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Chapter 14

THE ALPHA-FETOPROTEIN RECEPTOR (RECAF): CHARACTERIZATION AND POTENTIAL USES FOR CANCER DIAGNOSIS AND THERAPY

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ABSTRACT

Alpha-fetoprotein (AFP) was the first oncofetal antigen of clinical significance. Over 20000 papers have been published on this protein, covering from its physiology to its applications for cancer diagnosis and management. AFP has also been used as a tool to study cell differentiation and malignant transformation. During the course of those studies, AFP was found inside fetal and embryonic cells that do not synthesize it and therefore several groups started working on the hypothesis that AFP was internalized via a receptor mediated mechanism of endocytosis. This receptor for AFP (RECAF) would be expressed in most tissues and its expression would be related to the stage of cell differentiation.

Cancer cells are poorly differentiated and therefore, RECAF could be re-expressed in malignant cells, thus serving as a broad spectrum cancer marker. This was later confirmed, and since then, evidence has been accumulating supporting the use of RECAF in medical practice: (a) for detecting cancer cells on tissue sections, (b) as a circulating cancer marker for cancer diagnosis, (c) for scintigraphy, (d) for flow cytometry and (e) for cancer cell targeting. In this chapter, we review the literature on the AFP receptor and we present some original data on its biochemistry as well as on its potential uses in medicine.

Keywords: AFP, AFP fragment, AFP uptake, receptor, RECAF, binding, serum test, immunohistology, targeted therapy, cancer, tumor, malignant, leukemia, oncofetal, cell differentiation, monoclonal antibody, apoptosis, peptide, scintigraphy, immunoscintigraphy, imaging, glycan, glycoprotein

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INTRODUCTION

In the literature, the term "AFP receptor" has been abbreviated in many different ways such as AFPR, AFPr, AFPRp, AFP-BP, etc. which are difficult to pronounce and lead to confusion with AFP itself. Thus, throughout this chapter, the receptor for AFP is abbreviated as "RECAF," a term used by the author in previous publications.

Fifty years ago, Abelev et al. reported the discovery of the first oncofetal antigen, alpha-fetoprotein (AFP) [1]. After peaking in serum concentration during fetal life, AFP almost disappears after birth, with normal adult serum leveling at concentrations below 50 ng/ml [2]. However, in hepatocarcinomas and teratocarcinomas, serum AFP increases dramatically, attaining concentrations 1000 times higher [3]. These findings drew the attention of both the medical community, who foresaw new means for diagnosing and monitoring cancer patients, and basic research groups interested in studying the physiology of this protein during fetal life as well as in using AFP as tool to explore the process of cell differentiation and malignization.

While the main sites of AFP synthesis are the liver, stomach and yolk sac, using anti-AFP antibodies and the immunoperoxidase technique, Benno and Williams described the distribution of AFP in the developing rat brain [4]. Soon after, a series of reports confirmed the presence of AFP (and other plasma proteins) within embryonic neuroblasts in several species including rodents [5], sheep [6], primates [7], humans [8] and birds [9]. Since neuroblasts do not synthesize a significant amount of AFP, in the latter reference we proposed the existence of a receptor to explain AFP uptake by embryonic neural cells, and we suggested that the expression of this receptor could be related to the degree of cell differentiation[1].

At the time, it was noticed that as fetal development progresses, the AFP (and serum Albumin) staining in neural tissues follows a similar pattern from one species to another [10, 11]: Very immature neuroblasts in a given neural structure are initially AFP and Albumin negative. Then, rather abruptly, the staining becomes positive for both proteins, even within the same cell [12]. After some time, the staining, which is always restricted to the cytoplasm, fades both in intensity and in number of positive cells, first for AFP, and then for SA. This is not due to a decrease of AFP in serum because the AFP fading takes place before AFP reaches its peak in serum [13]. Nor is it due to the closing of the blood-brain barrier, which would prevent access of serum proteins to cells, since spinal ganglia neuroblasts turn immunohistologically negative for AFP while they remain positive for Albumin [10], which has a similar molecular weight. 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 fluid [13].

[1] This reference is difficult to find. The text reads: "*As an alternative to passive diffusion, the selectivity of the protein uptake could be due to specific receptors for AFP and for SA. These receptors would be only present at some intermediary stage of neuronal differentiation*."

AFP UPTAKE BY EMBRYONIC AND FETAL CELLS

A question arising from these initial observations was whether AFP and serum Albumin are incorporated from extracellular sources, thus validating the receptor hypothesis, or synthesized by neuroblasts. While it is not clear whether neural cells are capable of synthesizing these plasma proteins [14-17] it has been well established, both *in vitro* [18, 19] and *in vivo* [20, 21] that neuroblasts internalize AFP and serum Albumin from extracellular sources.

Injecting ^{125}I-AFP into pregnant rats [20] confirmed the accumulation of the labeled protein in neuroblasts but also in other fetal tissues that do not synthesize AFP and yet are positively stained for that protein by immunohistology [22]. Autoradiographies from these experiments also confirmed the exclusive cytoplasmic distribution of AFP.

Some of the initial *in vivo* uptake experiments were carried out with heterologous proteins [21]: When newborn rat serum containing a large amount of AFP was injected into the mesencephalic cavity of chicken embryos, the neuroblasts staining for rat AFP and rat Albumin was positive, with a color gradient fading from the lumen toward the peripheral layers. This staining pattern was identical to the one seen for the corresponding native proteins. On the other hand, the injection of rat IgG (MW ~150,000) or Ovalbumin (MW ~43,000) in the same cavity resulted in no uptake even when Ovalbumin was injected at 30 times its physiological concentration, which, at that embryonic stage, was two times the normal molar concentration of AFP in the cerebrospinal fluid [13]. These results indicate that (a) the uptake of AFP and Albumin is protein selective and (b) rat AFP and chicken AFP are similar enough to be internalized across species.

At the time, it was not clear whether the progressive disappearance of intracellular AFP at the end of gestation was a cell regulated process or just a consequence of the dropping concentrations of circulating AFP. The first possibility was proven correct since it was demonstrated, first in chicken, as mentioned above [10], and then in human embryos [23], that neural cells within spinal ganglia become AFP negative while the serum concentration of that protein is still rising.

AFP UPTAKE BY CANCER CELLS

The data at that point supported the following conclusions: (a) Fetal and embryonic cells from different tissues take-up AFP, (b) the uptake is protein-specific, (c) the uptake is related to the degree of cell differentiation and (d) embryonic cells from one species take up AFP from another species. All of these elements were consistent with the hypothesis of a receptor-mediated mechanism of AFP endocytosis whose expression would depend on the stage of cell differentiation [9, 21]. The uptake of heterologous AFP suggested that the structure of the binding sites on the receptor and the ligand are highly conserved across species.

Cancer cells and fetal/embryonic cells share a number of common biochemical and antigenic features [24]. Hence, we hypothesized that cancer cells (from tissues that incorporate AFP during fetal life) might regain the ability to internalize AFP and that the internalization would be carried out via its receptor, which would therefore qualify as a new 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-7 (human breast cancer) [25], a nickel induced rat rhabdomyosarcoma [26], the C1300 mouse neuroblastoma [27] and several human leukemias [28]. *In vivo* studies carried out in mice showed a significant accumulation of injected mouse ^{125}I-AFP in spontaneous mammary carcinomas when compared to normal tissues [29].

TUMOR IMAGING USING RADIOLABELED AFP

The injection of ^{125}I-AFP in 19 C3H/Bi mice bearing 31 breast spontaneous tumors resulted in a 3.6 tumor/liver ratio of radioactivity per mg of tissue [30]. Following injection with ^{131}I-AFP, two mammary carcinomas could be imaged by scintigraphy [30]. A more extensive study showed that after the injection of ^{131}I-AFP, eleven out of twelve spontaneous breast mouse tumors were clearly visualized despite using a standard gamma camera designed for ^{99m}Tc instead of ^{131}I [31]. Tumors as small as 3 mm in diameter could be seen, as shown in Figure 1. In yet another study, the C1300 mouse neuroblastoma tumor could be imaged when transplanted into mice [32]. Similar results were obtained by Line et al. [33, 34].

All of these findings, along with the high quality of the images obtained in animals, prompted us to scan patients after being injected with human ^{131}I-AFP. Since poorly differentiated cells take up AFP, we were concerned that gonad cells, bone marrow and normal stem cells could be killed or mutated 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 ^{131}I-AFP per animal, which is the equivalent, by weight, to 250 mCi for an adult human being. No abnormalities were detected in any of these mice, which had a normal life span. When mated, fertility was normal, and the two generations of their offspring that were kept under observation were also normal.

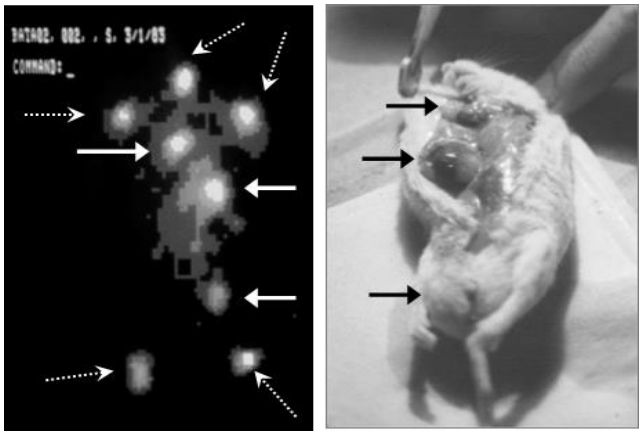


Figure 1. Scintigraphy of a mouse bearing 3 spontaneous tumors (histologically diagnosed as a primary mammary carcinoma and 2 metastases). On the left, the image obtained after injection of 72 uCi of ^{131}I-AFP. The dotted arrows point at radioactive markers used to locate the nose and extremities of the animal under the gamma-camera. The solid arrows show three positive lesions. The upper spot was initially thought to be the thyroid, even though the animals were given KI to drink for a week before injection. The autopsy on the right revealed that the spot corresponded to a metastasis 3 mm in diameter.

Thus, two healthy individuals (including the author) and three cancer patients were injected with 300-600 ug of ^{131}I-HuAFP (~0.6 mCi) and scanned 2 to 7 days later. In the normal patients, who were injected first, most of the radioactivity was localized in the liver, spleen and cardiac area. The first cancer patient (male, bearing a large abdominal tumor diagnosed as stomach cancer) showed a clear accumulation of radioactivity corresponding to the palpable mass. The second cancer patient was a female patient bearing bone metastases of a breast carcinoma. The original tumor had been surgically removed 2 years earlier. This patient was first injected with ^{99m}Tc-Pyrophosphate, which accumulates in regenerating bone tissue, and then was imaged according to routine procedures. As depicted in Figure 2 (left), the scintigraphy showed bone metastases in the lumbar vertebrae and the iliac crests. To track the location of the lesions, a radioactive pen was moved over the patient's back until the pen's spot observable on the gamma camera overlapped with the lesions spots. Their location on the patient's back was marked for future reference using a non-toxic marker. A week later, the patient was imaged again, fitting this time the gamma camera with a ^{131}I collimator. As a control, and before injecting the radioactive AFP, a new image was taken, which, as expected, showed no visible radioactivity given the short half life of ^{99m}Tc (~6 hours). The patient was then injected with human ^{131}I-AFP and scintigraphies were taken at 24 or 48-hour intervals. Figure 2 (right) shows the image obtained on the 6th day after injection. Repeating the lesion tracking process with the radioactive pen we were able to demonstrate that the 3 lesions pointed at by the solid arrows in both scintigraphies had the same location and therefore, that the lesions detected by ^{131}I-AFP were the same as the ones detected with ^{99m}Tc-Pyrophosphate. ^{99m}Tc-Pyrophosphate images show bone synthesis and therefore they are restricted to bone metastases, whereas ^{131}I-AFP images denote the presence of malignant cells and therefore they can be detected in other locations.

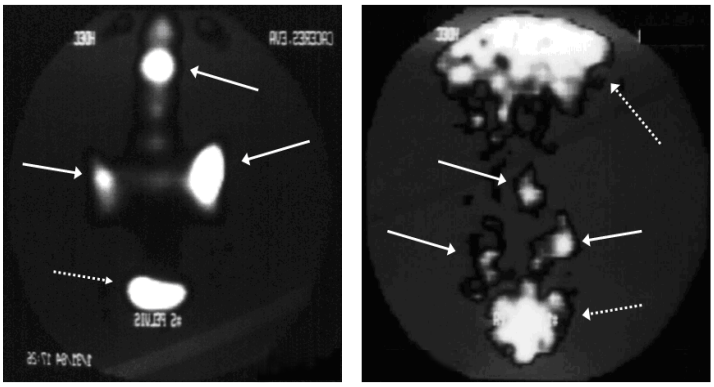


Figure 2. Scintigraphy of bone metastases of a human breast carcinoma. The patient was first injected with 99mTc-Pyrophosphate and then imaged according to routine procedures (left picture). The solid arrows show the areas of bone regeneration produced by metastases in a lumbar vertebra and the iliac crests. The dashed arrows show the liver area and the urinary bladder. Seven days later, the patient was injected with human 131I-AFP and scintigraphies were taken at 24-48 hour intervals. On the right, an image taken 6 days after the injection or radioactive AFP. The difference in magnification is due to the use of a different collimator for each isotope. The 3 lesions pointed at by the solid arrows coincided with those on the left picture.

The third patient was a 73-year old female who had had bone metastases of a breast carcinoma. Following chemotherapy and hormone therapy, the patient had been in remission for 18 months, at which time she was imaged as described above. Bone regeneration is a slow process and ^{99m}Tc-Pyrophosphate positive images can remain as such for many months after remission thus masking a complete remission. In effect, the ^{99m}Tc Pyrophosphate scintigraphy was still positive, whereas, as expected, no radioactive spots could be detected with ^{131}I-AFP.

These results, taken together with the images obtained in animals, strongly suggest that AFP might prove useful for tumor imaging in humans. Systematic studies involving a larger number of patients are necessary to fully ascertain the actual merits of this promising technique and the doses, as well as the time between radioisotope injection and imaging need to be optimized.

Using radiolabeled human AFP offers the advantage, over monoclonal antibodies, that it is a homologous molecule, thus virtually precluding anaphylactic reactions when used repeatedly. Also, only one radiopharmaceutical would be required to detect a wide variety of tumors, a practical consideration to be taken into account.

The discovery of the binding site of RECAF on the AFP molecule (see below) opens the possibility of using a radiolabeled AFP peptide for imaging. This offers several advantages over using whole AFP or a large fragment thereof: (a) Lower cost of production, (b) low risk of infection, and (c), the ability to tailor the peptide for: (i) radiolabeling, (ii) optimal tissue penetration, (iii) cell uptake and (iv) elimination/secretion, thus improving the image quality while reducing the radioactivity doses as well as the time between the injection and the image capture. This, in turn, could allow the use of ^{99m}Tc instead of ^{131}I or exotic isotopes such as ^{111}In or ^{123}I, etc. Using a peptide labeled with ^{99m}Tc would be practical since the latter is readily available in any nuclear medicine department, it would significantly reduce radiation exposure and it might enable the entire process, from injection to imaging, to be carried out in one session.

Something to be kept in mind when considering this type of scintigraphy (as well as in the evaluation of serum RECAF test results), is that AFP is accumulated in some acute inflammatory lesions [35, 36]. This does not seem to be the case in chronic inflammatory processes.

RECAF EXPRESSION AND DETECTION

Even though the expression of a receptor to explain AFP uptake was first proposed in 1981 [9], indirect evidence of this receptor-ligand system can be traced to earlier papers [37, 38]. The first of these articles concluded that 18% of normal peripheral T-cells bind AFP. However, later articles showed that peripheral T-lymphocytes do not bind AFP unless they are previously stimulated with Phytohaemagglutinin (PHA) [39]. The second article [38] reported the presence of two AFP rich ultracentrifugation fractions in immature rat uterine cytosols: A 4S fraction, detectable by an anti-AFP antiserum and an 8S fraction where AFP was not detectable. However, treatment of the 8S fraction with 0.4 M KCl, converted it to a 4S fraction, in which AFP could then be detected. It is likely that the 8S fraction corresponded to a receptor-AFP complex, which was dissociated at high KCl concentration. This is peculiar because in those experiments, AFP was detected using a polyclonal antiserum

and AFP is known to have at least 6 epitopes [40]. Thus, the AFP conformational changes required to void all of the epitopes when it binds to RECAF need to be very substantial. A similar phenomenon was 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 KCl [41]. Sarcione et al. also reported the presence of complexed AFP in cancer cell extracts, in a form that could be dissociated by KCl [42]. In addition, Sarcione's team demonstrated that AFP is synthesized by the MCF-7 human breast cancer cell line as a complex that is not recognized by anti-AFP antibodies unless it is first treated with a high concentration of KCl [43].

The first direct evidence of a receptor for AFP was described on MCF-7 cells by Villacampa et al. [44] who found results consistent with the presence of a two-site receptor model exhibiting positive binding cooperativity. The higher affinity site showed a Kd of 1.5 x 10^{-9} M and a number of 2000 sites per cell. The lower affinity site, with a number of 320000 sites per cell exhibited a Kd of 2.2 x 10^{-7} M. Similar AFP receptor systems were subsequently detected on the surface of the YAC-1 mouse T-lymphoma [45] (but not on normal adult mouse T-cells) and on the human U937 and THP-1 cell lines [46]. The number of sites/cell as well as the binding affinity vary from one cell line to another. The binding assays were not restricted to intact cells but also included MCF-7 and primary breast cancer cytosols thus evidencing a soluble RECAF fraction [47].

MONOCLONAL ANTIBODIES AGAINST RECAF

Monoclonal antibodies (Mabs) are more practical, consistent and inexpensive for detecting RECAF than AFP. Thus, we screened the supernatants of a fusion made against pooled human mammary carcinoma membranes and found 2 clones that inhibited the binding of AFP to malignant cells. Conversely, these Mabs were inhibited from binding to RECAF by an excess of AFP [48]. The Mabs also reacted with RECAF from human cord serum that was purified by AFP affinity. Paraffin sections stained with these Mabs showed strongly positive labeling of human fetal muscle and mammary carcinomas whereas benign breast tumors were negative.

Using one of these Mabs on placenta tissue sections, an independent study concluded that RECAF plays a role in moving AFP from the fetal circulation to the mother's blood stream [49], which is consistent with the earlier notion that AFP transports fatty acids to fetal (and cancer) cells [50-53].

These anti-RECAF Mabs also recognized mouse and dog cancer cells [54]. This cross reactivity is in agreement with the previous finding that AFP from one species is taken-up by cells from other species [21, 45] and it validates the use of syngeneic mouse tumor models for *in vivo* experimentation thus eliminating the need for xenografts in nude mice, which is a very artificial model.

More recently, we became interested in detecting circulating RECAF and therefore we made Mabs against the soluble RECAF fraction in MCF-7 cell extracts. One of these Mabs, named 1.4G11, complied with the conditions necessary to ascertain that it is directed against

RECAF[2]. As expected, this Mab also recognized mouse and dog RECAF. It is worth mentioning that all the anti-RECAF Mabs we have produced so far belong to the IgM class.

AN AFP PEPTIDE THAT BINDS TO RECAF

Both AFP and IgM Mabs are large molecules that are costly to produce and, given their size, are impaired when it comes to tissue penetration and secretion, an important point when considering targeted therapy. Moreover, as biologicals, they present some risks when injected to patients. These drawbacks could be overcome if the AFP binding site to its receptor consisted of a short peptide that could be synthesized chemically. To search for such a peptide, we digested human AFP with proteases following Dudich et al. methods [55] and we monitored the AFP binding of the fragments by binding inhibition of labeled AFP to microtiter plates coated with cancer cell extracts. Once the smallest RECAF binding fraction was identified, we applied a brute force approach: Starting from the C-terminal of the AFP molecule, we synthesized overlapping peptides covering the fragment[3] which we then tested for AFP binding inhibition,. We found an inhibiting peptide that we then trimmed until we established the shortest sequence that exhibited AFP binding inhibition activity [56, 57]. That sequence is KQEFLIN, starting at amino acid 549 of human AFP. For solubility reasons this sequence was extended to LQTMKQEFLIN (henceforth referred to as F4). It should be noted that this sequence does not correspond to other AFP peptides reported to have biological activity [58-64].

RECAF PURIFICATION AND CHARACTERIZATION

RECAF has been purified by several methods and from different sources: It has been purified from cord serum [48] and from the pleural effusion of a patient bearing lung metastases of a mammary carcinoma [54]. In both these cases the purification relied on the presence of AFP-RECAF complexes in the starting material, which is consistent with several studies showing the synthesis and release of these complexes in different types of fetal and cancer cells [41-43, 65, 66]. A more direct approach was taken by Severin et al. [67] who used AFP covalently coupled to Sepharose® to bind RECAF, and KCl at high concentration to elute it from the gel. Their results were virtually identical to ours using cord serum and Con-A Sepharose. Another method involved the use of AFP conjugated to a photoactivatable thio-cleavable cross-linker that was activated once the AFP was attached to RECAF on U937 cells (a human monocytic malignant cell line) [46].

The fractions purified with all of those methods exhibited a molecular weight of approximately 65 kDa, which is consistent with the molecular weight of the AFP-RECAF complex as measured by gel filtration after incubating radiolabeled AFP with materials rich in

[2] These conditions are: (a) an excess of the Mab must inhibit the binding of AFP to RECAF, (b) an excess of AFP must inhibit the binding of the Mab to RECAF and (c) on a Western blot of a cancer cell extract, both AFP and the Mab must detect the same bands.

[3] The peptides were 20 amino acids in length and the overlap was 10 amino acids. To test them, the peptides were incubated together with labeled AFP onto microtiter plate wells coated with RECAF containing material. Positive peptides inhibited the binding of whole AFP.

RECAF [68]. The molecular weight of the complex was found to be ~130 kDa, of which 65 kDa could be accounted for by AFP, with the other 65 kDa corresponding to RECAF. A molecular weight of ~65 kDa is also consistent with the detection, using anti-RECAF Mabs, of two adjacent bands weighing 62 and 67 kDa respectively, on SDS polyacrylamide transblots of human breast cancer extracts [48, 54].

Heavier RECAF fractions weighing 180 kDa [54] and 250 kDa [67, 69] have also been reported. The heavier fractions could correspond to complexes with anti-receptor auto-antibodies [70] or other cellular proteins.

Torres et al., using AFP immobilized on nitrocellulose membranes, reported the isolation of two smaller (31 kDa and 18 kDa) AFP binding glycoproteins from Raji cells (a human B-lymphoma) [71]. The 18 kDa fraction was also found in PHA stimulated human peripheral blood lymphocytes. No binding fractions were isolated from resting lymphocytes. Interestingly, the pictures in this article show a fainter band exhibiting a molecular weight of ~65 kDa, which is never discussed throughout the article. The authors stated: *"These observations strongly suggest that the isolated 31 kDa and 18 kDa glycoproteins are probably AFP receptors previously demonstrated in several neoplastic and normal cells undergoing growth and/or differentiation; indeed, they were identical to albumin-binding proteins described by others."* This is important because some previous studies also showed that Albumin inhibited the binding of AFP to its receptor, albeit to a lesser extent than AFP itself [44, 45]. However, this is inconsistent with the above mentioned scintigraphy results since the amount of injected radiolabeled AFP was minuscule compared to the concentration of Albumin in serum, which would have competed out any binding or internalization of the former into cancer cells. Also, Suzuki et al. [46] reported that serum Albumin did not compete with AFP for binding to RECAF. Thus, to elucidate whether Albumin competes with AFP for binding to RECAF, we carried out a series of experiments and we found that neither human Albumin nor bovine Albumin competed with AFP for binding to a RECAF rich MCF-7 extract (Figure 3). In previous experiments we had shown that neither Albumin nor Ovalbumin competed with our anti-RECAF Mabs [48].

An explanation to Torres et al. finding that Albumin and AFP bind to the same bands on polyacrylamide gels might be that the AFP binding activity is located on a glycan that could be attached to several proteins with different molecular weight. It is also possible that within the same band, some molecules are glycosylated with the AFP binding glycan while others carry a glycan that binds Albumin. This could reconcile the apparent binding cross reactivity on the gel bands with the lack of competition found in other experiments.

Yet, it remains to be explained why some experiments show AFP binding competition by Albumin while others do not. A possible explanation could be connected to the fact that different lots of AFP show different RECAF binding activity, which could, in turn, be explained by the proposed hypothesis that one of the functions of AFP is to carry essential fatty acids to fetal cells [17, 72, 73]. Once the AFP-fatty acid complex is internalized, the fatty acid is retained and the "empty" AFP molecule is released into the extracellular fluid intact [74], ready to fetch and transport another fatty acid molecule. It is reasonable to assume that fatty acid depleted AFP molecules are not internalized; otherwise, they would be secreted and taken up again in a vicious circle that would be detrimental to the system. The AFP lot binding differences could then be explained by differences in their fatty acid content. To test this hypothesis, we precipitated AFP with cold acetone, which also extracts the fatty acids from the protein. Next, we re-lipidated aliquots with a mixture of fatty acids as described for

native human AFP [75]. We then incubated the initial AFP, the delipidated AFP and the re-lipidated AFP with MCF-7 cells in culture. The results are shown in Figure 4, which clearly shows that the delipidated AFP is not taken up and that re-lipidation not only reverts the results of removing the lipids, but can also enhance AFP uptake depending on the amount of fatty acid added. These results have both practical and theoretical implications: From a practical point of view, the findings suggest that experiments involving AFP uptake by fetal or cancer cells could benefit from lipidating the protein with fatty acids. From a theoretical point of view, these results further support the notion that AFP functions as a carrier molecule for fatty acids and they could help explain the apparent competition between AFP and Albumin: Most commercial preparations of Albumin consist of Cohn fraction V, which is prepared using ethanol, a fatty acid solvent. The resulting de-fatted Albumin, when used in large concentrations, could sequester the fatty acids from the co-incubated AFP, thus greatly reducing the ability of the latter to bind to its receptor. In our competition experiments (Figure 3), we used pre-lipidated AFP (for both labeled and non-labeled AFP), and we added only 100 fold excess of Albumin (as compared to 5,000 fold excess in a previous paper [44]) since that amount was sufficient to show ~90% competition by unlabeled AFP.

It is worth noting that whole AFP requires fatty acids to bind to its receptor but the F4 peptide, which is part of AFP, does not. Hence, it appears that fatty acids induce AFP conformational changes [76] that expose the F4 sequence to the receptor rather than having the AFP-fatty acid complex binding directly to the receptor. It should be mentioned that the fatty acid binding site and the receptor binding site are far away on the AFP molecule; the fatty acid binding site is on or nearby amino acid 223 [77] whereas, as mentioned above, the receptor binding site is located nearby amino acid 549.

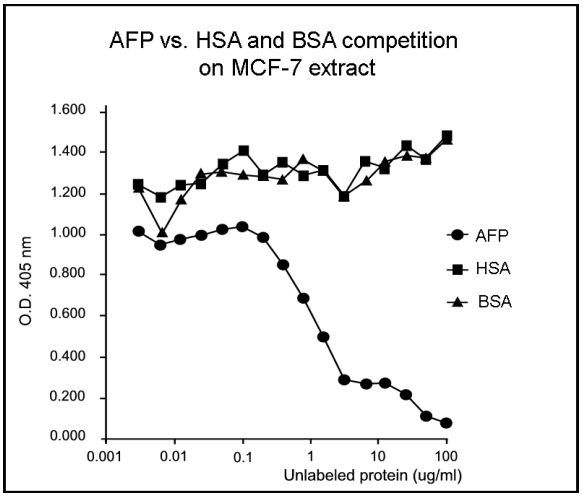


Figure 3. Binding competition between human serum Albumin (HSA), bovine serum Albumin (BSA) and AFP. The supernatant of an MCF-7 extract made without SDS (mainly 62 kDa RECAF) was coated onto microtiter wells. Biotin-AFP (1 ug/ml) was mixed with different concentrations of unlabeled AFP, HSA or BSA and incubated in the wells. After washing, Streptavidin-Peroxidase was added. The wells were washed, the reaction was developed with ABTS + H2O2 and the wells were read at 405 nm. Only unlabeled AFP competes with Biotin-AFP; there is no competition between Biotin AFP and HSA or BSA.

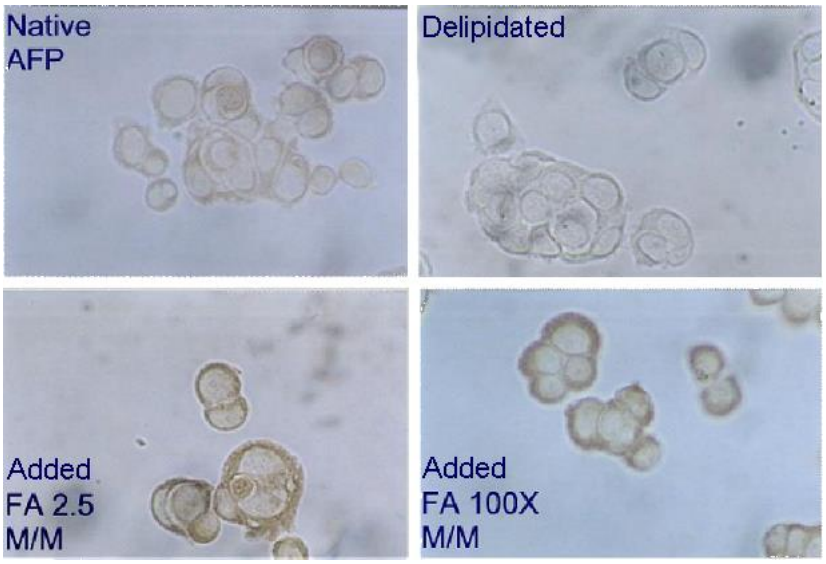


Figure 4. Native or treated AFP was incubated with MCF-7 cells in culture for 90 minutes at 37oC. After washing, the cells were fixed and immunostained using an anti-AFP antibody. AFP was delipidated by precipitation with cold acetone. To re-lipidate the delipidated AFP, we diluted a mixture of oleic, arachidonic and docohexanoic acids [75] in ether and then dried the solution on the wall of a rotating glass tube. The AFP was then incubated in the tube under rotation. The molar ratios (M/M) described in the figure correspond to the total amount of fatty acid dried on the glass tube wall over the total amount of AFP incubated in the tube. The delipidated AFP is not taken up, whereas the 2.2 M/M relipidated AFP exhibits stronger staining (and hence uptake) than the native AFP. Loading AFP with a fatty acids at 100X the molar ratio results in even stronger uptake.

In reference to RECAF being a carbohydrate, a previous report using glycan modification with sodium periodate or borohydrate showed that anti-RECAF Mabs recognize a structure that is compatible with an O-glycan [54]. Our own experiments show that AFP does not bind to an O-glycan but rather, it binds to an N-glycan on RECAF: We coated microtiter wells with K562 cell extracts and then we treated the wells with periodate (1-100 mM), followed by incubation with Biotinylated-AFP (B-AFP). As shown in Figure 5, the treatment with periodate results in a decrease of up to 90% of the binding activity and the decrease is proportional to the periodate concentration. As a control, wells coated with human albumin, which has no glycans, were treated in identical conditions and showed no decrease in binding to a polyclonal anti-HSA antibody.

To further demonstrate that only the glycan and not the RECAF peptidic chain is involved in the binding, we did the counter experiment: We terminally digested the RECAF containing material with pepsin and tested its binding inhibition activity. Since digesting the receptor coated onto a plate would result in detachment of peptidic fragments, we carried out the digestion in solution using 0.04% pepsin for 24 hours at 37^{o}C, pH 2.5 (a control PAGE of the digested material showed no bands aside from the pepsin band). After bringing up the pH, we mixed a fixed concentration of B-AFP with different concentrations of the digested RECAF extract. The mixture was then added to wells coated with the same extract, but not digested. Thus, the higher the concentration of RECAF left undigested in solution, the more B-AFP it would complex and therefore the lower would be the amount of free B-AFP

available to bind to the RECAF coating the plate. As a control, we subjected the same receptor preparation to the condition described above but without the pepsin. Another control was carried out using Albumin and an anti-Albumin antibody instead of the RECAF preparation and B-AFP respectively.

If the binding site were on a sugar, the digestion with pepsin would make no difference in the binding and therefore, the pepsin digested receptor and the control without pepsin should display similar binding curves for the receptor experiment.

Figure 6 shows that the inhibition curve obtained with the digested extracts was practically identical to that of non-digested extracts. This was not the case for the Albumin control, which showed a 5-fold difference in OD between digested and non-digested protein (data not shown).

Thus, the glucidic nature of RECAF has been established by two different methods; by destroying the glycan with periodate-borohydrate treatment and by destroying its peptidic backbone.

In addition, extracts from cells incubated with Tunicamycin, which specifically inhibits N-glycosylation [78], showed a ~90% decrease in AFP binding (Figure 7). Treating the cells with Galactosamide, which inhibits the linking of O-glycans, resulted in no change in binding.

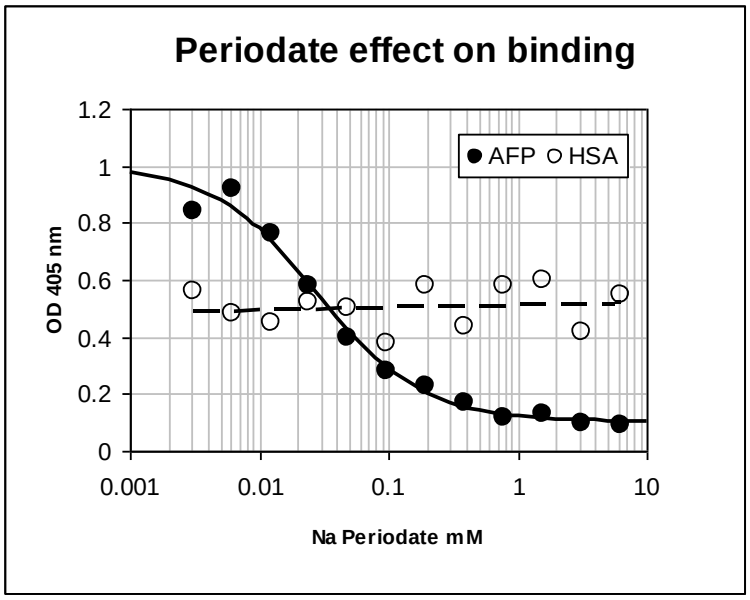


Figure 5. Sodium periodate treatment of RECAF demonstrating that RECAF is a sugar. Microtiter plates were coated with a K562 extract (black circles) or human serum Albumin (HSA, open circles) and then treated with different concentrations of periodate. The RECAF binding activity was tested using a constant concentration of Biotin-AFP. The HSA integrity was tested using an antibody. The Biotin-AFP and anti-HSA antibody were developed with Streptavidin-Peroxidase and anti-IgG-Peroxidase respectively. The Biotin-AFP binding decreases as the concentration of periodate increases, reaching a plateau above 10 mM. There is no effect of the periodate on HSA, indicating that the periodate is not affecting the RECAF peptidic chain but rather its glycans.

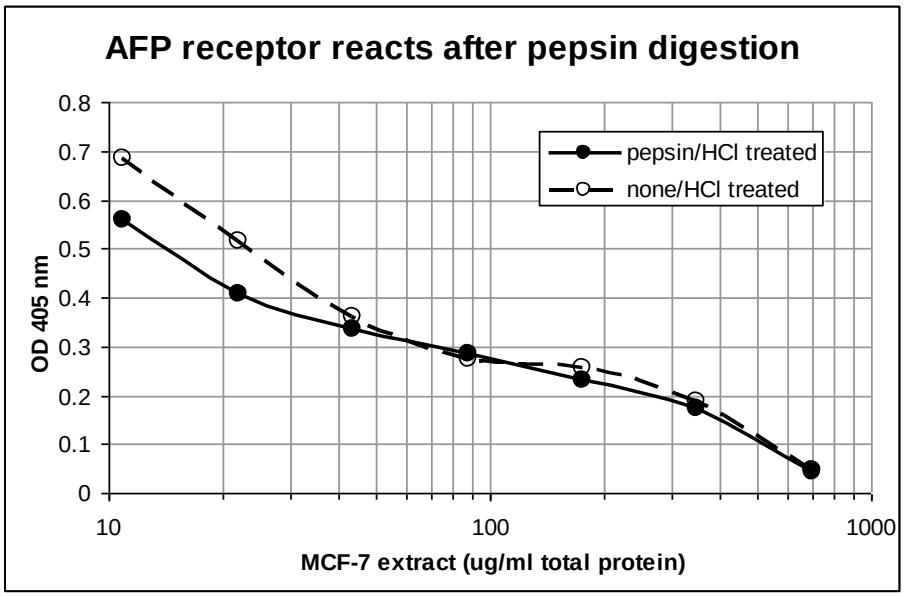


Figure 6. Pepsin treated RECAF behaves almost exactly like non pepsin treated RECAF. See text for the experiment's details.

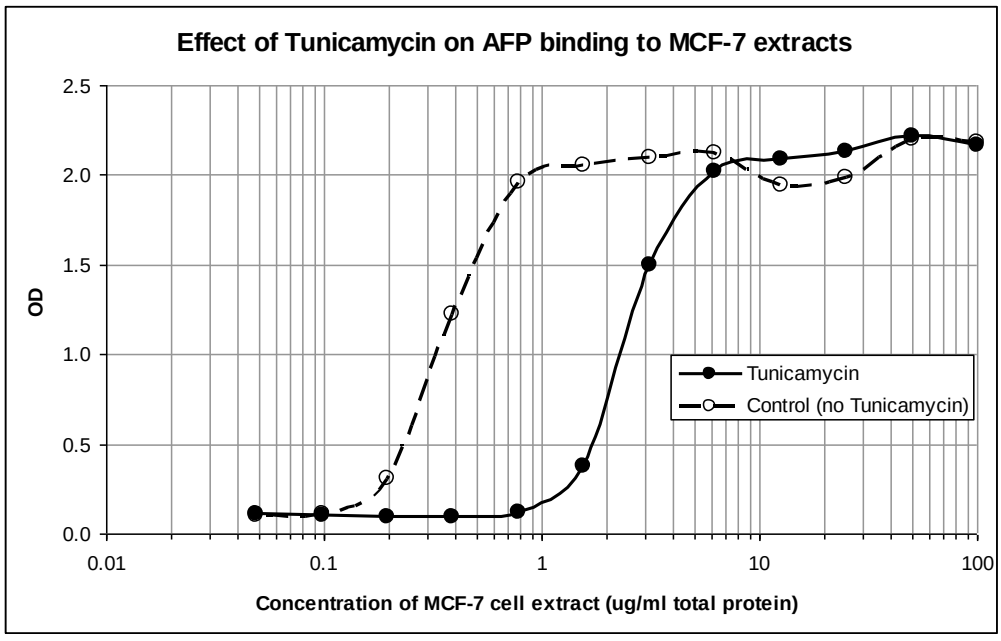
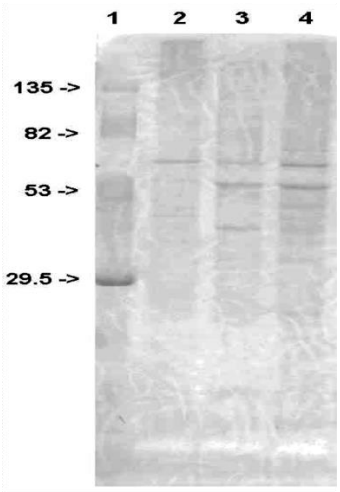


Figure 7. Binding of AFP to MCF-7 extracts from cells treated or not with Tunicamycin, a specific inhibitor of N-glycosylation. The cell extracts were coated at different concentrations onto ELISA plates, followed by incubation with 10 ug/ml AFP-Peroxidase. The AFP binding activity on Tunicamycin treated cells was significantly lower (~1/10) compared to not treated control cells, thus requiring a higher amount of MCF-7 extract coating to attain the same OD readings. These results indicate that RECAF is an N-glycan.



These results contradict the reports suggesting that the receptor is an O-glycan [54] or that the RECAF binding site is a widespread mucin [79, 80]. The author of the latter references also suggested that RECAF belongs to the scavenger receptor family [81]. The complex nature of this group of receptors and the general lack of information on the RECAF glycan structure makes this hypothesis difficult to ascertain. Perhaps once that structure is determined it might become apparent that RECAF belongs to some known family of receptors. Moreover, it is possible that there is more than one type of receptor involved: Pardee et al. have shown [82] that AFP is internalized by normal adult dendritic cells (DC), which is unexpected. They identified a mannose receptor (MR/CD206) as the primary DC uptake pathway for both normal cord blood-derived AFP and tumor-derived AFP. However, there is no indication that this internalization pathway is the same one that is present in cancer cells. In our own experiments, normal peritoneal mouse macrophages did take up AFP but they did not exhibit much protein specificity; they take up Ovalbumin and other proteins as well as AFP. This is not the case for cancer cells, which take up AFP but not Ovalbumin.

Thus, until more data are available, the author prefers to be cautious when speculating in a field in which information is scant. The evidence gathered so far supports the notion that RECAF is an N-glycan that is attached to a number of different proteins. Polyacrylamide gels of cell extracts show the ubiquitous 65/67 kDa doublet [48], which is water soluble and the 31 and 18 kDa bands, which require 0.5% SDS to be extracted [71]. In addition, we have observed two faint soluble bands in some of our Westerns; a 53 kDa and a 44 kDa band. The relative intensity of all these bands varies from one cell line to another. Moreover, extracts carried out in exactly the same conditions, from the same cells cultured, under identical conditions show variable RECAF bands patterns[4] as shown in Figure 8.

[4] Anecdotally, we observed that the receptor expression in cultured HL60 (a human acute promyelocytic leukemia), was modified by the plastic Petri dishes brand used for tissue culture.

Given our interest in a soluble band for cancer detection in serum, we concentrated our efforts on the 65/67 kDa band, which is consistently present in cancer cell extracts. While this fraction is soluble, it is also aggregated. When cancer cell extracts are passed through a gel filtration column, the AFP binding activity comes out in the void volume, even when using Bio-Gel A-5m, which has an exclusion size of 5 mega Daltons. Adding 0.5% SDS, or lowering the pH below 5.0 results in disaggregation with a single broad peak of AFP binding activity at ~70 kDA as measured by molecular weight calibrators.

POTENTIAL USES OF RECAF AS A TUMOR MARKER FOR *IN-VITRO* DIAGNOSIS

Aside from its potential for tumor scintigraphy, RECAF could prove useful as a widespread cancer marker to detect leukemia and malignancy on tissue sections, blood smears and bodily fluids.

RECAF on Leukemia Cells

Flow cytometry offers the possibility of monitoring residual disease in leukemia patients after treatment. The sensitivity of this method is far superior than detecting malignant cells under the microscope, thus prompting the administration of a new round of chemotherapy at an earlier recurrence stage. This technique showed strongly positive results when the 167H.4 Mab was used to stain HL60, Ramos and U-937 cells [54]. In those experiments, Molt-4 cells were not stained, which is consistent with the findings from other AFP uptake experiments [28, 83].

Immunohistology Using Anti-RECAF Mabs on Cancer Tissue Sections

Using an anti-RECAF Mab for immunofluorescence on paraffin sections, we reported the positive staining of 6 out of 6 human mammary carcinomas whereas 3/3 benign adenomas were negative [48]. Similar results were obtained with another antibody and the immunoperoxidase technique: We found positive staining in 21/23 mammary carcinomas, 14/15 lung cancers, 8/8 colon carcinomas and in approximately 90% of other malignancies. Tsuboi et al., using anti-RECAF antibodies found 34/47 positive stomach cancers and only 2 positive cases out of 61 benign specimens and non-malignant areas around cancer cells in specimens from cancer patients [84]. Interestingly, these authors found no statistical correlation between the staining intensity and the degree of cell differentiation. Also, they mention that a previous and unpublished study on colon cancer sections showed identical staining patterns between anti-RECAF Mabs and labeled AFP, which coincides with our own observations.

Figure 9 shows some examples of cancer and benign tissues stained by immunoperoxidase using an anti-RECAF Mab.

While paraffin sections stained with Hematoxylin and Eosin are usually clear enough for diagnosis purposes, there are some situations in which RECAF staining could prove helpful:

i. Frozen sections, where the sub-optimal quality of the specimen can lead to error.
ii. For detecting the presence of small metastatic foci within sentinel lymph nodes as currently done with cytokeratin immunostaining.
iii. Fine needle biopsies in which the lack of tissue architecture makes the diagnosis more difficult. Figure 10 shows the staining of fine needle biopsies from patients with benign and malignant breast lesions in which the difference between them becomes very clear after staining for RECAF.
iv. Smears, for the same reason as fine needle biopsies. To illustrate this point, we first spiked normal sputum or cervical smears with cancer cells and then we fixed them and stained them with an anti-RECAF Mab. The results are shown in Figure 11**.** Cervical smears are a special case because they are often examined with the aid of software. Some programs pinpoint the suspicious areas for the pathologist to focus on. Others carry out the whole diagnostic analysis, considering several parameters such as the nuclear/cellular surface ratio. In both cases, a great deal of microprocessing time is dedicated just to identifying cells and differentiating them from detritus and artifacts before analyzing whether they are malignant or not. On the other hand, detecting brown spots resulting from immunoperoxidase staining requires very little processing. Thus, pointing the pathologist's attention to the brown cells on a tissue section, or in fully automated systems, focusing processing time on the brown stained structures/cells should largely improve the speed and thus the efficiency of the system, even if the criteria used for diagnosis are kept unchanged and the positive staining is not considered as part of those criteria. Of course, the inclusion of positive RECAF staining as an additional criterion of malignancy should further improve the system's performance.

Serum RECAF and Its Use for Cancer Diagnosis

The soluble nature of some of the RECAF bands suggested that malignant cells could release the marker into the circulation, where it would be abnormally elevated. A first attempt to ascertain if circulating RECAF could be used for cancer detection compared sera from 17 cancer patients bearing assorted malignancies and 22 negative controls (normal donors or patients with hypertension or heart related conditions). RECAF was elevated in 16/17 cancer patients whereas 20/22 control sera were negative ($p < 2 \times 10^{-6}$ by t-test) [85]. Anecdotally, a female patient in the control group who consulted for severe hypertension had an extremely high level of circulating receptor. Given the statistical significance of the results, this patient was subjected to a CAT scan that revealed a previously unknown kidney tumor, which was soon after diagnosed as a hypernephroma.

Around that time, Sarcione et al., took a different and elegant approach for detecting RECAF in serum: Previous work [41-43] had shown that anti-AFP antibodies do no detect AFP bound to its receptor and it was well known that in breast cancer patients, AFP is not elevated [86, 87]. The authors hypothesized that the AFP in serum from cancer patients consisted of two fractions: Free AFP, which was detectable (and within normal values) and

RECAF bound AFP, which was elevated but undetectable. As a result, the *total* concentration of AFP would be higher in cancer patients than in normal individuals. To measure total AFP, they treated sera from both groups with 0.4M KCl to dissociate the RECAF-AFP complex[5] before measuring the AFP concentration, which had a mean of 26.4 ± 7.1 IU and a median of 28.0 IU in normal female serum. In the serum of breast cancer patients, the values were approximately twice as high; the mean AFP concentration was 53.0 ± 17.9 IU and the median was 50.0 IU [88]. It should be mentioned that the commercial kit used in this study was discontinued by the time we attempted to replicate those results. Thus, we tried using different antibodies and antisera, in both sandwich or competition assays without success; we found no difference between the serum AFP concentration in normal females and in breast cancer patients.

The development of Mabs first and RECAF binding peptides later allowed us to develop assays with which we have tested several thousands serum samples of the most prevalent types of cancer. We used radioimmunoassay [89], chemiluminescence [90, 91] and to a lesser extent ELISA [92]. We were also able to show proof of concept for lateral flow point-of-care tests [93]. The results obtained with the Mabs or the AFP peptides were similar [94].

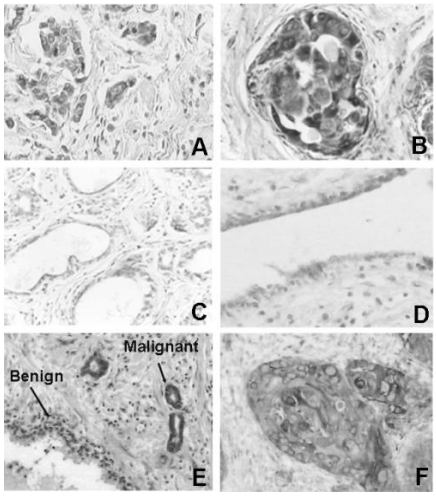


Figure 9. Immunoperoxidase of paraffin tissue sections using Mab 1.4G11. (A and B) Breast carcinomas, (C and D) negative breast benign fibroradenomas, (E) prostate carcinoma and (F) lung cancer. Staining is always cytoplasmic showing perinuclear accumulation. All but F are counterstained with Hematoxylin.

The preferred assay format consists of competing the RECAF in the sample with labeled RECAF for binding to the ligand (AFP peptide or Mab) coated onto a microtiter plate. This ensures that all proteins exhibiting the RECAF glycan are detected, which would not be the case with a sandwich assay in which one side of the sandwich would be the RECAF binding Mab and the other, a Mab against the peptidic chain of one of the proteins carrying the

[5] Chaotropic agents such as KCl at high concentration are known to split receptor-ligand bonds. The KCl needed to be diluted immediately before the assay because otherwise, the high salt concentration would prevent the assay's antibody from reacting with AFP.

RECAF glycan[6]. This is clearly illustrated in Figure 8: An assay in which the 2nd antibody were directed against the protein backbone of the 53 kDa band would detect samples 3 and 4 but not sample 2.

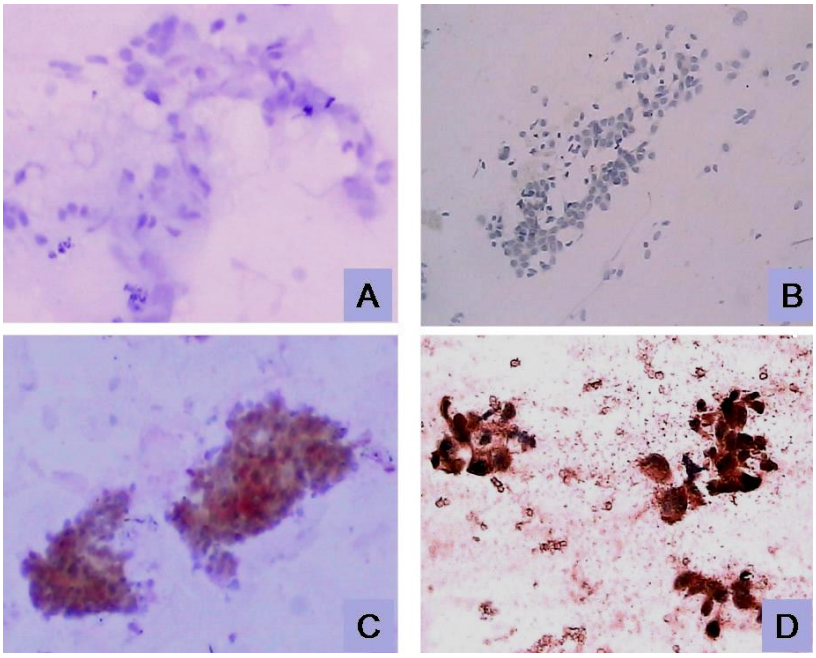


Figure 10. RECAF staining of breast fine needle biopsies. (A and B) negative benign lesions, (C and D) positive cancer cells. Counterstained with Hematoxylin.

Since we are measuring RECAF on several proteins with different molecular weights, the measurements cannot be expressed in ng/ml. As a result, the amount of RECAF is currently measured in arbitrary RECAF Units[7].

Figure 12 depicts some of our findings: At 95% specificity, sensitivity[8] values vary, from one cancer type to another, between 75% and 90%. Please note that benign lesions of the breast and prostate are in their vast majority negative. It remains to be determined whether the false positives in the benign groups are truly benign or if they correspond to premalignant lesions. In this regard, it is reasonable to consider that the biochemistry changes involved in the transformation process precede changes in the morphology and therefore a biochemical test might yield positive results before the pathologist can detect signs of malignancy under the microscope. This brings us to the issue of the "golden standards" used for evaluating the sensitivity and specificity of this kind of test. The so called "golden standards" consist of a set of serum samples from patients that were diagnosed as having cancer or benign lesions by a pathologist examining histological sections under the microscope. Yet, there is a percentage

[6] Our attempts at developing a sandwich immunoassay format resulted in a loss of 10-15% sensitivity (true positives) compared to the competitive assay. Using more than one Mab to capture all RECAF glycated proteins would be too noisy and impractical to manufacture and quality control.

[7] The 95 percentile of a population of normal donors used as cutoff value corresponds to 4,600 Units of RECAF.

[8] Sensitivity in this context does not refer to the amount of RECAF the test can measure but rather, it is the ability of the test to correctly identify patients with cancer. Specificity is the ability of the test to correctly identify individuals without the disease.

of cases in which pathologists err [95, 96]. This error is usually ignored because it is relatively small, but it becomes very significant when evaluating a test that approaches the highly sought 100% specificity and sensitivity necessary for screening. To yield those values, a blood test would have to replicate the pathologist's error, which is formally absurd.

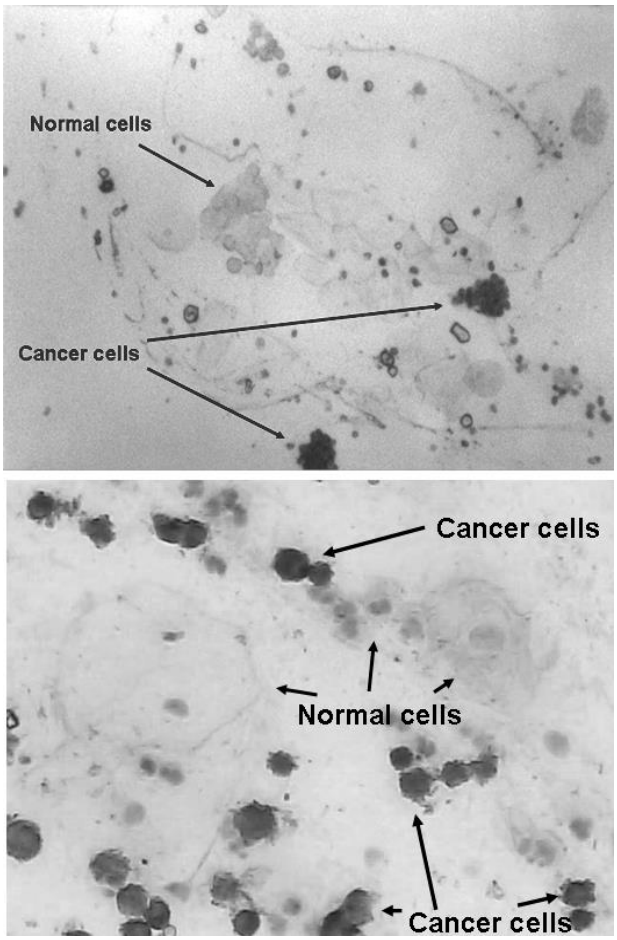


Figure 11. Sputum (top) and cervical (bottom) smears from normal individuals spiked with cultured lung cancer cells or cervical cancer cells respectively. After fixing, the slides were stained for RECAF. The cancer cells are notoriously stronger stained than the normal cells (including leukocytes in the bottom picture). Counterstained with Hematoxylin.

RECAF serum tests come close to the levels at which the pathologist error becomes relevant and this means that their actual sensitivity and specificity might be even higher. Also, it means that the test might be useful for cancer screening, which is very relevant given the fact that serum RECAF tests can detect a significant percentage of malignancies at early stages (I and II) [89, 91], when treatment is most effective. Furthermore, RECAF is not only elevated in the serum of cancer patients, but also in saliva, as shown in Figure 13. While the accuracy of the saliva test is lower than that of the serum test, saliva collection is remarkably simple and inexpensive. Thus, the saliva test should be set up with a cut-off value low enough to detect most of the cancer samples and then, on a second round, the saliva positive patients

would get a RECAF serum test with a high cut-off value to eliminate most of the false positives.

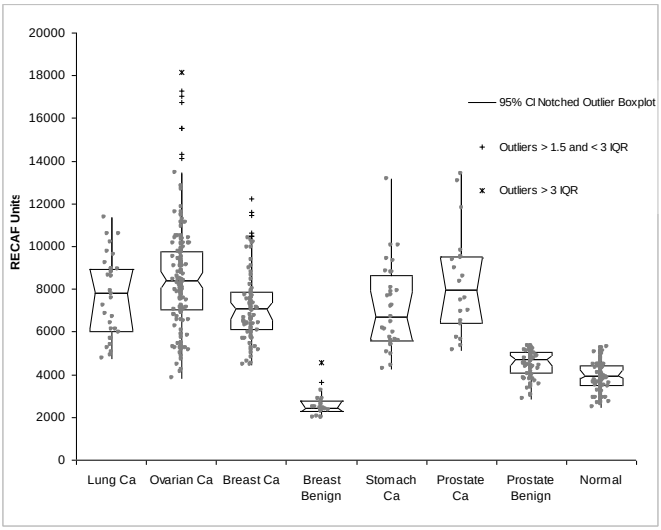


Figure 12. Serum RECAF concentration in patients with different types of cancer, benign lesions and normal donors. Ca = cancer. Circulating RECAF is elevated in cancer patients when compared to samples from patients with benign lesions or to serum from normal donors.

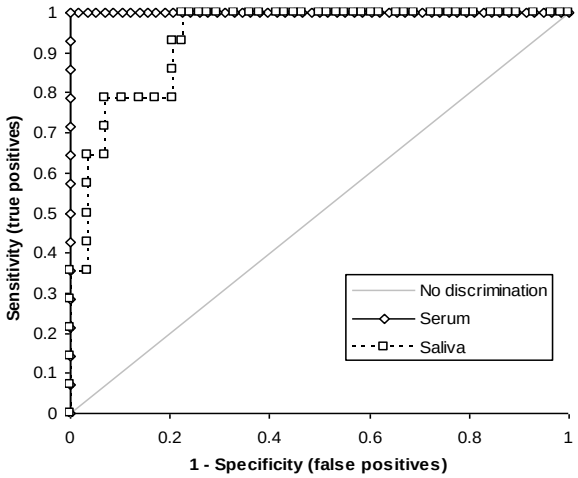


Figure 13. Receiver Operating Characteristic (ROC) curve of paired serum and saliva samples from 14 patients with breast cancer and 58 normal controls. The area under the curve (AUC) for serum = 1.0 and for saliva = 0.94.

The expression of RECAF in normal individuals is independent of age [89] and sex [unpublished results] and it seems to be unrelated to tumor mass. Moreover, there is no correlation in the expression of RECAF and CA125 [91] CEA [97] or PSA [98]. This allows combining the values of these common markers with the amount of RECAF thus increasing the overall sensitivity and specificity, in some cases, to the levels required for screening for

early stages of ovarian cancer [91]. Combining PSA with RECAF is of particular interest because it brings together the RECAF specificity for malignancy with the organ specificity of PSA.

An intriguing possibility is to use serum RECAF as an indicator for prescribing chemotherapy to previously treated cancer patients with no clinical manifestation of cancer recurrence. It has been found that waiting for clinical or imaging evidence of recidivism results in poor outcome whereas guiding therapy with traditional markers before clinical manifestation improves prognosis [99]. The general idea is to administer chemotherapy when the cancer marker increases over a certain threshold even if the patient shows no evidence of recurrence. The broad spectrum of RECAF expression offers the enticing possibility of using this marker for guiding therapy in many different malignancies rather than the limited types of cancer that can be managed this way using current cancer markers.

In addition to the human RECAF tests, we developed a canine version using slightly modified AFP peptides. The test performance in terms of sensitivity and specificity was essentially the same as for the human test[9].

POTENTIAL USES OF RECAF FOR CANCER THERAPY

Inhibition of Malignant Cell Proliferation by Anti-RECAF Mabs

Mouse Mabs directed against human RECAF recognize mouse malignant cells [48, 54]. The 167H.4 Mab reacts with EL-4 and YAC-1 (two mouse T-lymphomas), TA-3, (a mouse mammary carcinoma cell line) and P-388 (a mouse malignant cell line of dendritic origin). This is unusual but not totally unexpected since AFP from one species binds or is taken up by cells from another species [21, 45], which suggests that the binding sites on both AFP and RECAF are highly conserved and yet different enough to elicit an immune response. The cross reaction of anti-human RECAF Mabs with mouse malignant cells enables the use of simple syngeneic mouse tumor models for cancer targeting rather than transplanting human cancers into nude mice.

There is a plethora of literature ascribing biological activities to AFP, ranging from apoptosis to the stimulation of cell proliferation. The anti-RECAF 167H.4 Mab showed an inhibitory effect on the rate of cell division: P-388 cells incubated with the Mab showed a substantial inhibition in cell replication (Figure 14). The cells were not killed, as they continued to exclude Trypan Blue and would re-start replication after washing out the Mab. Complete DNA synthesis suppression was not achieved however, perhaps due to the presence of bovine AFP and bovine RECAF in the fetal calf serum required to obtain a measurable amount of cell replication in the controls. Bovine AFP binds to the mouse RECAF [45] and competes, to a certain extent, with the 167H.4 Mab [personal observations].

[9] While the overall sensitivity and specificity can be easily compared between humans and dogs, the comparison of the test performance within a given type of cancer is more difficult because the most prevalent types of cancer in dogs are different from the most prevalent cancers in humans: In dogs, the most common cancers are lymphomas, hemangiosarcomas, osteosarcomas and mast-cell-tumors. While breast cancer is also prevalent in dogs, it is less common than in humans.

A similar proliferation inhibition was obtained on LoVo cells (a human colon adenocarcinoma) [54]. In these experiments, it was shown that pure human AFP could reverse the inhibitory effect of the anti-receptor Mabs.

In addition, 167H.4 inhibited PHA transformation of normal human peripheral leukocytes.

All of these results point to a signal transduction pathway ending up on a cell proliferation control mechanism. In this context, it is worth mentioning that high concentrations of AFP can induce apoptosis [100, 101]. A great deal of work needs to be done in order to understand these control pathways, which could provide new strategies for cancer management.

These encouraging *in-vitro* results prompted a series of *in-vivo* experiments in which we inoculated C57 Black mice subcutaneously with 2 x 10^6 EL-4 cells followed, the same day, by the intravenous injection of 100 uL of either 167H.4 Mab ascites produced in Balb/c or normal Balb/c serum. In order to facilitate EL-4 cell grafting, the animals received a 300 Rads dose a week before injection. Four to five animals were included in each group and the experiments were repeated 6 times with consistent results: By the sixth day after injection, there was a clear difference between the control animals, which exhibited tumors 5-6 mm in size, and the treated animals, which had only a scar at the site of the injection. If allowed to progress, the tumors killed all of the control animals within 3 weeks. In 5 out of 6 experiments, all of the treated animals remained tumor free until euthanized. In one experiment we kept the treated mice for 8 months with no noticeable signs of disease or other abnormalities. Their offspring was normal. Figure 15 depicts 2 control and 3 treated animals from one of those experiments.

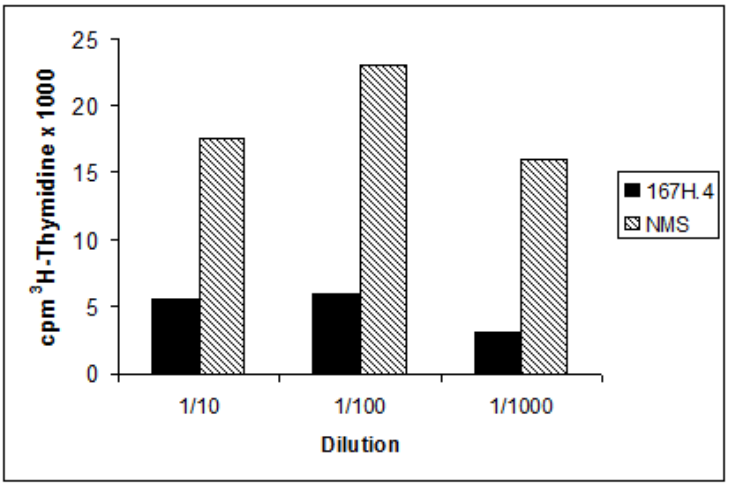


Figure 14 Inhibition of P-388 cell proliferation by the 167H.4 anti-RECAF Mab as compared to normal mouse serum (NMS). The incorporation of ^{3}H-Thymidine was reduced by 70%-75% in a 6 hour pulse chase experiment.

In the 6th and last experiment, the treated animals did developed tumors and eventually died of them, albeit they survived significantly longer than the controls (Figure 16). An assay conducted on the EL-4 cells in culture used for grafting these animals showed a drop to only 20% of specific ^{125}I-AFP binding. This unexplained and sudden drop in AFP binding, and

hence in RECAF expression, has been previously noticed in other cell lines, such as MCF-7 and Ichikawa when they are cultured over many passages [personal observations], and could be related to the above mentioned RECAF gel bands polymorphism (Figure 8)[10].

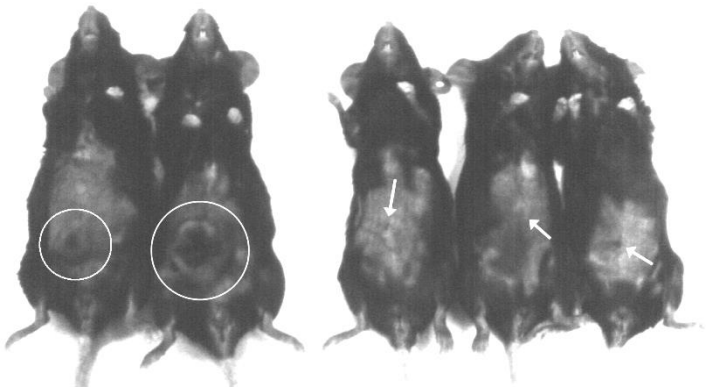


Figure 15. Tumor growth inhibition by the anti-RECAF 167H.4 Mab. Five mice on each group received an IV injection of either 100 uL of Mab ascites or normal mouse serum as a control, along with 2 x 10^6 EL-4 cells injected subcutaneously on the abdomen. After 2 weeks, all control animals exhibited large tumors (two of these animals are shown on the left, with a circle around the tumors). All treated animals were tumor free as shown on the three animals on the right. The arrows point to the small scars left where the EL-4 cells were injected.

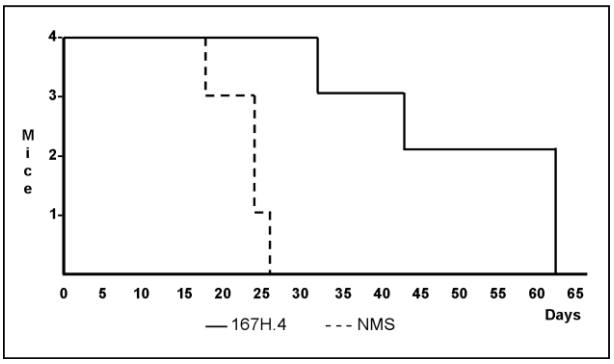


Figure 16. Tumor growth inhibition by the anti-RECAF 167H.4 Mab. This experiment was similar to the one depicted in Figure 15 with the difference that the specific AFP binding to the inoculated EL-4 cells had dropped by 80%. Nonetheless, the life span of the Mab treated animals was significantly longer than the control's.

These *in-vitro* and *in-vivo* results indicate that anti-RECAF antibodies might slow down or abrogate cell proliferation and makes our experience in carrying out over a dozen fusions to generate more anti-RECAF Mabs worth mentioning: After fusing the splenocytes of the immunized mouse with a myeloma partner[11], we allowed the resulting hybridoma colonies to grow in 96 well plates. Once they were visible to the naked eye, we screened the supernatants

[10] It must be stressed that this RECAF turn-off phenomenon is not related to selective pressure by the Mab or to *in-vivo* growing conditions since it takes place during cell culture under standard conditions and before the cells are injected into a mouse or exposed to Mabs, AFP or any other targeting molecule.

[11] SP2 or Fox-NY (the latter, was used for generating the previous round of anti-RECAF Mabs).

by ELISA against RECAF. We also screened for AFP binding inhibition as described elsewhere in this chapter. We usually found 5-15 positive clones in a 10 plate fusion. As time went by (and the concentration of Mab in the wells increased), the cells from those positive wells would stop growing, they would become picnotic and finally die. However, the cells could be kept alive by changing the medium every 48 hours. This 'suicidal' behavior could be explained by the presence of RECAF on the hybridoma cells, which would be carried over from the fusion partner. As the concentration of anti-RECAF secreted by the hybridoma increased, the cells would stop proliferating in a sort of autocrine negative feedback loop.

Another worth mentioning fact is that not all anti-RECAF Mabs inhibit binding. For example, Mab 1.4G11 has no *in-vitro* effect on cell proliferation despite (a) recognizing all the same bands that AFP recognizes on a Western (including the membrane associated 31 kDa and 18 kDa bands), (b) inhibiting the binding of AFP to its receptor and (c) being inhibited from binding to RECAF by an excess of AFP. These are the same criteria used for discovering 167H.4, which *did* inhibited cell proliferation. The main difference between the two Mabs is the immunogen used: 167H.4 was generated from a mouse immunized with a pool of human breast cancer biopsies solubilized with NP-40 and retained on Agarose-PNA (Peanut Agglutinin), which was then injected into a mouse [54]. 1.4G11 was produced from a mouse immunized against the soluble RECAF fraction from an MCF-7 extract.

It should be pointed out that a 1/300 dilution of the serum from the mouse used to generate the 167H.4 Mab also inhibited the binding of ^{125}I-AFP to Ichikawa cells by 71% [48] (as a reference, the supernatant of the 167H.4 clone inhibited ^{125}I-AFP binding by 73%). This is of practical interest: If a 1/300 dilution of the serum could inhibit cell proliferation *in-vitro*, it is likely that inside the animal the neat serum should inhibit cell proliferation even better, providing the mouse with some protection against cancer whether it was grafted of spontaneously generated. In essence, the animal would have been vaccinated against most cancer types given the broad spectrum of RECAF expression.

At present, the mechanisms underlying the proliferation inhibition are unknown but they must involve a message from the AFP receptor(s) on the cell surface to the nucleus. It seems reasonable to consider that the receptor molecules carry the message to the cytoplasm or even to the nuclear membrane, where immunohistology shows strong RECAF accumulation and capping. A thorough analysis of the possible pathways involving AFP binding proteins has been advanced by Mizejewski [102].

RECAF Targeted Chemotherapy

The discovery of cancer markers rekindled Paul Ehrlich's dream of a "magic bullet" which would target and kill cancer cells without destroying healthy cells, thus significantly improving standard chemotherapy.

The most common type of cancer-targeted compound consists of two parts, the delivery moiety (usually an antibody), which should be as cancer specific as possible, and the toxic moiety, which, once delivered into the cell, kills it. The latter can consist of chemotherapy agents such as Doxorubicin, Chlorambucil, Taxol, etc. or biological toxins such as Ricin, Abrine and others. Radioactive isotopes have also been used for this purpose [103]. The delivery moiety and the toxic moiety can be linked together in a variety of ways. The type of link is important because it must fulfill certain requisites: It should split only within the

targeted cell and once inside, cancer cells must be able to break it down effectively to allow the toxic component to reach its target(s).

In general, targeted therapy has not delivered on its promise mainly because of the lack of suitable delivery vehicles: There are not that many cancer markers available and most – AFP is a typical example – move in the wrong direction, from within the cell outwards. The toxic moieties have also exhibited problems. For example, at tolerable doses, many chemotherapy agents do not achieve 100% cell killing, which leads to recurrence and in many cases, to selection of drug resistant cancer cells. Powerful cell toxins such as Ricin or Abrine are exceptions, since they exhibit phenomenal killing efficiency but their large size and immunogenicity are problematic.

RECAF offers several interesting features as a cancer cell target: Firstly, the receptor moves in the right direction; it carries AFP into the cell. This favors the intake of toxic payloads. Secondly, AFP, or a peptide thereof can be used as the delivery molecule with some obvious advantages over using antibodies such as: (i) no immune response triggered against the injected material (thus, no need to humanize Mabs), (ii) small size with faster tissue penetration and elimination if an AFP fragment or peptide is used and (iii) the possibility to tailor a peptide to optimize cell penetration and/or conjugation to the toxic moiety. The major difficulty associated with using RECAF as a target used to be the high cost and ethical issues related to purifying AFP of fetal origin in large quantities but this has been addressed by developing recombinant polypeptides [104, 105] or short peptides [56, 57] containing the AFP amino acid sequence that binds to RECAF.

As early as 1983, Deutsch et al. conjugated Daunomycin to arachidonic and docosahexaenoic acids and showed, both *in-vitro* and *in-vivo*, that the conjugates were effective against AFP producing hepatomas but ineffective against hepatomas that did not synthesize that protein. *In-vitro*, cell killing by Daunomycin-fatty acid conjugates was ~3 times higher than using Daunomycin alone. *In-vivo*, 100 days after grafting rats with an AFP producing hepatoma, all non-treated animals as well as the animals that received Daunomycin alone were dead, whereas 3 out of 5 animals injected with the conjugates were still alive [106]. These authors also found that the Daunomycin exhibited a "marked diminution" in toxicity when conjugated to fatty acids as compared to the free drug. They speculated that their results depended on the affinity of AFP for arachidonic acid, which would selectively bring the Daunomycin complex to the hepatoma cells.

A more direct approach was taken by Feldman et al., who conjugated Doxorubicin directly to AFP. This resulted in a 5-fold increase of the conjugate toxicity compared to the drug alone [107].

As mentioned above, the conjugation chemistry is important because AFP is known to enter and exit cancer cells unscathed [45] whereas the toxic payload must be retained and reach its cellular target. To address this issue, Yabbarov et al. used an acid-labile aconitil link between rAFP3D (a recombinant 3rd domain fragment of AFP) and Doxorubicin [108]. They found that while Doxorubicin alone was slightly more cytotoxic to SCOV3 cells (an ovarian cancer cell line sensitive to the drug) than the conjugate, the latter was significantly less toxic to normal lymphocytes than Doxorubicin alone, thus allowing the use of a higher concentration of the drug. Interestingly, the conjugate was far more effective on SKVLB ovarian cancer cells, which are resistant to Doxorubicin, than the drug alone. This suggests that the resistance mechanism might be related to poor cell internalization of the drug, which would be enhanced by the delivery mechanism provided by the AFP fragment. If this is

representative of a more general situation, then AFP-drug conjugates might help treat some drug resistant cancers.

Similar results were obtained with Paclitaxel loaded nanoparticles attached to rAFP3D [109].

Besides drugs or toxins, RECAF could be used as a target for the intracellular delivery of interference RNA, as suggested by Glebova et al. [110].

The literature and our own experiments strongly support the idea that conjugating cytotoxic agents with AFP or fragments thereof moderately increases toxicity, but its main merit comes in the form of a substantial reduction of the drug cytotoxicity to normal cells. It is the cancer/normal cytotoxic ratio that is much improved. This indicates that the amount of conjugated drug could be greatly increased without generating the dreadful secondary effects of chemotherapy, thus rendering the treatment more effective.

A potential problem in using RECAF as a target is that its expression is related to the degree of cell differentiation and therefore, poorly differentiated normal cells, such as stem cells and reproductive cells, could be targeted as well. This, however, does not seem to be the case: As mentioned above, the administration of anti-RECAF Mabs completely abrogated the growth of tumors in mice but the animals did not seem to be affected by the treatment despite being followed up for several months. Also, mice injected with massive doses of ^{131}I-AFP were unaffected by the radiation and had normal litters indicating that the reproductive cells were not affected.

In a series of experiments *in-vitro*, we tried to circumvent the difficulties related to the drug-ligand[12] conjugation chemistry by coupling the anti-RECAF 1.4G11 Mab to Thimerosal®, a mercury based antimicrobial that attaches loosely to free thiols in proteins [111]. The conjugation method and cytotoxicity test were extremely simple: The Mab was incubated with Thimerosal® for 4-6 hours and the free antimicrobial was then removed by gel filtration on Sephadex® G-25. Immediately after, the conjugate was incubated for 48 hours with the target cells in serum free medium and the cell viability was determined by the MTT method [112]. As shown in Figure 17, the conjugate killed cancer cells very effectively while having little effect on MRC-5, which is considered as a normal human diploid lung cell line [113].

Figure 18 shows the effects of a 1.4G11-Thimerosal® conjugate and two irrelevant Mabs conjugated to Thimerosal® in identical manner, on JC mouse breast cancer cells [113]. Only the 1.4G11-Thimerosal® conjugate killed the cells, indicating that cell demise was not the result of Thimerosal® leaking from the conjugate into the medium but rather due to the intracellular delivery of the toxic payload by the anti-RECAF Mab.

It is unlikely that Thimerosal® will be used for *in-vivo* cancer targeting since its loose binding to proteins would result in serum proteins sequestering most of the injected chemical. Moreover, mercury is a toxic compound. However, these conjugates could be used *in-vitro* for purging bone marrow of leukemia cells prior to using it for auto-transplantation.

[12] The ligand in this case could be AFP, or an anti-RECAF Mab, an AFP peptide, etc.

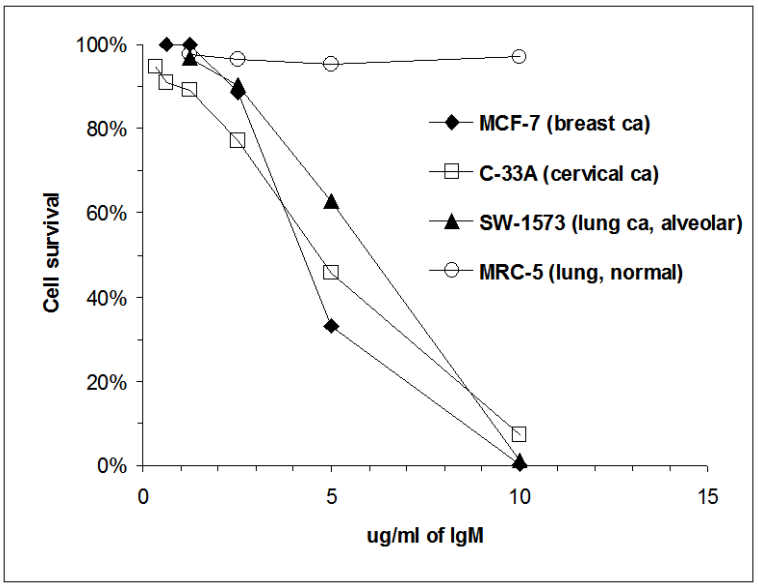


Figure 17. Incubation of cancer and normal human cell lines with different doses of the 1.4G11 anti-RECAF Mab conjugated to Thimerosal®. Cells were incubated with the conjugate for 48 hours in serum free medium. Cell killing was determined by the MTT assay. The conjugate's effect on MRC-5 (a normal cell line) is negligible compared to the effect on a lung cancer cell line (SW-1573).

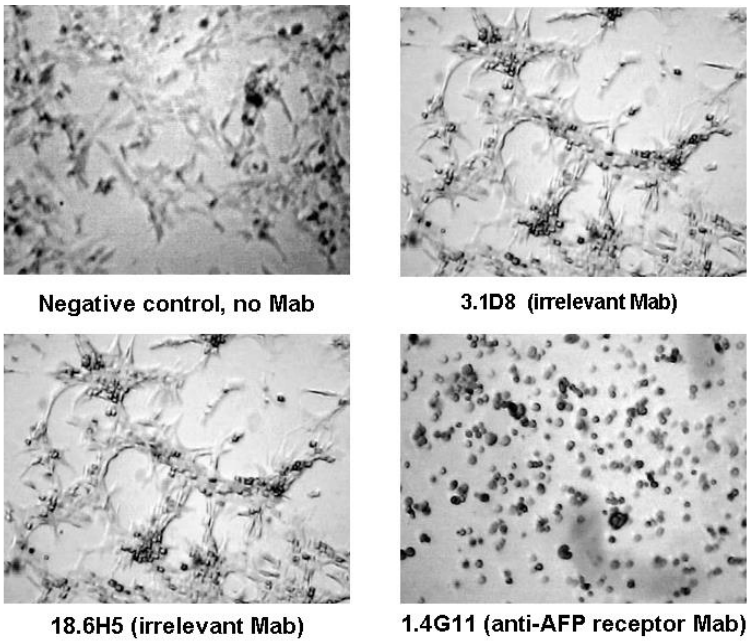


Figure 18. JC cells were incubated under identical conditions (48 hours, serum free medium and 10 ug/ml of IgM) with two irrelevant IgM Mabs and 1.4G11 (an IgM producing Mab against RECAF). The 3 Mabs were conjugated to Thimerosal® under identical conditions. Only the 1.4G11- Thimerosal® conjugate killed the cells, showing that cell demise is not due to leaking of conjugated Thimerosal® into the medium.

CONCLUSION

There is a substantial amount of research done on RECAF, ranging from its characterization and physiology, to its potential uses in medical practice.

On the characterization side, it is clear that a number of different proteins in cancer cell extracts bind to AFP. It is equally clear that the receptor for AFP is an N-glycan. Until proven otherwise, it seems logical to assume that the same glycan is attached to all these proteins. Thus we used, throughout this chapter and in previous articles, the acronym RECAF as a representation of the site, on that glycan, where AFP binds. This makes the term RECAF a functional descriptor rather than a protein name, which greatly simplifies things, particularly when working with complex mixtures such as cell extracts or serum where more than one protein may be binding to AFP. In addition, to denote a particular protein or gel band with RECAF activity, one can add the molecular weight at the end of RECAF. For example, the 65 kDa RECAF band would be RECAF-65.

The potential medical applications based on RECAF are particularly enticing: There is a body of evidence showing that RECAF could be used as a marker for detecting cancer cells on tissue sections and more importantly, on smears. Elevated serum RECAF has been shown to detect early stages of cancer, when conventional therapy yields 80-90% survival rates. The high sensitivity and specificity of the marker leaves open its potential for screening, particularly when used in combination with other cancer markers. On a more speculative basis, serum RECAF could be used for monitoring patients in remission, and perhaps prompting, if values increased over time, the administrating of therapy before recurrence became clinically evident, at which time the prognosis is usually ominous.

Several authors have demonstrated that RECAF has potential for scintigraphy, with which, small malignant tumors have been detected. This type of imaging could be used to confirm the malignant nature of a lesion in a suspicious mammography or to evaluate the presence of metastases in patients already diagnosed with cancer.

Finally, there is substantial evidence, both *in-vitro* and *in-vivo*, showing that cancer cells can be targeted and destroyed using anti-RECAF Mabs by themselves or coupled to cytotoxic agents, as well as by AFP and fragments or peptides thereof coupled to drugs.

Given the span of applications in which RECAF has yielded positive results and the medical significance of some of those applications, it appears as evident that this system warrants a great deal of future work.

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