

THE ALPHA-FETOPROTEIN RECEPTOR: A WIDESPREAD CANCER MARKER OF CLINICAL POTENTIAL

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Introduction:

Thirty years ago, Abelev *et-al* reported the existence of the first oncofetal antigen, alpha-fetoprotein (AFP)¹. After reaching the highest concentration among circulating proteins during fetal life, AFP almost disappears after birth, attaining a normal adult serum concentration of less than 50 ng/ml². However, in some malignant diseases such as hepatocarcinomas and teratocarcinomas, AFP plasma levels can be one thousand-fold higher³. These findings drew the attention of clinicians, who envisaged new means for diagnosing and monitoring cancer patients, and of basic researchers who started collecting data on the physiology of AFP during fetal life. Using the immunoperoxidase technique, Benno and Williams described the distribution of AFP in the developing rat brain⁴. Soon after, a series of reports confirmed the presence of AFP and other plasma proteins within embryonic neuroblasts in several species including rodents⁵, sheep⁶, birds⁷, primates⁸ and humans⁹. It was noticed that as fetal development progresses, the distribution of AFP and serum albumin (SA) in neural tissues follows a similar pattern from one species to another¹⁰: When a given nervous system structure is very immature, no intracellular AFP or SA is visualized. Then, rather suddenly, the staining becomes positive for both proteins, even within the same cell¹¹. The cells remain positive for a certain period of time, depending upon the animal species and the neural structure in question. After some time, the staining, which is always cytoplasmic, gradually fades both in intensity and in the number of positive cells, first for AFP, and then for SA. Neither protein is found in fully mature nervous structures. Other serum proteins, such as IgG or ovalbumin in chicken embryos, are never present in neuroblasts despite being conspicuously present in the cerebrospinal fluid¹².

AFP uptake by embryonic and fetal cells:

A question arising from these initial observations was whether AFP and SA are incorporated from extracellular sources or synthesized by neuroblasts. While it is not clear whether neural cells are capable of synthesizing these plasma proteins^{13, 14, 15, 16}, it has been well established, both *in-vitro*^{17,18} and *in-vivo*^{19, 20} that neuroblasts can readily incorporate AFP and SA from extracellular sources.

The injection of homologous ¹²⁵I-AFP into pregnant rats¹⁹ resulted in an accumulation of radioactivity in the same fetal tissues in which native intracellular AFP had been previously evidenced. Autoradiographies from these experiments also confirmed the exclusive cytoplasmic distribution of the internalized AFP seen with immunoperoxidase and most importantly that most other fetal tissues also take up AFP²¹. Some of the initial *in-vivo* uptake experiments were carried out with heterologous proteins²⁰. When newborn rat serum was injected into the mesencephalic cavity of chicken embryos, the staining for rat AFP and rat SA showed a gradient (from the lumen outwards), identical to that displayed by their native counterparts. By contrast, the injection of rat IgG (MW ~150,000) or OA (MW ~43,000) resulted in no uptake even when OA was injected at 30 times its normal concentration (twice the normal molar concentration of AFP) in the chicken embryo cerebrospinal fluid.¹² These results indicated that the uptake of AFP and SA is selective and it is not species specific.

It was not clear, at the time, whether the progressive disappearance of intracellular AFP at the end of gestation was a cell regulated process or was it just a consequence of the dropping

concentrations of circulating AFP. The first possibility appears to be right since it was demonstrated, first in chicken²² and then in human embryos²³, that spinal ganglion neural cells become AFP negative while its serum concentration is still rising. On the other hand, the staining for SA in the same structures remains positive for a significant period of time after AFP becomes undetectable thus suggesting that there is no access impairment of serum proteins to ganglion neuroblasts.

AFP uptake by cancer cells:

The data at that point supported the following conclusions: (a) Embryonic and fetal cells from a variety of tissues take-up AFP, (b) The uptake is protein-selective, (c) The uptake is related to the degree of cell differentiation and (d) AFP from one species can be taken-up by embryonic cells from a different species. All of these elements were in agreement with the advanced hypothesis of a specific receptor mediated mechanism of endocytosis^{7,20} whose expression would be regulated according to the degree of cell differentiation^{10,22}. The uptake of heterologous AFP suggested that the binding site in both the receptor and the ligand is conserved across species, perhaps in the form of short aminoacid sequences that remained almost unchanged throughout evolution within the AFP/Albumin family of proteins²⁴.

Cancer cells share a number of common biochemical and antigenic features with fetal cells²⁵. Hence, the hypothesis was proposed that malignant cells, derived from tissues which incorporate AFP during fetal life, might re-express the ability to take-up AFP, via a receptor which would therefore qualify as a new widespread oncofetal antigen. To verify this hypothesis, a number of studies, both *in-vitro* and *in-vivo* were undertaken. *In-vitro* experiments evidenced the uptake of AFP by a variety of cancer cell lines such as MCF-7²⁶ (a human mammary carcinoma), a nickel induced rat rhabdomyosarcoma²⁷, the C1300 mouse neuroblastoma²⁸ and several human leukemias²⁹. *In-vivo* studies carried out in mice showed a significant accumulation of injected ¹²⁵I-MoAFP in spontaneous mammary carcinomas when compared to normal tissues³⁰.

Tumour imaging using radiolabeled AFP:

Following the injection of ¹³¹I-MoAFP, spontaneous mouse mammary carcinomas could be clearly imaged³¹ allowing, in some cases, the visualization of tumours as small as 3 mm in diameter³². In the latter study, eleven out of twelve tumors were detected using a standard gamma camera linked to a computer. Another study in mice showed that transplanted C1300 neuroblastoma tumours could be imaged as well³³.

These considerations, along with the high quality of the images obtained in animals, prompted us to scan patients injected with ¹³¹I-HuAFP. As the uptake of AFP is related to the degree of cell differentiation, it was possible that undifferentiated cell precursors in organs such as the gonads and the bone marrow could be adversely affected by the accumulation of radioactivity. In order to address this possibility, we injected twelve female and six male mice with a dose of 0.1 mCi of ¹³¹I-AFP per animal, the equivalent, weight-to-weight, to 250 mCi for an adult human being. No abnormalities were detected in these mice, which had a normal life span. When mated, fertility was normal, and two generations of their offspring were also normal.

Two healthy individuals (including the author) and three cancer patients were thus injected with 300-600 ug of ¹³¹I-HuAFP (~0.6 mCi) and scanned 2 to 7 days later³⁴. Figure 1 shows the image of a male patient bearing a large abdominal tumour (diagnosed as a stomach carcinoma). The arrows point to large radioactive spots on the area of the palpable mass.

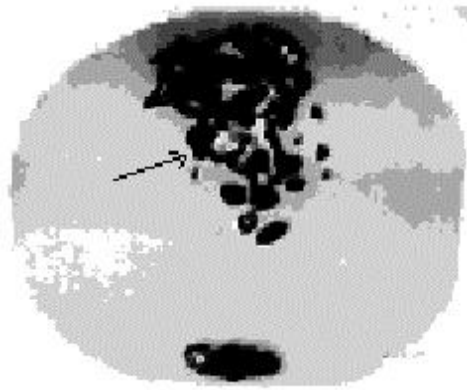


Figure 1: Stomach cancer scintigraphy using ^{131}I -AFP. This patient had a palpable abdominal mass on the area pointed at by the arrow. The carcinoma had infiltrated the transverse colon.

The second case was a female patient bearing bone metastases of a breast carcinoma. The original tumour had been surgically removed 2 years before. This patient was first injected with $^{99\text{m}}\text{Tc}$ -Pyrophosphate (which accumulates in regenerating bone tissue) and then imaged according to routine procedures. The image thus obtained showed several bone metastases in the lumbar vertebrae and the iliac crests (Figure 2a). Before moving the patient, the location of the metastases was pinpointed on her back with the aid of a radioactive pen which was moved about until its image on the screen overlapped with the previously acquired lesions. Each spot was then marked on the patient's back using a non-toxic felt. A week later the patient was imaged again, fitting the gamma camera with a ^{131}I collimator. As expected, no image could be collected due to the short half life of $^{99\text{m}}\text{Tc}$ (approximately 6 hours). The patient was then injected with ^{131}I -HuAFP and scintigraphies were taken at 24-48 hour intervals. Figure 2b shows the image obtained 6 days after the injection. With the aid of the radioactive pen, we were able to demonstrate that the lesions detected with ^{131}I -HuAFP did in fact coincide with the marks on the patient's back and therefore with the metastases detected using $^{99\text{m}}\text{Tc}$ -Pyrophosphate.

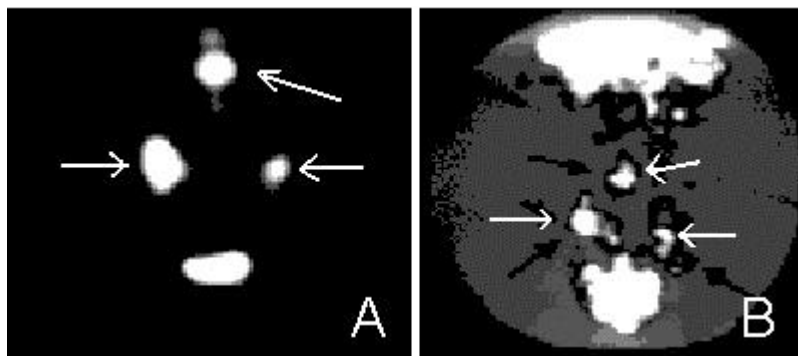


Figure 2: Scintigraphy of bone metastases of a breast carcinoma. The patient was first injected with $^{99\text{m}}\text{Tc}$ -Pyrophosphate and then imaged according to routine procedures. In A, the arrows show the areas of bone regeneration (metastases in the lumbar vertebrae and the iliac crests). Seven days later the patient was injected with ^{131}I -HuAFP and scintigraphies were taken at 24-48 hour intervals. In B, an image obtained 6 days after the injection of radioactive AFP. The 3 lesions pointed at by the arrows coincide with those in A.

The third patient was a 73 year old female who had a history of iliac metastases of a breast carcinoma. Following chemotherapy and hormone therapy, the patient had been in remission for 18 months when she was imaged as described above. Bone regeneration is a slow process and it is generally accepted in nuclear medicine practice that $^{99\text{m}}\text{Tc}$ -Pyrophosphate positive images can

remain as such many months after tumour remission. In this third patient, the ^{99m}Tc pyrophosphate scintigraphy was still positive, whereas no radioactive spots could be detected with ^{131}I -HuAFP thus suggesting that AFP accumulation in bone metastases is not related to bone regeneration. Chronic inflammatory processes are also unlikely to accumulate AFP as shown in experimental animal models³⁵.

These results, taken together with the images obtained in animals, strongly suggest that AFP might prove useful for tumour imaging in humans. Systematic studies involving a larger number of patients are necessary to fully ascertain the actual merits of this promising technique. AFP mediated scintigraphy offers several interesting features: Employing a homologous molecule virtually precludes anaphylactic reactions and the fact that only one radiopharmaceutical is required to detect a wide variety of tumours is an important practical consideration. Using an AFP fragment containing the binding site for its receptor rather than the entire molecule could result in a faster clearance thus favoring the use of ^{123}I and other isotopes better suited than ^{131}I for human imaging. Producing such a fragment by synthesis or by genetic engineering would also reduce its cost while allowing to tailor the molecule for optimal uptake, clearance and radiolabelling.

AFP receptor detection:

Even though the presence of an AFP receptor to explain AFP uptake was first proposed in 1981⁷, indirect indications of the existence of this receptor-ligand system can be traced to earlier papers^{36,37}. The latter reference reported the presence of two AFP rich ultracentrifugation fractions in immature rat uterine cytosols: A 4 S fraction, positive when tested with an anti-AFP antiserum and an 8 S fraction in which the immunological detection of AFP was only possible after treatment with 0.4 M KCl, which also converted the 8 S fraction into a 4 S fraction. It is likely that the 8 S band corresponded to a receptor-AFP complex, which was dissociated at high KCl concentration. This dissociation was also observed by Smalley and Sarcione, who reported that the uterus of immature rats synthesizes AFP in a form that was not recognized by anti-AFP antibodies unless it was treated with 0.4 M KCl³⁸. Sarcione *et al*³⁹ also reported the presence of AFP in cancer cell extracts, in a complexed form which could be dissociated by KCl. These authors also demonstrated that AFP is synthesized by the MCF-7 human breast cancer cell line as a complex which is not recognized by anti-AFP antibodies unless it is dissociated from its receptor by KCl treatment⁴⁰.

The first direct evidence of a receptor for AFP was provided by Villacampa *et al*⁴¹ using MCF-7 cells. The results that we found on this human mammary carcinoma cell line are consistent with a two-site receptor model exhibiting positive cooperation. The higher affinity site exhibited a Kd of 1.5×10^{-9} M and a number of 2000 sites per cell. The lower affinity site, with a number of 320000 sites per cell showed a Kd of 2.2×10^{-7} M. A similar receptor system was subsequently detected on the surface of the YAC-1 mouse T lymphoma⁴² (but not on normal adult mouse T cells) and on the human U937 and THP-1 cell lines⁴³. The number of sites/cell as well as the binding kinetics and affinity vary from one system to another. Binding assays were not restricted to intact cells but also extended to MCF-7 and primary breast cancer cytosols thus evidencing the soluble nature of the AFP receptor⁴⁴.

Monoclonal antibodies against the AFP receptor:

Although the AFP receptor can be detected using labeled AFP, this approach suffers from a number of drawbacks: In the first place, the receptor needs to be functionally intact. As an example, frozen tumour sections and malignant cells in suspension bind fluoresceine labeled AFP, but routine paraffin sections do not (unpublished results). Secondly, receptor molecules already complexed to AFP might not be detected efficiently since labeled AFP has an equal or lesser affinity for the receptor than the native AFP present in the complex. Thirdly, the relatively

low affinity constants reported mean that the signal/noise ratio in binding assays is also low. This results in an unusually high background. Forthly, procuring pure human AFP in large quantities poses severe practical difficulties.

Monoclonal antibodies (Mabs) are an appealing alternative for tracing the AFP receptor. Thus, we screened the supernatants of a fusion made against a pool of human mammary carcinoma membranes and found 2 clones that inhibit the binding of AFP to malignant cells and which conversely are inhibited from reacting with the AFP receptor by an excess of AFP⁴⁵. We also purified the receptor from human cord serum and verified that the isolated fraction reacted both with the Mabs and with labeled AFP. Immunohistological studies carried out with one of these Mabs (167H.4) on paraffin sections, showed a strongly positive staining of human fetal muscle and mammary carcinomas. Interestingly enough, the Mabs also recognized mouse and dog cancer cells⁴⁵. This cross reactivity is in agreement with the previous finding that AFP from one species is taken-up by cells from other species^{20,42} and it provides a useful tool for *in-vivo* experimentation as discussed later.

AFP receptor purification and partial characterization:

At present, a number of AFP receptor purification methods have been reported. Most of them rely on the relatively weak interaction between the receptor and AFP. For example, when the pleural effusion from a patient bearing lung metastases of a mammary carcinoma was loaded onto a hydroxylapatite column, a large AFP receptor peak, traceable by the 167H.1 and 167H.4 Mabs, came out in the void volume of the column, followed by a steady leakage into the washing buffer⁴⁶ and personal observations. Increasing the molarity of the $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer resulted in a second large peak also detectable by the anti-AFP receptor Mabs. Presumably, part of the receptor is complexed to AFP which in turn binds to the column matrix and the receptor slowly "leaks" from the complex into the washing buffer. The presence of an AFP-receptor complex in the pleural effusion agrees with a number of studies showing the synthesis and release of AFP-AFP receptor complexes in different types of immature and cancer cells^{38,39,40,47,48}.

Another purification method is based on the binding of AFP to Concanavalin-A (Con-A). This binding can be used to purify AFP under high NaCl concentrations⁴⁹. Since KCl (0.4 M) has been reported to split the AFP-receptor bond³⁹ and human cord serum is known to contain a significant amount of AFP (part of which is presumably complexed to its receptor), we devised a strategy consisting in capturing the complex (via the AFP side) onto a Con-A Sepharose column under a high NaCl concentration. Then the NaCl was substituted by KCl which would split the complex, with the consequential release of AFP receptor⁴⁵.

A more direct approach was taken by Severin *et al*⁵⁰, who used AFP covalently coupled to Sepharose and KCl to elute the receptor. Their results were virtually identical to those obtained using Con-A Sepharose.

Yet another method involves the use of AFP conjugated to a photoactivatable thio-cleavable cross-linker which is activated once the AFP is attached to its receptor on U937 cells⁴³ (a human monocytic malignant cell line).

The fractions purified with all of the above mentioned methods exhibited a molecular weight of approximately 65 kD. This was also the case when the molecular weight of the AFP-AFP receptor complex was measured by gel filtration after incubating radiolabeled AFP with receptor-rich material⁵¹. The molecular weight of the complex was found to be 130 kD, of which 65 kD can be accounted for by AFP. A molecular weight of ~65kD is consistent with the detection, using anti-AFP receptor Mabs, of two adjacent bands weighing 62 and 65 kD respectively on SDS polyacrylamide transblots of human mammary cancers^{45,52}.

On the other hand, Torres *et-al*, using immobilized AFP on nitrocellulose membranes reported the isolation of two smaller (31 kD and 18 kD) AFP binding glycoproteins from Raji cells (a human B-lymphoma). The 18 kD fraction was also found in PHA stimulated human peripheral blood lymphocytes (HPBL). No binding fractions were isolated from resting HPBL. The molecular weight discrepancy between this report and the others could be related to the statement, by the authors, that the recovered fractions were identical to a previously described receptor for serum albumin. This, however, was not the case in the work by Suzuki *et-al*⁴³, who reported that serum albumin did not compete with AFP for binding to the isolated AFP receptor. Albumin did not compete with our anti-AFP receptor Mabs either⁴⁵.

Heavier AFP receptor fractions weighing 180 kD⁴⁶ and 250 kD⁵⁰ have also been reported. These fractions might correspond to complexes with anti-receptor auto-antibodies (Laderoute^{personal communication} and Severin *et-al*⁵³).

Potential use of the AFP receptor as a tumour marker for *in-vitro* diagnosis:

Aside from its potential for tumour scintigraphy, the AFP receptor could prove useful as a widespread cancer marker to detect malignancy on tissue sections, blood smears and bodily fluids.

Immunohistology using anti-AFP receptor Mabs on cancer tissue sections:

Using the 167H.4 anti-AFP receptor Mab and immunofluorescence on paraffin sections, we reported the positive staining of 6 out of 6 human mammary carcinomas. Three out of three benign adenomas were negative. Similar results were obtained using immunoperoxidase. Positive staining was found in 21/23 mammary carcinomas, 14/15 lung cancers, 8/8 colon carcinomas and in approximately 90% of other malignancies. Figure 3 shows some examples of immunoperoxidase stained cancer sections as well as a negative benign mammary adenoma (all pictures correspond to paraffin sections).

Future systematic studies will establish the usefulness of the AFP receptor for immunohistochemical diagnosis. There are a number of particular situations in which the pathologist might also find this marker helpful:

1. Border-line cases (or pre-malignant lesions e.g. colon polyps) in which the diagnosis of malignancy might not be easily established by conventional histology.
2. For detecting, by immunofluorescence, the presence of small metastatic foci within lymph nodes. It is conceivable that a few malignant cells, which could otherwise escape the pathologist, would this way appear clearly visible on the negative background. Figure 3B shows the immunofluorescence staining of a mammary carcinoma section. Small positive fluorescent spots dispersed over the negative normal tissue clearly pinpoint the location of small malignant cell foci.
3. For immuno-cytology, in particular on Papanicolau and sputum smears where the cell architecture is often disrupted making the diagnosis difficult.
4. In clear-cut malignancies, when it might still be necessary to know whether the patients cancer cells do actually express the AFP receptor, prior, for example, to using radiolabeled AFP for tumour imaging as described above.

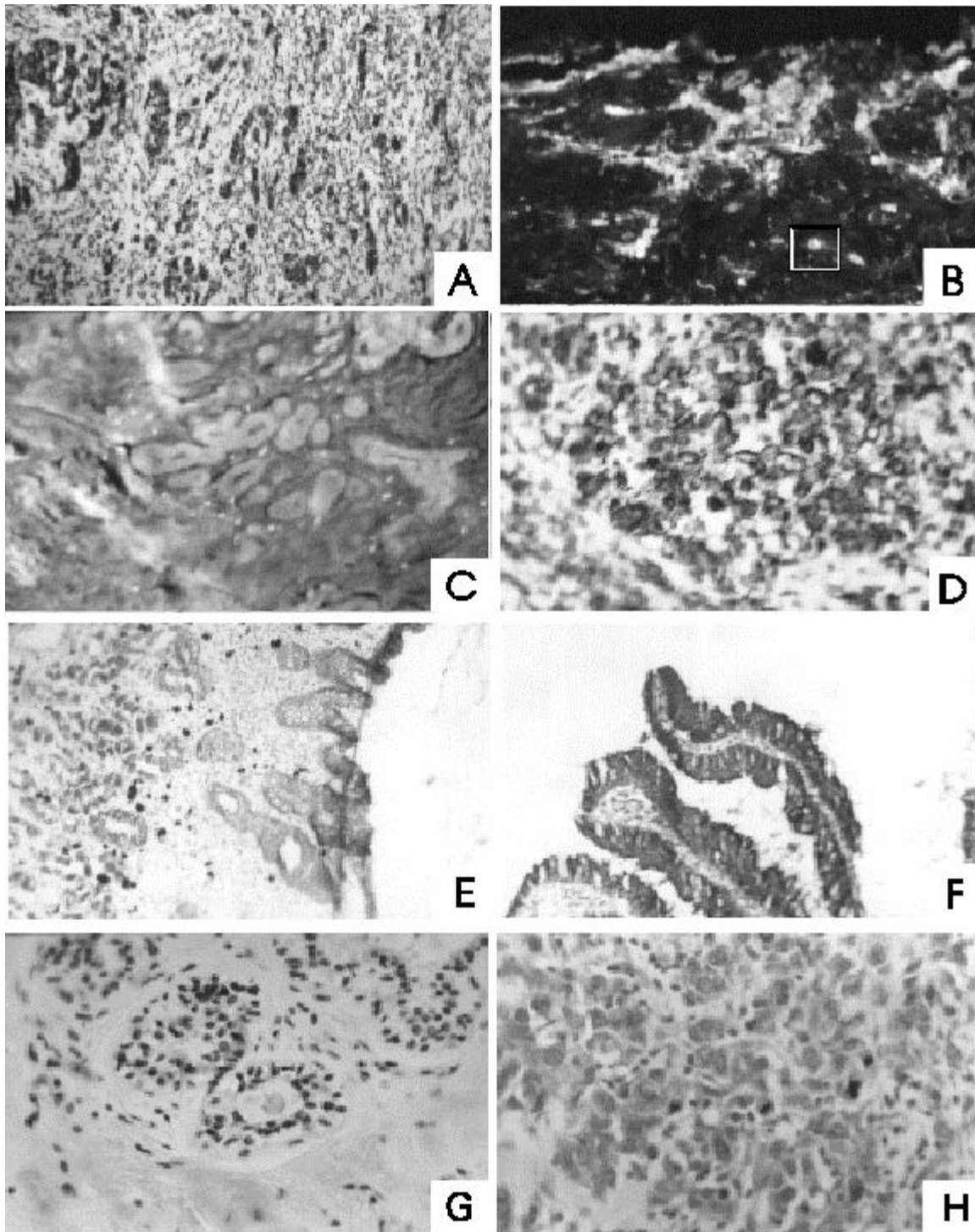


Figure 3: Immunofluorescence (B and C) and immunoperoxidase (all others) staining of different types of tumours and normal tissues with anti-AFP receptor Mab 167H.4. In A and B, two mammary adenocarcinomas. Inside the square in B, a small foci of malignant cells which, if present in a lymph node, might remain unnoticeable with standard stainings. In C, a breast benign adenoma. D is a lung carcinoma. Notice the capping on many of the positive cells, a feature often seen in AFP uptake experiments. In E, a gastric carcinoma and in F a colon

carcinoma. In G another negative benign mammary adenoma (with peroxidase) and in H negative normal liver.

AFP receptor on leukemia cells. Diagnostic and therapeutic potential:

Figure 4 shows the FACS results obtained using the 167H.4 Mab on several human leukemia cell lines. HL60, Ramos and U-937 were strongly positive. It is worth mentioning that Suzuki *et-al*⁴³ isolated the AFP receptor from the latter with a method unrelated to the Mab used in our FACS analysis. The negative result found on Molt-4 is consistent with the findings from AFP uptake experiments using these cells^{54,55}.

The above mentioned principle of using immunofluorescence to visualize a small percentage of malignant cells within a large number of normal cells also applies to leukemias. Using cytofluorometry, it might be possible to monitor a patient under treatment and decide upon the administration of a new round of chemotherapy as soon as a significant, albeit small, number of malignant cells re-appears in the blood stream. This could lead to an increase in therapy efficiency since the sensitivity of cytofluorometry is considerably higher than that of conventional blood smear microscopy. Another intriguing possibility is to use anti-receptor antibodies to clean-up a patient's bone marrow *in-vitro*, either by cell sorting or with the help of immunotoxins⁵⁶. Cleansed marrow could then be used for auto-grafts following whole body radiation or chemotherapy. It is worth noting that the use of anti-receptor antibodies is not mandatory for these assays since AFP itself can be successfully used for delivering drugs or toxins into cancer cells as proposed elsewhere³² and demonstrated by Nakachian *et-al*⁵⁷.

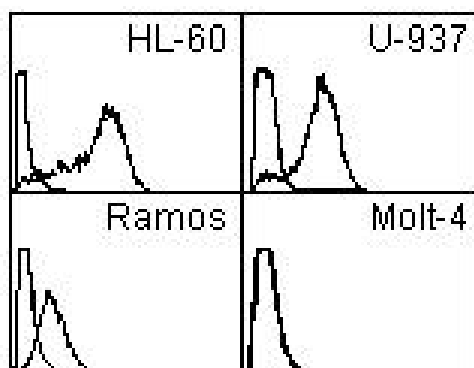


Figure 4: Flow cytometry of 4 leukemia cell lines stained with the 167H.4 anti-AFP receptor Mab. The relative fluorescence intensity corresponds to the X axis. The Y axis depicts the number of cells. The leftmost curve on each quadrant corresponds to a negative control using an irrelevant Mab. Notice that whereas HL-60, U-937 and Ramos are positive, Molt-4 is negative. These results are consistent with findings using FITC-AFP.

AFP receptor in serum. Potential use for cancer diagnosis and follow up:

The AFP receptor can be isolated from human cord serum⁴⁵ and is detectable in fluids such as a pleural effusion from a patient with lung metastases of a mammary carcinoma^{45,46} and in breast cancer cytosols⁴⁴. The soluble nature of the molecule suggests that it might be released from cancer (and fetal) cells into the extracellular fluid and eventually into the blood stream, either actively or following cell death. Thus, it could be possible to detect an elevated concentration of AFP receptor in the serum of cancer patients. In order to explore this possibility we tested sera

from 16 patients bearing different types of malignancies and 22 cancer free individuals. The assay posed some difficulties since the two available Mabs react against the same site thus ruling out a sandwich immunoassay. Practical reasons prevented us from using pure labeled AFP receptor in a competition assay. Hence, the tests were carried out as follows: Each well on a standard ELISA plate (NUNC) was coated for 3 hours with a 1/16384 dilution of patient's serum in PBS. The wells were then blocked with 1% OA (Sigma), washed with PBS and incubated with a 1/2000 dilution of 167H.4 Mab ascites⁴⁵. After washing again with PBS, the assay was continued in the standard fashion using a peroxidase labeled anti-mouse IgM conjugate and ABTS as the substrate. In order to compare results from one test to another, each plate contained several samples of the above mentioned pleural effusion (referred to as PE). Thus, optical density values were normalized as the percentage of AFP receptor in PE for comparison purposes. The non-cancerous samples used as controls were from patients hospitalized for varicose veins surgery, hypertension and other non-malignant disorders as well as from healthy individuals. Figure 5 shows the results obtained. Table I shows the statistics corresponding to these patients. There is a very significant difference in the circulating concentration of AFP receptor between cancer patients and non-cancer individuals ($p < 0.000002$ by t-test). Table II depicts the clinical diagnosis corresponding to each cancer patient.

Anecdotally, an abnormally high level of circulating receptor was found in a female patient who was admitted with severe hypertension (this case is not included in the data shown). In view of the high significance of the results obtained, this patient was subjected to a CAT scan which evidenced a kidney tumour, eventually diagnosed as a hypernephroma. Even though these are preliminary results, the significance of the p value cannot be ignored. A comprehensive study using a better enzyme immunoassay as well as a larger number of patients grouped by tumour type should provide more conclusive data.

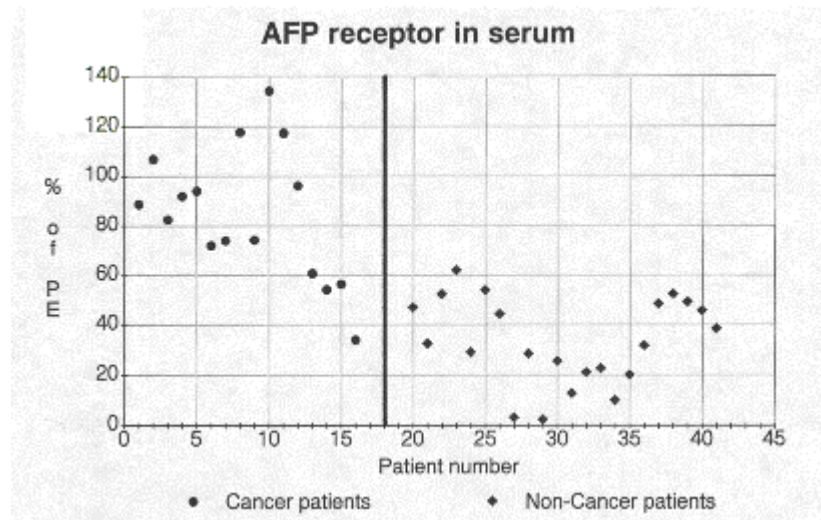


Figure 5: Enzyme immunoassay showing the relative concentration of AFP receptor in serum from cancer bearing and cancer free patients. All values were normalized as the percentage contained in PE, a pleural effusion produced in a patient with lung metastases of a mammary carcinoma. Notice the clear difference ($p < 0.000002$ by t-test) between malignant and non-malignant cases. Table II below shows the assortment of tumours used in this study.

Table I Statistics corresponding to patients studied for circulating AFP receptor.

Values are expressed as the percentage of PE found in patients sera.

Parameter	Cancer positive	Cancer negative
Mean	85	33
SD	26	17
N	16	22
P = 2.2 x 10 ⁻⁶		

Table II

Tumour types corresponding to patients studied for circulating AFP receptor.

Patient	Cancer Type
1	Mucinous adenocarcinoma
2	Lymphoid leukemia
3	Angiosarcoma
4	Adenocarcinoma
5	Sarcoma
6	Cystic adenocarcinoma
7	Adenocarcinoma
8	Carcinomatosis
9	Carcinomatosis
10	Astrocytoma
11	Neoplasia
12	Primary Hepatoma
13	Carcinoma
14	Metastatic carcinoma
15	Carcinoma
16	Osteosarcoma

Inhibition of malignant cell proliferation by anti-AFP receptor Mabs:

Mouse Mabs directed against the human AFP receptor cross-react with mouse malignant cells^{45,46}. In all, we were able to show that the 167H.4 Mab reacts with EL-4, YAC-1 (two mouse T-lymphomas), TA-3, (a mouse mammary carcinoma cell line) and P-388 (a malignant cell line of dendritic origin). This is surprising but not totally unexpected since AFP from one species binds to, or is taken up, by cells from another species^{20,42}, which suggests that the binding sites on both AFP and its receptor are conserved, at least partially. The aforementioned Mab recognizes the AFP binding site on the receptor (it competes with AFP). Hence, small differences between the structure of human and mouse receptor binding sites could still make the former immunogenic to mice while the conserved portions of the binding site would explain the cross reactivity. The cross

reaction of anti-human AFP receptor Mabs with mouse malignant cells is a fortunate circumstance which allows to develop simple tumor targeting experimental models in animals without the drawbacks of using nude mice.

The interaction between anti-AFP receptor antibodies and malignant cells is not limited to a passive binding; the antibodies also modulate the rate of cell replication: When P-388 cells were incubated with the 167H.4 Mab, we found that cell replication was drastically inhibited (Figure 6). Cells were not killed, as they excluded trypan blue and would re-start replicating after being washed with Mab-free medium. Total DNA synthesis suppression was not achieved, a fact perhaps related to the presence of bovine AFP (and likely bovine AFP receptor) in the fetal calf serum added to the medium in order to obtain a measurable amount of cell replication. Bovine AFP binds to the mouse AFP receptor⁴² and competes, to a certain extent, with the 167H.4 Mab

personal observations

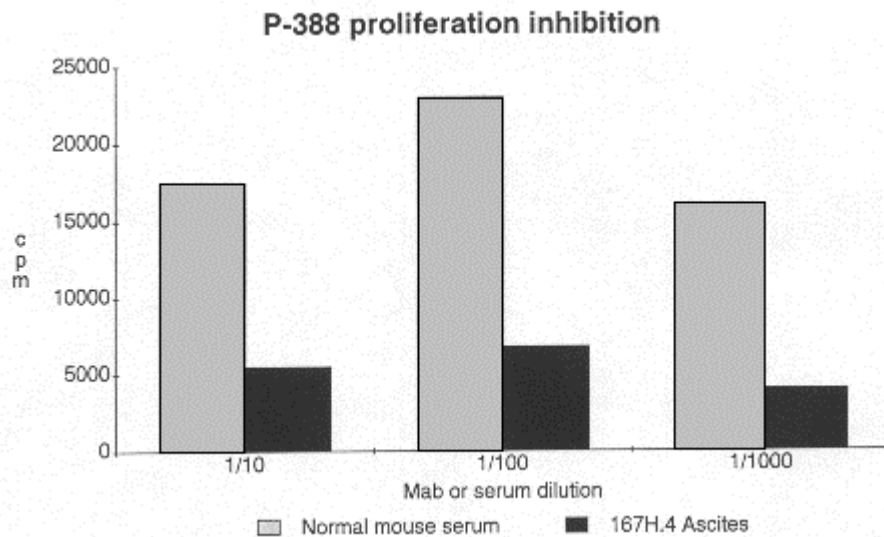


Figure 6: Inhibition of P-388 cell proliferation by the 167H.4 anti-AFP receptor Mab as compared to normal mouse serum. The incorporation of ³H-Thymidine is reduced by 70%-75% in a 6 hour pulse chase experiment.

A similar proliferation inhibition was obtained on LoVo cells (a human colon adenocarcinoma) in serum free medium⁴⁶. In these experiments, it was shown that pure human AFP could reverse the inhibitory effect of the anti-receptor Mabs. PHA transformation of normal human peripheral leukocytes is also inhibited by 167H.4⁴⁶.

These encouraging *in-vitro* results prompted us to carry out a series of *in-vivo* experiments in which C57 Black mice were injected with 2×10^6 EL-4 cells subcutaneously the same day they received a 100 uL intravenous injection of either 167H.4 Mab ascites or normal Balb/c serum (Balb/c mice were used to produce the Mab ascites). In order to facilitate graft take, all animals were exposed to 300-500 Rads from a Cs source a week before the injections. The experiments were repeated 6 times, using 4-5 animals in each group. The results were consistent: Five to six days after the injection, there was a clear difference between the control animals, which exhibited tumours 5-6 mm in size, and the treated animals which had only a scar at the injection site. If allowed to progress, the tumours killed the control animals within 3 weeks. In 5 out of 6 experiments, all of the treated animals remained tumor free until they were sacrificed, in one case, 8 months later. No noticeable signs of disease or other abnormalities were detected in

these animals, or in their progeny. Figure 7 shows 2 controls and 3 treated animals from one of these experiments. The mice were sacrificed 2 weeks after receiving the injections.

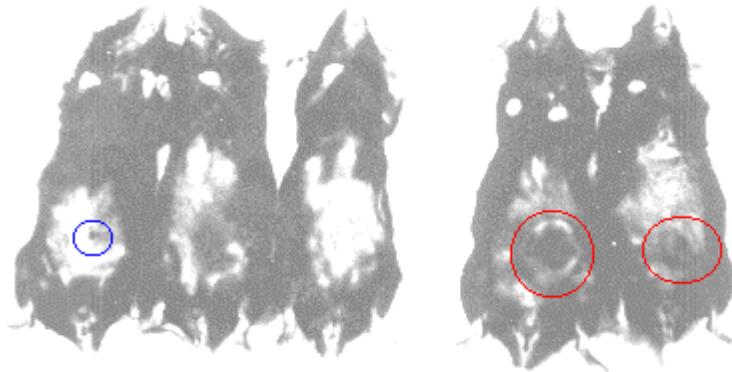


Figure 7: Tumour growth inhibition by the anti-AFP receptor 167H.4 Mab. Five mice received 100 uL of Mab ascites IV the same day they were injected with 2×10^6 EL-4 cells SC on the abdomen. Another five mice received 100 uL of normal mouse serum as a control. Animals received 300 rads a week prior to the experiment to facilitate graft take. All treated animals were tumour free 2 weeks after injection. All controls bore large tumours. In the picture, the three animals on the left belong to the treated group. Notice a small scar at the point of injection (blue). On the right, two of the control animals showing large tumours (red). Similar results were obtained in 5 other experiments.

In the 6th experiment, the treated animals did developed tumors and eventually died of them, albeit their life span was significantly longer than that of the controls (Figure 8). An assay conducted on the EL-4 cells in culture used for grafting the animals in this experiment showed that only 20% of ^{125}I -AFP binding was specific (displaceable by cold AFP) as opposed to the 60-80% normally encountered. This unexplained sudden drop in AFP binding and uptake has been previously noticed on other cell lines, such as MCF-7, Ichikawa and YAC-1 when they are kept in culture for many passages^{Uriel and Villacampa, personal communications and personal observations} (Some very preliminary data suggest that the brand name or type of plastic Petri dish used for cell culture might have an influence in the expression of the AFP receptor).

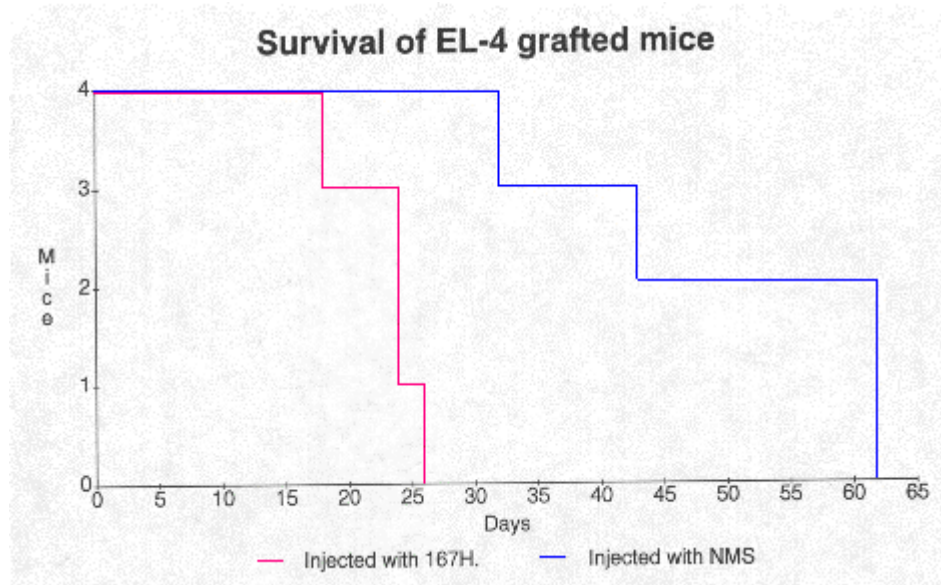


Figure 8: Tumour growth inhibition by the anti-AFP receptor 167H.4 Mab. This experiment was similar to that depicted in Figure 7 with the difference that the specific binding of AFP to the EL-4 cells in culture used to graft the mice had decreased to 20% of total binding as compared to the usual 60-70%. Notice however, the longer life span of the animals treated with Mab 176H4 as compared to the controls.

These *in-vitro* and *in-vivo* results indicate that antibodies directed against the AFP receptor might slow down or abrogate cell proliferation. A great deal of work needs to be done in order to further explore this possibility. If confirmed in animal models, the obvious goal would be to try this approach in cancer patients. Bearing this objective in mind, it is important to consider that using mouse monoclonals in humans is not desirable and seeking a better alternative from the onset might save a significant amount of work. A possibility could be to generate human/mouse chimerical antibodies⁵⁸, even though they not always work as well as expected^{59,60}. Another approach could be to actively immunize cancer patients with a heterologous AFP receptor. The circulating antibodies thus produced would cross react with the human AFP receptor and inhibit malignant cell proliferation in a system similar to the mouse model used in our experiments (that is, a system in which homologous antibodies directed against a heterologous AFP receptor cross-react with the homologous AFP receptor on the surface of cancer cells). It is worth noting that a 1/300 dilution of the serum from the mouse used to create the 167H.4 Mab inhibited ¹²⁵I-AFP binding to Ichikawa cells by 71% as compared to the 73% inhibition obtained with the supernatant from the 167H.4 clone⁴⁵. The relatively slow progression of malignancy would provide the time required to reach significant levels of circulating antibodies before the host succumbs to the disease. Active immunization against the AFP receptor could also be used as an adjuvant therapy to surgery and/or medical treatments such as radiation, chemotherapy and hormone therapy. In the realm of pure speculation, should this type of vaccination protect against a broad variety of cancers, it is conceivable that it could be profilactically used in high risk populations or even on the general public (above certain age).

At present, the mechanisms underlying the proliferation inhibition results are unknown. On the other hand, we do know that the Mab used binds to the AFP receptor and that some kind of message must be delivered from the AFP receptors on the cell surface to the nucleus in order to explain the cell proliferation inhibition. It seems reasonable to assume that the receptor itself carries the message at some point, e.g. from the cell surface to the cytoplasm or even to the nuclear membrane. A thorough analysis of the possible pathways in which the AFP binding proteins might be involved has been recently advanced by Mizejewski⁶¹.

Conclusions:

As in most scientific fields, there is a gradient of certainty in the knowledge we have about the AFP receptor.

There is solid evidence showing AFP internalization by fetal and cancer cells and it is safe to assume that the uptake is mediated by a receptor with a molecular weight close to 65 kD. It is also well documented that the internalization of AFP as well as the expression of the receptor by fetal and cancer cells is a widespread phenomenon, common to many types of malignancies and present in all studied species. Moreover, several independent reports show that AFP from one species is taken up by cells from another species and that anti-human AFP receptor antibodies recognize murine cancer cells. These findings are consistent with a conserved structure among species, an indication of a basic biological mechanism. There is also ample evidence of the soluble nature of the AFP receptor.

Some of the results presented here show, albeit with a lesser degree of certainty since they must be independently confirmed, that AFP or antibodies against the AFP receptor might be useful for cancer detection. The specificity of the detection methods, which include tumour scintigraphy, serum screening and immunohistochemistry needs to be determined, since it is not known whether the AFP receptor is expressed in conditions other than malignancy or pregnancy.

There is also preliminary data suggesting that anti-AFP receptor antibodies can prevent tumour growth and that conjugates of AFP and anti-mitotic drugs kill cancer cells more efficiently than the drugs alone.

The firmly established knowledge basis on the AFP receptor, together with its enticing potential for cancer diagnosis and therapy should stimulate future research in this area. It is worth noting that the potential for cancer diagnosis in humans is not just an abstract concept; it is supported by positive results already obtained on tissue sections, in serum samples, in leukemias (by fluorocytometry) and for tumour imaging. The potential use of the AFP receptor extends to the field of cancer therapy, an area in which promising results have been obtained both *in-vitro* experiments and in animal studies.

Given the span of positive results, it would be very surprising indeed, once all and each one of these areas are thoroughly explored, to reach the conclusion that the AFP receptor is of no clinical significance.

Addendum: The author has noticed that the term "AFP receptor" sometimes leads to confusion with the AFP molecule itself. Also, besides being impractical, the "AFP receptor" term renders the molecule "identity challenged" in favor of AFP. Thus, the author wishes to propose the use of the term RECAF to identify the receptor for AFP in the future.

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