An Enzyme-linked Immunosorbent Assay for Cancer Procoagulant and Its Potential as a New Tumor Marker

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ABSTRACT

Cancer procoagulant (CP) is a M, 68,000 cysteine proteinase that initiates blood coagulation and is expressed by a variety of malignant cells but not by normally differentiated cells. Polyclonal immunoglobulin G and monoclonal immunoglobulin M antibodies were developed to purified CP and used to develop an enzyme-linked immunosorbent assay to analyze the antigen in human serum samples. The purpose of this preliminary study was to determine whether or not the analysis of CP in the serum might be a useful tumor marker.

Polycarbonate (PC) was added to normal serum to establish a quantitative standard curve; the correlation coefficient of seven standard curves was 0.99. The upper limit of the normal range was established with 46 normal sera (mean ± 2 SD = 0.57 μg/ml). A total of 128 blinded serum samples were analyzed: 54 were from cancer patients (29 with gastrointestinal cancer, 22 with lung cancer, and three with uterine cancer); 20 were from benign disease patients; and 54 were from normal individuals. All of the 13 early stage cancers were >0.57 μg/ml (positive), 31 of 41 (76%) of the late stage cancers were positive; overall, 44 of the 54 cancers (81%) were positive. Forty-nine of 54 (91%) of the normal sera and 16 of the late stage cancers were positive; overall, 54 sera (80%) of the benign disease sera were negative. Overall, the assay had a specificity of 81% and a specificity of 88%.

INTRODUCTION

Despite many therapeutic advances, early detection of malignancy has great potential as a means of affecting outcome and survival of cancer patients. All too often we find the disease too far advanced and therapeutic options limited. Earlier diagnosis and treatment can affect outcome as evidenced by screening Papanicolaou smears for squamous cell carcinoma of the cervix (1).

The development of an assay with similar potential for early detection of other malignancies would be of considerable aid to clinical oncologists. Recently, investigators have identified several substances that are produced by tumor cells for use as diagnostic markers of cancer. These include the germ cell markers human chorionic gonadotropin and α-fetoprotein, which are very specific markers, and less sensitive and specific markers such as CEA, CA-12,5, CA-19.9, CA-15.3, PAP, and others. Recent reviews describe the use of these and other tumor markers in diagnostic and therapeutic applications (2–6).

During our studies to identify a substance that initiated the abnormal blood coagulation associated with malignant disease, we discovered (7), purified, and partially characterized CP (8, 9). To determine the distribution of CP activity in various malignant and normal tissues, a variety of extracts of serum-free culture media and cell suspensions of malignant or transformed cells as well as their normal counterparts were examined (10–13). It was found that CP activity existed in the extracts of malignant cells, but not in extracts from normal tissue or serum-free media from normal cells in tissue culture. Careful enzymatic and immunological characterization of extracts of malignant and benign melanocytic tissue revealed CP in the melanoma extracts and not in the benign nevi extracts (14). In acute nonlymphocytic leukemia, CP was identified in most of the cytological subtypes but not in control mononuclear cells from either peripheral blood or bone marrow aspirates (15). In addition, a virtually identical procoagulant protein has been purified from human amnion-chorion tissue (fetal origin), implying that CP may be an oncofetal protein (16, 17). These data were sufficiently supportive of the unique expression of CP by malignant cells to warrant the preliminary study to evaluate the potential of CP as a tumor marker. A double antibody "sandwich" ELISA was developed and used to assay for CP in the serum of cancer patients and of noncancerous control individuals. These preliminary results suggest that CP has potential as a tumor marker, and they are sufficiently encouraging to warrant additional work on the assay and to expand the study.

MATERIALS AND METHODS

Purification of Cancer Procoagulant Antigens. Cancer procoagulant was purified from extracts of rabbit V2 carcinoma and human amnion-chorion by immuno- and p-chloromercurial benzoate affinity chromatography as described previously (9). Briefly, CP was purified by immunoaffinity chromatography in which the immunoaffinity resin was prepared by coupling purified goat polyclonal IgG to cyanogen bromide-activated Sepharose. Extracts were equilibrated overnight at 5°C, the column was washed thoroughly with 20 mM barbitol buffer (pH 7.4), and bound antigen was eluted with 3 M NaSCN (Sigma, St. Louis, MO). Trace contaminants were removed by adsorbing CP to an organosomeric resin, washing contaminants off, and eluting CP with 2 mM and 5 mM HgCl2. The CP sample was a single band on SDS-PAGE and PAGIF. It had the enzymatic properties of CP, including procoagulant activity in normal and Factor VII-deficient human plasma (7, 8); it directly activated human Factor X in a 2-stage assay (8); and its activity was inhibited by mercury, iodoacetamide, and peptidyl diazomethyl ketone and was reactivated by KCN (8, 9, 18). The purified CP preparation was used to immunize mice and to screen hybrid cells for antibody.

Polyclonal Antibodies to Cancer Procoagulant. The polyclonal goat antibody was raised by standard methodology (9). Briefly, the goat was immunized and boosted with s.c. injections of between 50 and 100 μg of purified enzyme from rabbit V2 carcinoma suspended in Freund’s adjuvant. The goat IgG fraction was partially purified from the serum by standard purification techniques, including ammonium sulfate fractionation and DEAE-cellulose ion exchange chromatography. A trace contaminant of an antibody to normal rabbit serum protein was removed by coupling normal rabbit serum proteins to cyanogen bromide.
activated Sepharose and passing the antibody preparation over this "normal rabbit serum column." Potential cross-reactivity to normal human serum proteins was eliminated in the same way, by adsorbing the antibody preparation to human serum protein-resin. The purified antibody was tested for cross-reactivity with normal rabbit serum and normal human serum by crossed immunodiffusion; it was not immunoreactive with either.

Hybridoma Antibodies to Cancer Procoagulant. BALB/c mice (Charles River) were immunized with cancer procoagulant to induce a B-cell immune response, according to standard procedures (19, 20). Briefly, spleen lymphocytes from immunized mice were hybridized with the P3/X63-AG8.653 variant of mouse myeloma cells with 50% polyethylene glycol (21). The hybrids were plated with 2 x 10^5 normal mouse spleen cells/ml as a feeder layer, and the unhybridized myeloma cells were eliminated by hypoxanthine aminopterin thymidine suicide. Cells from antibody-positive wells were expanded and cloned at 0.1 to 0.5 cells per well with a feeder layer of normal spleen cells. Antibodies from positive wells were classified using specific subclass anti-mouse immunoglobulins according to the technical information provided in the Zymed Lab Mono Ab-ID EIA kit (22). Antibodies were screened by a standard single antibody microtiter plate immunoassay using purified CP absorbed to microtiter wells. Alkaline phosphatase-labeled anti-mouse IgM antibody was used as the signal antibody, and the color was read at 405 nm on a Dynatech microtiter plate reader.

Antibody Production and Purification. The anti-CP IgM was produced in the ascites of Pristane-treated BALB/c mice (23). During the analysis of immunoglobulin in the ascites fluid, procoagulant activity was discovered in the cell-free ascites. This procoagulant had the characteristics ascribed to cancer procoagulant, including inhibition by mercury, initiation of coagulation of factor VII-depleted human plasma, and immunoprecipitation with the polyclonal goat antibody (9). To effectively utilize the antibody in immunoassays or other immunological experiments, it was necessary to separate the antigen from the antibody. This was accomplished by making the ascites 6 M urea, adding to 50% Polyethylene Glycol 6000 to precipitate the IgM, and running the sample over a 1.5-m agarose (Bio-Rad, Richmond, CA) column (1.5 x 60 cm) that was pre-equilibrated in 6 M urea. The fractions containing the IgM protein peak were pooled, dialyzed against 3 changes of 20 mM barbitur buffer saline (pH 7.4), and concentrated over an Amicon PM-50 membrane.

An alternative procedure used to separate antigen from the IgM was to make the ascites 6% Polyethylene Glycol 6000 to precipitate the IgM. The precipitate was recovered by centrifugation, decanting the supernatant, and redissolving it in a minimal volume (about 1 ml/10 ml of ascites) of 0.1 M phosphate-buffered saline (pH 7.4). The solubilized IgM was made 50% with ethylene glycol and stored at 5°C until it was further purified by HPLC (Waters, Inc.). A semipreparative GF-450-XL (DuPont) was pre-equilibrated in 0.1 M sodium phosphate buffer in 50% ethylene glycol, up to 2 ml of the antibody preparation were applied and eluted at 1 ml/min, and fractions were collected to separate IgM from the antigen.

The purified IgM was concentrated on an Amicon PM-50 ultrafiltration membrane, made 50% with glycerol, and stored at —20°C until it was used in an assay. Testing of the ascites IgM antibody and the purified IgM antibody clearly demonstrated that the purified IgM had greater immunoreactivity, as expected.

Development of a Double Antibody Immunoassay. The wells of an Immulon II 96-well microtiter plate were coated with 0.19 μg/well of monoclonal antibody (1:80 dilution of stock IgM) for 2 h at 25°C, and the plate was washed 3 times with PBT. Open sites in the wells were blocked with 0.1% normal human serum albumin in 0.1 M phosphate buffer (pH 7.6) and stored in a vacuum desiccator at 5°C until it was used in an assay. Serum (25 μl) was diluted in an equal volume of PBT. In the microtiter wells and incubated for 2 h at 5°C. The wells were emptied and washed 3 times with PBT, and 50 μl (0.26 nm) of alkaline phosphatase-conjugated goat anti-CP antibody (24) in phosphate-buffered saline were added and incubated for 1 h at 37°C. Finally the p-nitrophenyl phosphate substrate was added, incubated at 37°C for between 30 and 60 min, and read on a microtiter plate reader at 405 nm.

Each step of the assay was checked to determine the optimum conditions for the assay. The dilution of monoclonal antibody was checked from 1:1 dilution to 1:50,000 dilution. Human serum albumin (0.1%) was as good a blocking agent as if not better than, the same concentration of bovine serum albumin, ovalbumin, normal goat serum, normal human serum, and normal rabbit serum. Higher (to 5%) and lower (to 0.01%) concentrations of each of the protein solutions were tested without improving the assay results. The time (1, 2, 4, and 16 h) and temperature (5°, 25°, and 37°C) for coating and blocking the wells were determined as well as each incubation. A variety of washing buffers and concentrations of nonionic detergents were tested to determine the most effective way to remove substances that increased nongspecific binding. Thus, each of the conditions was carefully checked to arrive at the sequence of steps that provided the best assay system.

Standard Curve and Calibration for the Assay. A standard curve was prepared by adding known amounts of pure CP to normal serum. Concentrations from 0 to 30 μg/ml were used. Each microtiter plate contained a standard curve, 6 to 8 normal control sera and a high and a medium control pooled sera, and the unknown serum samples.

Analysis of Human Serum Samples. The initial serum samples included 46 normal volunteers from the Health Sciences Center that were processed to establish the normal range. The remaining samples were blinded and analyzed without knowing the patient's identity or diagnosis. After the assay was complete, the results were analyzed, and the samples were identified and compared with the results of the analysis. In the 128 serum samples that were analyzed in this blinded fashion, there were 54 sera from cancer patients, 54 from normal individuals, and 20 from individuals with benign disease (Table 1). The samples were usually analyzed in triplicate in at least 3 different assays.

A small group of samples were collected in duplicate or triplicate to determine the effect of anticoagulants and hemolysis on the assay. Heparin, citrate, and NaF plasma samples had essentially the same CP values as did their serum counterparts, but the results had greater variability. EDTA-plasma samples had assay results that were about 50% lower than their serum counterparts and greater variability. Samples with slight hemolysis had comparable results to unhemolyzed serum, but extensive hemolysis resulted in high assay results. Lipemic serum had high analysis results, but the addition of clearing agents, such as Liposolve, provided samples that appeared to have reliable assay values.

RESULTS

The identification of procoagulant activity in the ascites indicated the need to purify the antigen from the antibody. Ascites was made 6 M urea to dissociate antigen-antibody interactions.

![Fig. 1. An elution profile from a 1.5-m agarose chromatography column showing the separation of hybridoma IgM antibody from CP antigen. Four ml of ascites were made 6 mol with crystalline urea, applied to the column that was pre-equilibrated in 6 M urea, and eluted with 6 M urea in 20 mM barbitral buffer. Protein was monitored by measuring absorbance at 280 nm (-----). Fractions were dialyzed against 20 mM barbitral buffer, and IgM was measured in a microtiter plate assay using alkaline phosphatase-labeled goat anti-mouse IgM antibody (-----). The clotting activity was measured with the recalcification clotting time of citrated normal human plasma (------).](image-url)
The two proteins were then physically separated on a 1.5-m agarose gel filtration column (Fig. 1) with an exclusion limit of $1.5 \times 10^7$ daltons which can separate $M_c$, 850,000 IgM from $M_c$, 68,000 cancer procoagulant. The HPLC chromatographic profile of the GF-450-XL purification of IgM was essentially the same as that in Fig. 1; using ethylene glycol as the dissociating agent provided IgM with better stability and immunoreactivity, so this procedure was adopted as the method of choice. The purified IgM fractions were assayed for CP by (a) crossed immunodiffusion against polyclonal anti-CP goat antibody, (b) assaying the sample for iodoacetamide sensitivity of triplicate wells and SDS-PAGE.

The CV for 44 duplicate samples analyzed in the same assay was 8.6 ± 5.9%. The interassay CV for 35 samples that were assayed in from 2 to 5 different assays was 13.2 ± 9.4%. There were 7 pairs of samples that were blinded with different code numbers and integrated into the pool of samples; the mean CV was 18.1 ± 5.8%. Hand pipetting of all steps in the assay probably contributed to the elevated CVs in the assay.

A standard curve was prepared from the samples of normal serum that contained known amounts of CP. This standard curve was used to establish linearity of the assay, to quantitate the level of CP in the unknown serum samples, and to determine the reproducibility of the assay. Fig. 2 shows the mean ± standard error of the mean of 7 standard curves. The regression analysis of the data shows a correlation coefficient of 0.99. The results of each plate were analyzed by checking the linearity of the standards.

The mean and one standard deviation of the 46 normal serum samples was 0.36 µg/ml ± 0.11 µg/ml. Thus, the mean + 2 standard deviations was used to establish the normal range of 0 to 0.57 µg/ml; results above 0.57 µg/ml were considered positive.

Analysis of Blinded Samples. A total of 54 normal samples from volunteers were “blinded” with code numbers and integrated into the pool of 54 blinded cancer patient samples and 20 benign disease patient samples. Of the 54 normal sera, 49 (91%) were negative, while 16 of 20 benign disease sera were negative. In the overall noncancer population, 65 of the 74 sera (88%) were negative; i.e., the results were <0.57 µg/ml.

Table 1 shows some of the clinical details of the 54 cancer patients and 20 benign disease patients from whom serum samples were obtained. Fig. 3 shows the distribution of data for each of the groups of samples. In the 29 samples from patients with gastrointestinal cancers, all of which were colorectal adenocarcinoma, 25 (86%) were negative; i.e., the results were <0.57 µg/ml.

![Figure 2](https://example.com/figure2.png)

Fig. 2. The mean of seven standard curves in which pure CP antigen was added to normal human serum and analyzed by the ELISA procedure described in the text. The blank signal of the normal serum was subtracted from each sample to calibrate the signal for each level of antigen. Each standard was assayed in triplicate on each microtiter plate, as described in the text. These standards were used to quantitate the level of antigen in the normal and blinded serum samples. The correlation coefficient was 0.99, and the equation of the least-squares fit line was $y = -9.43 + 7.69x$. Points, mean; bars, SEM.

![Figure 3](https://example.com/figure3.png)

Fig. 3. Analysis of 128 cancer and noncancer sera, the results of the blinded samples analyzed with the immunoassay. The normal ± 2 SDs (0.57 µg/ml) was used as the upper limit of normal. The samples with CP levels less than 0.57 µg/ml were considered normal, and those above that level were considered positive.

The left column shows the 54 normal serum samples that were assayed, and 49 of them were normal. The second column from the left shows 29 gastrointestinal cancer patient sera and 3 urogenital cancer patient sera; 22 of the GI samples and all of the GU samples were positive. Three gray tone dots with white centers represent the GU cancer samples. The last column on the right shows 22 lung cancer patient serum samples; 19 of them were positive. X represents the early stage (Stage I and II) cancers.

**Table 1. List of clinical profiles of patients that provided blood for the blinded serum samples analyzed in the study, including information about the patient’s type of cancer, the patient’s type of benign disease, and the number of samples in each group.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Malignancies</strong></td>
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<tr>
<td>Gastrointestinal</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>2</td>
<td>1</td>
<td>25</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Lung</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Squamous cell</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td></td>
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<tr>
<td>Urogenital</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Ovarian</td>
<td>1</td>
<td>1</td>
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<tr>
<td><strong>Benign disease</strong></td>
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<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Asthma</td>
<td>4</td>
<td>(1 patient also had BPH)*</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pneumonia</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>COPD</td>
<td>6</td>
<td>(1 patient also had asbestos)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Emphysema</td>
<td>5</td>
<td></td>
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<td></td>
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<tr>
<td>Other</td>
<td></td>
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<tr>
<td>Inflammatory bowel</td>
<td>1</td>
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<tr>
<td>Deep vein thrombosis</td>
<td>1</td>
<td></td>
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</table>

* BPH, benign prostatic hypertrophy.
rectal cancers, there were 22 (76%) positive (>0.57 μg/ml) sera. There were 22 serum samples from patients with lung tumors, 7 with squamous carcinoma and 15 with small cell carcinoma. Nineteen of these samples (86%) were positive.

All 3 sera from patients with urogenital cancer were positive.

Table 2 shows the data analyzed by stage of disease. All of the Stage A and B gastrointestinal cancer patients, Stage I and II lung cancer patients, and the patient with Stage II ovarian carcinoma were positive. Thus, 13 of 13 sera (100%) from early stage cancers were positive. In contrast, only 19 of 27 (73%) of the later stage gastrointestinal cancer patient sera and 10 of 13 (77%) of the Stage III and IV lung cancer patient sera were positive. Both of the late stage urogenital cancer patient sera were positive.

There were 20 serum samples from patients with benign disease; 18 of them had various types of pulmonary disease. Two patients had more than one type of benign disease; one patient with asthma also had benign prostatic hypertrophy. A second patient with COPD had a diagnosis of asbestosis and was a 2-pack/day smoker; this was one of the 4 benign disease patients that tested positive in the assay. A second positive benign disease patient had severe COPD and was also a heavy cigarette smoker.

Statistical analysis of the results (25) indicates that the ability of the ELISA to correctly identify patients with cancer (i.e., the sensitivity of the test) was 76% for colorectal tumors, 86% for lung tumors, and 100% for the urogenital malignancies, with an overall sensitivity of 81%. The sensitivity for early stage cancers was 100%. The specificity of the test (the percentage of noncancer patient sera that were negative) was 88%.

DISCUSSION

It is widely believed that malignant cells produce substances that are not produced by normal cells, but finding and identifying these substances has been very difficult. With the enormous medical promise and human effort devoted to the quest for reliable tumor markers, it is not surprising that many tumor antigens have been identified and partially characterized during the past 20 yr in the hope of providing methods for early detection and therapeutic monitoring of cancer. However, many substances that are found in cancer cells are also found in some normal and noncancerous (benign) diseases. Thus, many of the antigens that have been identified to date are also present in sera from some normal individuals and from a spectrum of benign disease conditions, making them inadequate for acceptable detection or screening procedures. However, they have been clinically useful for monitoring cancer therapy; most notable among these are CEA and α-fetoprotein. Recently the list of tumor markers has grown substantially with the addition of PAP, tissue polypeptide antigen, pancreatic oncocytic antigen, CA-19.9, CA-12.5, CA 15.3, and others. There are recent reviews of tumor markers and tumor-associated antigens (2-6).

Carcinoembryonic antigen, first described by Gold and Freedman (26) and by Fuk and Gold (27), is a high-molecular-weight (M, 180,000 to 200,000) glycoprotein that is present in various types of malignant disease, including tumors of the GI tract (30%), stomach (72%), pancreas (88%), breast (24%), and respiratory tract (30%) (28). Elevated plasma CEA is associated with a variety of nonmalignant disorders, such as chronic obstructive pulmonary disease (57%) and most inflammatory diseases, including pneumonia (46%) and hepatitis (40%). CEA is elevated in about 10% and 15% of normal nonsmokers and smokers, respectively.

α-Fetoprotein is a M, 70,000 protein (29, 30) that is elevated in the serum of about 80% of patients with hepatic tumors, almost all those with teratocarcinomas (31, 32).

CP was originally found during studies to identify a substance that initiated the abnormal blood coagulation associated with malignant disease. Abnormalities of the coagulation system (33-35), including disseminated intravascular coagulation (36, 37), have been repeatedly identified as pathological features of malignant disease. Furthermore, fibrin is deposited in and around solid tumors (38-42), is associated with blood-borne malignant cells (43, 44), and is thought to participate in various aspects of the malignant process (45-50).

These documented abnormalities in the coagulation system in malignant disease led investigators to look for procoagulants in neoplastic tissue (51-54). We identified, purified to homogeneity, and partially characterized CP, a unique cysteine protease (7-9, 55) that initiates coagulation by directly activating Factor X in the coagulation cascade. CP is a single polypeptide protein with a molecular weight of 68,000 and an isoelectric point of 4.8. It is rich in serine and glycine and contains no measurable carbohydrate (9). Its physical, chemical, and enzymatic properties are different than other coagulation enzymes (9).

During the course of these studies, it became important to establish whether or not CP was found in association with normal tissues (11) and cells (10, 12); it was found in neither intact normal cells, extracts, nor culture media. Furthermore, a procoagulant protein was purified from human amnion-chorial tissue (of fetal origin), and its properties are virtually identical, with the exception of minor differences in amino acid composition, to CP from rabbit V2 carcinoma (16, 17), B16 melanoma, and all the human tumor extracts that have been analyzed. This suggests that the procoagulant protein is highly conserved across species lines and tumor types and that CP may be an oncofetal protein.

During the development of the hybridoma antibody, we had trouble obtaining consistent results with the screening assay

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Table 2 Summary of the results of the study

<table>
<thead>
<tr>
<th>Stage</th>
<th>Early</th>
<th>Late</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI tumors</td>
<td>3/3 (100)*</td>
<td>19/26 (73)</td>
<td>22/29 (76)</td>
</tr>
<tr>
<td>Lung tumors</td>
<td>9/9 (100)</td>
<td>10/13 (77)</td>
<td>19/22 (86)</td>
</tr>
<tr>
<td>GU tumors</td>
<td>1/1 (100)</td>
<td>2/2 (100)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>13/13 (100)</td>
<td>31/41 (76)</td>
<td>44/54 (81)</td>
</tr>
<tr>
<td>Noncancer patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>49/54 (91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>16/20 (80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65/74 (88)</td>
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</table>

* Numbers in parentheses, percentage of samples correctly identified.
and getting an IgM with adequate immunoreactivity from ascites samples that contained from 5 to 7 µg of IgM/ml of ascites. The ascites contained a procoagulant that had all the enzymatic properties of CP (8, 9) and cross-reacted with anti-CP polyclonal goat antibody on an Ouchterlony immunodiffusion plate (9). Subsequently, it found that the tissue culture medium from both the parent P3/X63-AG8.653 cell line and other hybrid cell clones from colleagues in the Health Sciences Center contained CP with immunoreactive and enzymatic identity. Although unexpected, it was logical since both the parent cell line and the hybrid cell lines have the malignant phenotype and should produce the CP antigen if CP is an oncofetal protein as suggested above.

It was necessary to separate the antigen and IgM so that the IgM could be used in an immunoassay. We chose to take advantage of the difference in the physical size of the two molecules for the separation. Two methods were used to dissociate CP from IgM: 6 M urea and 50% ethylene glycol; both methods facilitated dissociation so the IgM could be resolved from the CP on gel filtration chromatography (1.5-m agarose and GF-450 HPLC). However, IgM purification in 50% ethylene glycol resulted in more stable IgM that was free of CP.

In this preliminary study, antibodies to CP were developed and used in an immunoassay to measure CP in serum from cancer and noncancer patients. The assay correctly identified 44 or 81% of the 54 serum samples from cancer patients. Ninety-one % of the normal sera and 80% of the benign disease sera were negative; 88% of the 74 noncancer sera were correctly identified. The mean + 2 standard deviations seems to be a reasonable estimator of the 90th percentile of the sample distribution (the limit excludes the upper 10% of the observations). Thus, the percentage of false-positive results for the normal sera is within the limits of the expected value.

In a previous pilot study (56), 140 blinded serum samples were assayed from patients with colorectal (26), lung (27), breast (35), prostate (6), lymphoma (6), osteosarcoma (4), and gall bladder cancer (3) and from 70 normal individuals and 10 patients with benign disease. Approximately 90% of the samples were correctly identified. Thus the results of both studies are similar and support the hypothesis that CP may be a tumor marker.

Comparison of these data with data from an extensive 1974 clinical trial of CEA (57) shows that CEA had a sensitivity of 72% for colorectal cancer, 91% for pancreatic cancer, 74% for lung cancer, and 47% for breast cancer (58). CEA had low specificity (ranging from 30% to 82%) due to the large number of benign conditions that led to positive serum CEA levels. In contrast, CP had a sensitivity of 76% for colorectal cancer and 86% for lung cancers. The specificity of the CP assay was 88%. In addition, the assay correctly identified 100% of the early stage cancers. These statistics compare favorably with CEA. Thus, the data support the hypothesis that CP may be an oncofetal protein with the distribution necessary to be an effective tumor marker.

There was a substantial percentage of false-negative cancer patient sera in both the gastrointestinal and lung cancer groups of patients with late stage cancer. It was noted that several of these "false negative" patients were terminal at the time the blood samples were obtained and that they died from their malignancy shortly thereafter. We examined the blood samples for endogenous anti-CP antibodies because such antibodies in the serum of these cancer patients would probably interfere with the immunoassay. These serum samples were reacted with pure CP on Ouchterlony-crossed immunodiffusion, and 3 of the 4 showed immunoprecipitation bands, suggesting the presence of endogenous antibodies. Alternatively, there is evidence for a decreased production of CP activity in the serum of rats with late stage epithelioma (59), suggesting that CP production in late stage cancers may decrease. In the false negative samples, CP may be associated with a serum protein or other substance produced by the tumor that binds CP, blocks the epitope, and inhibits the immunoreactivity of CP in the assay. The false negative results in late stage cancer will be the subject of continued studies.

The objective of this study was to develop a basic immunoassay system and to obtain adequate preliminary evidence from blinded cancer and noncancer sera to test the concept that CP may be a useful tumor marker. Although we concluded that the immunoassay for CP had reasonable clinical potential as a useful tumor marker test, there were several difficulties that need to be resolved by refinements of the assay. These include the following: (a) developing a more stable monoclonal IgG hybridoma antibody to replace the IgM antibody used in the assay; (b) establishing a method to screen serum samples for endogenous anti-CF antibody; (c) doing follow-up studies on the normal and benign disease populations if they have high CP levels in their blood to determine whether or not they have malignant disease; and (d) studying a large number of benign diseases to develop a list of noncancerous conditions associated with elevated serum CP levels.

These are questions that cannot be answered in the present study but will be examined very closely in the next phase of evaluation of CP as a tumor marker. In spite of these problems, we feel that the immunoassay for CP in serum may have reasonable clinical potential as a cancer diagnostic or monitoring test for cancer.

REFERENCES

An Enzyme-linked Immunosorbent Assay for Cancer Procoagulant and Its Potential as a New Tumor Marker

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