

During ontogeny in mammals, proteins termed oncofetal antigens are produced at various stages of embryonic, fetal and neoplastic growth. Since these oncofetal proteins may appear, disappear or fall to extremely low levels, they have also been referred to as phase-specific proteins. In adults, these proteins may reappear in the course of acute infection, tumor growth, organ dysfunction and/or regeneration. The term "oncofetal" has been derived from the dual appearance of these proteins in both fetal and tumor-associated growth. One of the most widely studied oncofetal proteins to date is AFP (1).

AFP was first discovered in human fetal blood in the 1950s by Bergstrand and Czar using paper electrophoresis (2). In 1963, Abelev *et al*. (3) demonstrated the dual appearance of this protein in fetal mouse blood and in mice bearing transplantable hepatomas. One year later, Tatarinov described a similar fetal protein in human patients with primary hepatocellular carcinoma (PHC) and testicular carcinoma (4). In the 1970s, investigation in the use of AFP in the clinical laboratory for cancer and birth defect detection and diagnosis expanded at an amazing rate. The resulting reports eventually led to the use of AFP in postsurgical monitoring of cancer patients and to the prenatal diagnosis of neural tube defects. Concurrently, reports on the synthesis, sites of origin and amino acid and carbohydrate structure of AFP rapidly emerged. By that time, it was recognized that AFP was produced in large quantities by the yolk sac and then by the fetal liver, with small amounts coming from the gastrointestinal tract and the brain during mammalian early embryogenesis. Following birth, serum levels of AFP were observed to gradually decline until the low nanogram amounts associated with adult life were achieved. Significant elevations of AFP were found to recur in adults as a normal physiological event during pregnancy and in certain malignant and nonmalignant pathological conditions.

In the early 1980s following the advent of the monoclonal antibody (MAb), antibodies against AFP were successfully used as investigational probes and as therapeutic tools (5). Detection of high and sustained serum AFP concentrations in adult humans was considered to be a reliable diagnostic indicator of AFP-producing tumors (6, 7). The conjugation of drugs (antibiotics, antimetabolites) to antibodies added further importance to the targeting of antibodies to AFP-secreting tumors (8). It was also in this time period that complementary DNA (cDNA) and genomic clones of human AFP were isolated. By the late 1980s and early 1990s, considerable interest was directed towards the regulation of AFP gene expression and the possible biological roles of AFP. It was recognized that the regulation of the expression of AFP genes was primarily transcriptional, thus providing an excellent model for the study of both tissue specificities and developmental regulation of eukaryotic gene expression. At the same time, AFP was found to exert various functions by different mechanisms.

Based on our investigations, the present review will cover several aspects of the current knowledge on AFP, especially its biological roles and significance in the development of new therapeutic agents.

Regulation of synthesis

Recent studies have identified a number of *cis*-acting regions within the promoters of mouse, rat and human

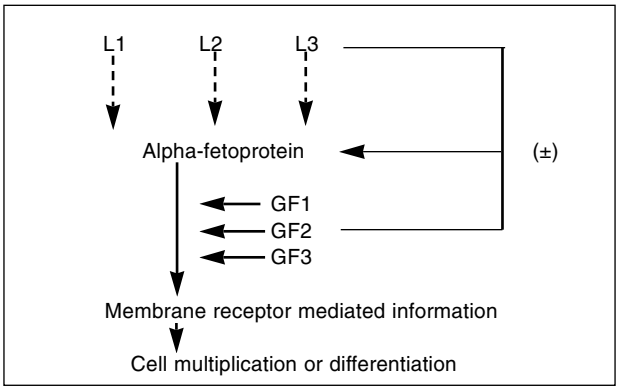

Fig. 1. Hypothetical diagram representing the regulatory loop of α-fetoprotein biosynthesis and action. L = ligands; GF = growth factors.

AFP genes that bind *trans*-acting factors and are important for the regulation of AFP gene expression (9). For example, the rat AFP gene proximal promoter was shown to be positively regulated by AP-1, the product of *c-jun* and *c-fos* oncogenes (10). AFP gene expression was also found to be regulated by glucocorticoids (11). Figure 1, representing the regulatory loop of AFP biosynthesis and action, illustrates that AFP biosynthesis can be negatively or positively regulated by its ligands and some growth factors. Ligands and growth factors can also regulate the biological roles of AFP (12).

Physiochemical properties

The application of recombinant DNA technology has resulted in rapid progress in the structural analysis of the AFP gene. In 1983, Tamaoki *et al.* isolated cDNA and genomic clones of human AFP (13). The nucleotide sequence of AFP was deduced from 3 overlapping cDNA clones, and the amino acid sequence revealed 19 amino acids in the signal sequence and 590 amino acids in mature AFP (14). This oncofetal protein is a single-chain polypeptide containing at least 15 histidine residues and 14 cysteine-cysteine disulfide bridges. Structural analysis of both human and bovine AFP has also been performed by electron microscopy, image processing and circular dichroism, revealing that the molecule is U-shaped, apparently monomeric and has outside dimensions of approximately 80 Å (15).

AFP is a glycoprotein containing 3-5% carbohydrate, depending on the animal of origin. The carbohydrate moiety is composed of a neutral and two acidic oligosaccharides whose structures have been determined (16). The oligosaccharides side chains on AFP produced by various tissues have been shown to differ in their concanavalin A (Con A) binding properties. AFPs from some animal sources have been subdivided into a multitude of isoprotein forms depending on their binding affinity to a variety of lectins, including Con A.

AFP, comprised for the most part of acidic amino acids, migrates anodally as an α_1-protein in the postalbumin region of an electrophoretogram. The molecular weight varies from 64 to 72 kD, depending on the animal of origin and the method used for purification. The isoelectric point of mammalian AFP ranges from 4.6-5.2 and the sedimentation constant is 4.5-5.0 Svedburg units with a stokes radius of 3.26 nm. The half-life of mammalian AFP is 3-5 days as compared to 15-19 days for albumin. AFP in the fetal circulation readily diffuses or is actively transported across the placental barrier and enters the maternal circulation. Thus, the rise in maternal serum AFP in the second and the third trimester of pregnancy is attributed to fetal sources.

AFP from various mammalian species has been found to exhibit significant antigenic cross-reactivity (17). Denatured peptides of AFP and albumin are immunologically cross-reactive (18). This finding, coupled with amino acid sequence data demonstrating considerable structural homology between AFP and albumin (19), supports the belief that these two serum proteins share a common ancestral gene and AFP is in fact “embryonic albumin “. However, many biological properties of AFP, which will be described later, are not at all similar to those of albumin.

Biological activities

AFP is a molecule of extremely variable biological activities. The main biological roles of AFP include: (i) osmotic pressure regulation and carrier-transport of ligands; (ii) immunoregulation; (iii) growth regulation; (iv) hematopoietic factors; (v) sexual differentiation; and (vi) antiprotease activity. Two functions of AFP have withstood the test of time, namely the carrier-transport of various ligands and the regulation of certain aspects of the immune responses. AFP binds to various ligands, each with a different binding affinity. The concept of AFP as an immunoregulatory agent is attractive in explaining immunologic tolerance during pregnancy since the fetus may be considered an allograft in the mother’s body. Furthermore, it is possible that tumor cells escape host antitumor defense by producing AFP that has a negative effect on the immune system. The third function, only recently described, is the regulatory control of cell growth in normal and neoplastic tissues. Expression of AFP is frequently seen during periods of rapid cell growth as observed in the developing fetus. Additionally, certain benign liver abnormalities, such as cirrhosis, chronic active hepatitis or carbon tetrachloride intoxication, where liver damage and subsequent regeneration occur, are also associated with increased production of AFP. Thus, growth regulation by AFP is conceivable. From the above descriptions of the various roles in which AFP is involved, it becomes increasingly apparent that no single function can be attributed to AFP.

Immunoregulatory activities

AFP molecules are capable of exerting powerful and selective regulatory forces on the immune system. No

Table I: In vitro *inhibition of mitogen-induced proliferation and one-way mixed lymphocyte reaction (MLR) of mouse spleen cells by AFP.*

	Concentration (mg/l)	Inhibition (%)
Con A reactivity	3	17.5
	6	27.1
	12	39.8
	25	49.5
	50	59.2
	100	66.0
PHA reactivity	1	19.2
	10	47.6
	50	54.8
	100	79.3
One-way MLR	5	10.5
	10	26.3
	20	34.6
	40	45.9
	80	53.4

Con A-concanavalian A; PHA = phytohemagglutinin.

such function has been reported for immunochemically similar albumin molecules.

AFP was shown to exert selective inhibitory signals to T cell-dependent immune reaction *in vitro*. AFP suppressed the phagocytosis of human macrophage and depressed mobility (20, 21). Con A and phytohemagglutinin (PHA) responses and allogenic mixed lymphocyte reaction (MLR) of mouse splenic cells were significantly inhibited by various concentrations of AFP (22-24) (Table I). In addition, AFP inhibited T cell proliferation induced by autologous (self) antigen and E-rosette formation (25, 26). AFP also suppressed interferon (IFN) or interleukin (IL)-2-stimulated natural killer (NK) cell activities (27). The formation of cytotoxic lymphocytes (CTL) could also be suppressed by *in vitro* treatment of AFP (28). AFP was found to inhibit primary and secondary IgM, IgG and IgA antibody responses to T cell-dependent antigens *in vitro* (29). However, T cell-independent antibody synthesis was not inhibited. The mitogenic response to protein A expressing *Staphylococcus aureus* bacteria (strain Cowan I), a known human B lymphocyte mitogen, was normal after treatment with AFP (23). AFP also did not affect the antibody response to 2,4-dinitrophenyl (DNP)-Ficoll, a T cell-independent antigen (30). AFP administration *in vivo* actively reduced mitogen activity of both PHA and Con A as well as secondary responses to sheep red blood cells (31).

The mechanisms through which AFP exerts its immunoregulatory influence are largely unknown. However, the mechanism of immunosuppression mediated by AFP-induced suppressor cells has been demonstrated (32-34). These suppressor cells were found to be nylon-wool nonadherent and their effect could be almost completely abolished by treatment with anti-Thy 1,2 serum plus complement. Suppressor cell precursors were found to be sensitive to cyclophosphamide (*in vivo*) and to hydrocortisone (*in vivo* and *in vitro*). Suppressor cells are resistant to different doses of radiation (Table II). In adoptive transfer experiments *in vivo*, these AFP-precultured suppressor cells were shown to reduce the humoral response of mice to sheep red blood cells and the cell-mediated cytotoxic response to allogeneic tumor cells. However, in contrast to Con A-induced suppressor cells, AFP-induced suppressor cell activity was overcome by mitogen enhancement of the proliferative response in mixed lymphocyte culture, suggesting that the suppressor cell activity induced by AFP is quantitatively weaker than that induced by Con A.

Immunoregulatory activities of AFP are also related to its effect on immune molecules. AFP was capable of decreasing macrophage Ia expression (35). Recently, human AFP was found to cause selective and rapid downregulation of monocyte MHC class II molecules, which are key molecules in antigen presentation on cells of monocyte lineage (36). AFP significantly suppressed phorbol 12-myristate 13-acetate (PMA)-induced tumor necrosis factor (TNF)-α and IL-1β production by U937, a human monocytic cell line, in a time- and dose-dependent manner, with 58% and 67% maximal inhibition observed, respectively. AFP also inhibited IFN-γ plus lipopolysaccharide (LPS)-induced TNF-α and IL-1β production. Results from Northern blot analysis showed that AFP suppressed PMA-mediated TNF-α and IL-1β mRNA expression. Moreover, PMA-induced prostaglandin PGE_2 production by U937 cells was enhanced by AFP. Pretreatment with indomethacin reversed AFP-inhibited TNF-α production by 78%. Thus, it was suggested that AFP downregulates TNF-α and IL-1β production in monocytic cells via a PGE_2-dependent mechanism (37).

Both transforming growth factor-β1 (TGF-β1) and -β2 display immunomodulatory activities and have been widely reported to regulate immunocompetent cells, including human T cells, B cells, NK cells and LAK cells. Amniotic fluids were found to reduce PHA-induced peripheral blood mononuclear cell (PBMC) proliferation in serum-free cultures by 68%. Pretreatment of amniotic fluids with anti-TGF-β1 and -β2 antibodies used alone resulted in a mean percent loss of inhibition of 33% and 52%, respectively, indicating that the immunomodulatory activity of human AFP was correlated with TGF-β1 and -β2. To investigate the mechanism of AFP-mediated inhibition, the effect of AFP on IL-2 production by Con A-stimulated PBMC blasts was also determined using the CTLL-2 cell bioassay. IL-2 production was reduced by

Table II: Properties of AFP-induced suppressor cells.

Suppressor cells pretreated by	% Inhibition of cytotoxicity
–	92
Nylon column fractionation	79
Anti-Thy 1,2 + complement	26
Low dose of irradiation (400 R)	82
Higher dose of irradiation (700 R)	61
Hydrocortisone 25 mg *in vivo*	45
Hydrocortisone 10^{-6} M *in vitro*	34
Cyclophosphamide 100 mg/kg *in vivo*	47

Table III: Summary of immunoregulatory activities of AFP-

Immunoregulatory activities	Ref.
Inhibition of macrophage function	20, 21
Inhibition of T cell mitogen-induced lymphocyte proliferation	22, 23
Suppression of allogenic mixed lymphocyte reaction	24
Suppression of T cell proliferation induced by autologous (self) antigen	25
Inhibition of E-rosette formation	26
Suppression of activated NK cells	27
Inhibition of cytotoxic lymphocyte generation	28
Suppression of T cell-dependent antibody synthesis	29
Induction of suppressor cells *in vitro* and *in vivo*	32-34
Inhibition of cellular expression of macrophage Ia antigen	35
Downregulation of monocyte MHC class II molecules	36
Downregulation of cytokine activities in immune cells	37-39

56% in amniotic fluid-treated blasts and by 61% in AFP-treated blasts, as compared with controls (38).

Murine listeriosis is a valuable model for investigating the role of leukocytes, macrophages and cytokines in the development of cellular immunity. The *in vivo* immunosuppressive effect of AFP was demonstrated in infection experiments in which transgenic mice expressing human AFP were infected with *Listeria monocytogenes*, a facultative intracellular pathogen. The transgenic mice displayed a diminished ability to eliminate bacteria during the early stage of infection. In addition, a significantly reduced production of IFN-γ was observed in liver and sera and a decrease of TNF was noted in spleen. These data indicate that AFP suppresses the production of IFN-γ and TNF in NK cells and macrophages so that the mice could not eliminate the bacteria. This is the first report showing that AFP has an *in vivo* immunosuppressive effect on cytokine production which is related to early host defense against microorganism infection (39).

The effect of AFP on immune function is mediated by a cell surface receptor on the immune cells. AFP may bind to specific receptors on monocyte/macrophage as well as on activated T lymphocytes with malignant or blastic transformation, but not to resting T lymphocytes (40, 41). AFP appears to bind directly to its receptors on the cell membrane, resulting in a variety of changes in the immune function of cells. Studies are in progress to determine the precise mechanisms of how the receptor-AFP interaction mediates these changes in immune functions.

In conclusion, AFP has been reported to be a multifunctional immunoregulatory molecule with stimulatory as well as inhibitory activities. Examples of the reported immunoregulatory effect of AFP are summarized in Table III.

Regulation of tumor growth

It has been observed that the passive administration of anti-AFP serum inhibits the growth of AFP-producing hepatoma cells *in vitro* and *in vivo*. This finding has potentially important implications that AFP may function as a tumor-promoting agent. Our investigations indicated that human AFP did indeed stimulate the growth of mouse ascites hepatoma-22 (H-22) cells *in vitro* independent of immunosuppressive effects. To further confirm the specificity of action of AFP, H-22 cells were incubated with a combined AFP/anti-AFP antibody. The growth-stimulatory effect of AFP was obviously abolished by anti-AFP antibody (42) (Table IV). In another *in vitro* experimental system, growth stimulation by AFP in H-22 cells was found to be associated with an increase in RNA synthesis. Results showed that human AFP (350 mg/l) enhanced RNA synthesis in mouse H-22 cells by 253%, while human serum albumin had no effect. The effect of AFP was also significantly abolished by treatment with AFP antiserum(43).

Table IV: Effect of AFP and anti-AFP antibody on hepatoma-22 cell growth in vitro.

	Concentration (mg/l)	% Stimulation
AFP	9	29.6
	18	62.9
	50	118.5
	75	125.9
	100	140.7
AFP + anti-AFP	50 + 55	22.2
	100 + 110	18.5

In addition to its direct stimulation of growth in hepatoma cells, AFP dose-dependently enhanced human mammary tumor cell proliferation induced by platelet-derived growth factor (PDGF) *in vitro*; ablation of endogenous AFP by affinity chromatograph significantly reduced the proliferative activity of human mammary tumor cells (44). Human AFP also promoted the growth of mouse Ehrlich ascites carcinoma (EAC) cells (42) and increased RNA synthesis in mouse sarcoma-180 cells *in vitro* (43).

The mechanism by which AFP exhibits direct regulation of growth remains to be explained. It was demonstrated that the observed effect of AFP on cell growth was not simply due to nonspecific addition of exogenous protein since equivalent doses of human serum albumin did not affect tumor cell growth. Direct exposure in culture of AFP-secreting mouse tumor cells to heterologous anti-AFP IgG produced a cytotoxic effect, suggesting that AFP

Table V: Effect of AFP on the natural history of Moloney sarcoma virus-induced tumors.

Group	No. tumors/No. mice inoculated	No. progressors/No. tumors	% Survival at 100 days
PBS	13/15	2/13	80
Albumin	16/18	3/16	85
Transferrin	15/19	2/15	75
AFP	16/19	13/16	20
AFP + anti-AFP	12/16	1/12	80

resides at the tumor cell surface (45), and AFP cell-surface receptors have been detected in human breast cancer cells (46). The following possibility may be considered: AFP may bind to the tumor cell membrane for purposes of self-controlled growth by the autocrine mechanisms. Thus, the tumor cell membrane surface could be a major target of analysis. It has been known that some growth factors such as PDGF also stimulate tumor cell growth. Considering that heterologous serum which contains high levels of growth factors was used to supplement the culture medium, it is also possible that AFP functions to modulate self-controlled growth of tumor cells by increasing the efficiency of the paracrine cycle of growth factor-mediated cell proliferation. In addition, it has been found that AFP inhibits cell apoptosis, an activity which may contribute to its regulatory effect on *in vitro* tumor growth (47).

The effect of AFP administration on the development of experimental tumors in mammals has been investigated *in vivo* with the use of chemical and virus carcinogens. Studies have shown that AFP accelerated the appearance of plasmacytomas in pristane-primed mice while albumin and transferrin were ineffective (48). In addition, after inoculation with Rous sarcoma virus, quails treated with chicken AFP developed tumors with shorter latent periods as compared to the tumors developed in untreated quails (49). The influence of murine AFP on the threshold dose, mean tumor size, regression time and number of progressors for Moloney sarcoma virus (MSV)-induced tumors in mice was also studied. AFP-treated mice developed larger tumors which required a longer period for regression and displayed a significantly higher mortality rate. Furthermore, AFP, but not murine albumin or transferrin, allowed the growth of tumors when normally subthreshold doses of virus were injected. These effects of AFP were abolished by pretreatment with anti-AFP antibody (50) (Table V).

The mechanisms of AFP regulation of *in vivo* tumor growth are not very clear. In addition to direct stimulatory action on tumor cells, the immunosuppressive activities of AFP in relation to its occurrence in certain malignant conditions may also be associated with the *in vivo* tumor-promoting effect. It was found that the influence of AFP on the natural history of MSV-induced tumors involved the generation of suppressor cells (50). Indeed, the immunosuppression produced by AFP was demonstrated in unmanipulated mice following transfer of spleen cells from AFP-treated donors. This transfer of immunosuppression was noted in IgG and IgA secondary responses to sheep red blood cells as well as by quantitating the response of T cell mitogens on spleen cells. These results suggest that immunosuppression accompanying certain tumors may be attributable to significant levels of AFP.

Other biological activities

AFP synergized growth factors such as epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) cause a marked increase in the proliferation of porcine granulosa cells. In the monolayer culture system, AFP alone did not stimulate the proliferation of porcine granulosa cells from small ovarian follicles. However, when combined with EGF and IGF-I or PDGF, AFP significantly enhanced growth factor-mediated proliferation. Equivalent doses of purified human serum albumin or transferrin had no effect (51).

An intracellular pool of AFP has been detected in the brain, suggesting a role for this protein in the development of neuronal cells in processes such as cell proliferation, differentiation and migration. Moreover, the observation that high brain AFP levels correspond with periods of critical hypothalamic differentiation suggests a role for AFP in processes of differentiation and/or maturation (52).

In addition, placental cells underwent increased proliferation *in vitro* in response to AFP (53). It is conceivable that AFP may regulate the differentiation of stem cells into more specialized cells of the erythrocyte series. Hence, AFP may function indirectly in hematopoeisis (54).

Significance in the development of new agents

Just as in the 1980s when investigation focused on the widespread use of MAb against AFP in the detection of human AFP-secreting tumors, the 1990s has become a period in which the potential therapeutic uses of human AFP itself have been explored. There are several explanations to indicate why AFP is a good candidate for therapeutic use. First, AFP is a normal native constituent of the body and is capable of traversing both the intercellular and intracellular fluid compartments. Second, the body is immunologically tolerant to AFP because small amounts (ng levels) are constantly being secreted into the circulation. Therefore, AFP is not considered a foreign antigen when injected into the body and dose not pose a

threat during its metabolic removal. Third, AFP can operate in either a fetal, neonatal or adult environment and is rapidly eliminated by the body. Finally, the novel strategy of treatment of tumors through an AFP target is innovative and attracting the attention of the world's scientists (55).

Potential therapeutic uses

1) Immunotherapy

Many patients with autoimmune disorders experience a period of remission during pregnancy. Such cases may well represent a "natural experiment" which may provide evidence supporting the theory that AFP, serving in an immunoregulatory capacity, is the biochemical agent responsible for the temporary inhibition of these clinical manifestations. It is conceivable that AFP could someday be employed to treat certain autoimmune disorders such as rheumatoid arthritis (RA), myasthenia gravis (MG), allergic encephalomyelitis, acquired hemolytic anemia and lupus erythematosus-like disease.

Studies have demonstrated that human AFP functioned as an immunosuppressant to ameliorate the development of arthritis. Experimental arthritis was induced in the knee joint of transgenic mice expressing human AFP and normal mice by immunization with methylated bovine serum albumin in Freund's complete adjuvant. Results showed that only 21% of the transgenic mice developed definite arthritis as compared to 56% observed in normal mice (56).

Preliminary evidence, although yet unconfirmed, has suggested that antibodies to acetylcholine receptor (AChR) from patients with MG could be inhibited from binding *in vitro* by the addition of crude or purified AFP. Preliminary studies showed that intraperitoneal injection of AFP into animals immunized against AChR prevented the induction of experimental autoimmune MG; moreover, after treatment with AFP, muscle weakness disappeared in animals immunized with purified basic (brain) protein (57). These findings support the possibility of using AFP therapeutically in autoimmune demyelinating disease.

Other studies involving experimental allergic encephalomyelitis-like disease in NZB mice have demonstrated that pharmacological doses of murine AFP significantly altered and delayed the immunoglobulin-class appearance of thymic-dependent autoantibodies (58).

These results indicate that AFP, with its selective immunoregulatory functions and lack of toxicity, may be a potential clinical immunosuppressant.

2) Miscellaneous uses

Just as drugs can be conjugated to MAbs to produce a "magic bullet", a protein which targets a particular target organ can be utilized in a similar fashion. Therefore, AFP can be conjugated to compounds such as alkylating agents, antibiotics and antimetabolites to act on hepatoma cells. Another approach would be to construct a radiolabeled AFP molecule conjugated to an antitumor drug to benefit both tumor detection and therapy. Moreover, since it is known that a number of plant and bacterial toxins kill sensitive cells by catalytically inhibiting protein synthesis, AFP could be conjugated to the A-chain of either ricin or diphtheria toxin and used therapeutically to destroy target cells providing that the cell bears the proper AFP receptor. Such a system utilizing AFP/toxin heteroconjugates would be restricted to hepatoma cells thus leaving normal hepatic cells unharmed. Finally, human and mouse AFP are capable of binding to the α-subunit of human trophic hormones such as FSH, LH, TSH or hCG. This binding phenomenon has been demonstrated by both electrophoretic and column chromatographic procedures. Because of enhanced uptake of hormones into cells, one could conceive of a therapeutic application of such an AFP-hormone conjugate to be used for patients suffering with various cell surface receptor diseases including reduced numbers of receptors or receptors with altered affinities (59).

It is known that a high level of AFP expression is transcriptionally controlled by the 5'-flanking sequence of the AFP gene. Using the 5'-flanking sequence as a promoter for suicide genes such as herpes simplex virus thymidine kinase gene, *E. coli* cytosine deaminase gene or varicella-zoster virus thymidine kinase gene, the therapeutic efficacy of virus-mediated suicide gene transduction followed by administration of noncytotoxic prodrugs such as ganciclovir, 5-fluorocytosine or 6-methoxypurine arabinoside, has recently been studied in tumors *in vitro* and *in vivo*. Viral vector transduction demonstrated that the suicide gene was expressed only in the AFP-producing cells and not in cells not producing AFP. The suicide molecules expressed by the suicide gene enzymatically converted these prodrugs into potent inhibitors of DNA synthesis, resulting in the selective death of transduced tumor cells. Thus, it is suggested that the AFP promoter sequence alone can provide enough tumor-specific activity for therapeutic gene expression and induce selective growth inhibition in virus-infected, AFP-producing human tumor cells (55, 60, 61).

3) Methods of administration

Novel methods of administration of AFP must be devised. For example, the classical methods of intravenous, intramuscular and subcutaneous injections have been and will probably remain the most commonly used methods of AFP administration. However, the use of encapsulated glycerin implants of AFP may also be considered. These implants allow AFP to be released in low doses over prolonged periods of time. Another method of continuous delivery may be the use of osmotic minipumps which release a predetermined amount of AFP at a given rate. Methods of oral administration are also possible. AFP could also be encapsulated in lipoidal

micelles for intravenous administration. Methods of topical cream or oil application in ointment form are feasible if AFP could be formulated into a lipoidal derivative. However, studies are necessary to devise a simple, safe and effective means of administering therapeutic levels of AFP over prolonged periods of time.

4) Potential risks

As a normal constituent of mammalian serum, AFP at physiological levels would have no toxicity *in vivo*. However, the other potential risks or disadvantages of employing AFP as a therapeutic agent must be considered.

Purified human AFP has never been administered to a syngeneic host and there is presently considerable lack of knowledge in this field. Also, it is not known whether different isoproteins of human AFP will evoke an immune response in humans. Lastly, hybrid molecules formed by the fusion of animal with human AFP will undoubtedly stimulate antibody production in the host treated with this compound. Another risk of human AFP administration to a syngeneic host is the growth regulatory potential of AFP. Both rodent and human AFP have been shown to exhibit growth regulatory properties in reproductive tissues. Such effects will have to be assessed according to doses first in animal models, then in human cell cultures and finally in human volunteers. In a similar manner, the immunoregulatory properties of AFP will have to be examined for dose effects on host immune responses.

These disadvantages of AFP administration may be rendered minimal by the judicious application of dose, protein purity and timing and route of administration. Since AFP has been likened to “fetal albumin” and enters the maternal circulation, it appears unlikely that it adversely affects adult tissues.

New target for tumor therapy

In many conventional chemotherapeutic regimens of tumors, drugs display their activities at the level of DNA. However, these drugs are often deleterious for other rapidly dividing cells and cause well-known toxicities. Therefore, attempts aimed at other pharmacological targets are especially interesting. Taking previous investigations into account, results strongly infer that AFP, as a tumor-promoting factor, contributes to the generation and development of certain tumors such as PHC and is an important target of tumor therapy. Suppression of gene expression and biological activities of AFP would become a new strategy for the treatment of AFP-associated tumors.

Prostaglandin PGJ_2, a potent inhibitor of proliferation of tumor cells, was shown to inhibit the growth of HuH-7 human hepatoma cells and dose-dependently reduce AFP concentrations. Studies using Northern blot analysis have demonstrated that PGJ_2 caused a marked reduction in AFP mRNA levels while levels of actin-β mRNA remained unchanged. Results from transient chloramphenicol acetyltransferase plasmid transfection experiments showed that PGJ_2 did not suppress the enhancing activity of AFP which may regulate both AFP and albumin gene expression in HuH-7 cells. Instead, a selective repression of the AFP promoter activity was observed. These results suggest that PGJ_2 suppressed not only cell growth but also expression of the AFP gene through the repression of its promoter activity at the transcriptional level in human hepatoma cells (62). TGF-β1 was also found to downregulate AFP gene expression without affecting albumin gene expression in HuH-7 cells (63). Specific transfer factor (STF) prepared from the spleen of mice immunized with AFP antagonized the promotive effect of AFP on DNA, RNA and protein synthesis of H-22 hepatoma cells and reversed the AFP-mediated immunoinhibition in H-22-bearing mice. This effect of AFP was antigen-specific and donor-dependent (64, 65).

Hepatocyte growth factor (HGF) is a potent mitogen for hepatocytes. However, in certain human hepatoma cell lines, growth is inhibited by HGF. It was found by Northern blot analysis that HGF dose-dependently suppressed AFP mRNA expression in hepatoma cells without altering actin-β mRNA expression (66).

When BEL-7404, a human hepatoma cell line, was grown in a medium containing dimethyl-4,4’dimethoxy-5,6,5’,6’-dimethylenedioxybiphenyl-2,2’-dicarboxylate (DDB), a new antihepatitis drug, the secretion of AFP was significantly lower as compared to control cells, whereas albumin secretion was not markedly changed. DDB also suppressed the expression of AFP mRNA and *c-myc* mRNA and enhanced the expression of p53 mRNA (67). L-4-Oxalysine (OXL) is a new antitumor and immunoregulatory agent of natural origin first discovered in China (68, 69). Studies showed that OXL (12.5 mg/l) had no marked antagonistic action on the AFP-stimulated growth of H-22 cells. However, when the same concentration of OXL was used in combination with 5-fluorouracil (3.1 mg/l), there was an increase in the percent of suppression on H-22 cell growth stimulated by AFP. In contrast, OXL was inhibitory on the activity of T cells induced by AFP (34). AFP-suppressed splenocyte proliferation and one-way MLR were also antagonized by treatment with OXL. Using an *in situ* hybridization technique with digoxigenin-labeled AFP cDNA probes and an immunocytochemistry method, we found that exposure of BEL-7404 hepatoma cells to OXL resulted in an obvious decrease in AFP mRNA and cytoplasmic AFP protein, indicating that OXL inhibited AFP gene expression in human hepatoma cells (70, 71). OXL is also effective against H-22 hepatoma growth *in vivo*. Preliminary clinical studies indicated that treatment with OXL induces improvement of symptoms in PHC patients. Therefore, according our investigations, the antihepatoma activity of OXL is suggested to be associated with its influence on AFP synthesis and action. Thus, OXL has the potential of being developed as a new anti-PHC drug based on the AFP target.

The recent use of antisense oligonucleotides as a therapeutic tool in modulating gene expression represents a newly established strategy for treating tumors.

Using this approach, oligonucleotides can serve as effective blockers of translation through sequence-specific hybridization with targeted mRNA. Hence, designing antisense oligonucleotides for blocking translation of AFP mRNA may be another important approach for the treatment of AFP-associated tumors. On the other hand, the cellular mechanisms responsible for AFP gene expression have been widely investigated. Proteins encoded by nuclear oncogenes such as *c-jun*, *c-fos* and *c-myc* are transcriptional factors that initiate cellular AFP gene expression (9). Therefore, oncogene inactivation by antisense oligonucleotides may also be required so that AFP gene expression and further tumor cell growth are successfully inhibited by AFP antisense oligonucleotides. Ribozymes are naturally occurring RNA enzymes that catalyze RNA cleavage and RNA splicing reaction in a sequence-specific manner. Ribozymes that catalyze RNA cleavage have the potential to be developed for gene therapy application as inhibitors of AFP gene expression. However, major problems in gene therapy are the stability of the therapeutic antisense/ribozyme *in vivo* as well as the specific cellular targeting (55, 72, 73).

Conclusions

AFP, a member of the so-called oncofetal proteins family, does not appear to be a mere carrier protein. Many other biological activities, especially possible roles in the immune system and tumor growth regulation, have been tentatively attributed to the protein (74-77). However, it must be noted that progress in the elucidation of mechanisms has been relatively difficult to achieve since the various biological roles of AFP depend on many factors. There are factors related to the protein itself including its isoforms, its known and unknown ligands, as well as the effective concentration to be used. There are also factors related to the conditions of cells on which the AFP acts (78) and the cell cycle must be taken into account. Thus, there are still many fields to investigate.

Current efforts have focuses on several major research areas such as the understanding of autoimmune diseases and the targeting of drugs. AFP is situated at the crossroads of these fields of research. Present genetic engineering techniques might enable the synthesis of native AFP and modified analogs with enhanced potency. Therefore, we are currently on the threshold of an exciting era in which the therapeutic potential of AFP may be fully appreciated and utilized. However, to do this, we must first recognize the complex biological activities of AFP. Novel AFP molecules must be developed to improve target cell selectivity and reduce cell cytotoxicity in normal tissues. In addition, further studies are necessary in order to devise a simple, safe and effective means of administering therapeutic levels of AFP over prolonged time periods.

During the past 50 years numerous compounds have been used in the treatment of cancer. Some have been successful in improving remission in a wide range of malignant diseases and in some cases, for example lymphomas and testicular cancers, have resulted in cures. All of these agents which interact with DNA, however, have a low degree of specificity. Thus, the resulting side effects have limited the effectiveness of these agents and cancer still remains one of the major causes of premature death. Therefore, it is necessary to find new methods to improve therapeutic effectiveness and reduce the toxicities. It has been recognized that AFP is an important stimulator of tumor growth. Therefore, opportunities are now emerging in which anti-AFP strategies can be translated into effective approaches for developing cancer-specific agents with a greater selectivity. In particular, the identification of oncogenes and their protein products has not only given us an insight into how changes in the biochemistry of cells can lead to oncogenesis but also has presented potential targets for novel anticancer therapies. Considering that RAS and SRC proteins are typical of the intracellular signaling pathway and MYC and AP-1 proteins are important transcription factors that regulate genes encoding for proliferation-associated proteins, a number of oncoproteins may be involved in the mechanisms by which AFP receptors transduce their signals to the nucleus of tumor cells. It is therefore likely that anti-oncoprotein-based therapeutic regimens will also play an important role in tumor therapy aimed at the AFP target for the next years (79, 80).

Figure 2 summarizes the AFP-mediated tumor cell proliferation regulatory pathways and the possible areas where agents can be designed to have therapeutic effect.

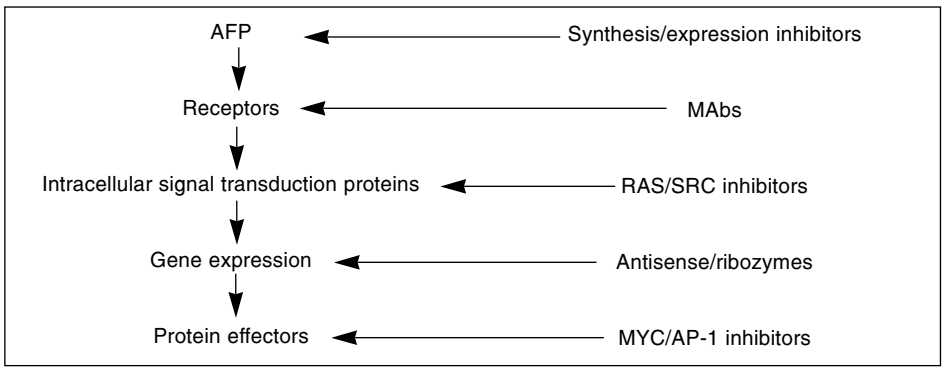

Fig. 2. AFP-mediated tumor cell growth regulatory pathways and new therapeutic strategies aimed at the AFP target.

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