Isolation and Characterization of Cancer Procoagulant: A Cysteine Proteinase from Malignant Tissue†

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ABSTRACT: Cancer procoagulant, a proteolytic procoagulant enzyme, has been purified from rabbit V2 carcinoma extracts by two procedures. In the first, the protein was purified by benzamidine–Sepharose affinity chromatography, gel filtration chromatography, and phenyl-Sepharose hydrophobic chromatography. Antiserum was raised against the purified protein and was used to prepare an immunoadsorbent column. In the second, tumor extracts were purified by immunoaffinity chromatography followed by *p*-(chloromercuri)benzoate affinity chromatography. The second procedure was substantially quicker and easier. The final product of both procedures was homogeneous on the basis of analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing. The molecular weight was 68 000 and the isoelectric point 4.8. The proteinase activity of cancer procoagulant directly activated factor X, in the absence of factor VII, and was inhibited by 1 mM iodoacetamide and 0.1 mM mercury which are classic cysteine proteinase inhibitors. A carbohydrate analysis showed less than 1 mol of hexose or sialic acid/mol of protein. The amino acid analysis showed that serine (19.1%), glycine (18.77%), and glutamic acid (12.5%) were the prevalent amino acids. The amino acid composition of cancer procoagulant was substantially different than other known factor X activating proteinases or other cysteine proteinases including cathepsin B.

The well-documented association between fibrin metabolism and malignant disease has been the subject of recent reviews (Laki, 1974; Rickles & Edwards, 1983; Markus, 1984). There is increased deposition of fibrin in the vasculature (Nusbacher, 1964; Whitecar, 1973) and within solid tumors in animals and patients with cancer (O'Meara & Jackson, 1958; Dewey & Bale, 1963; Ogura et al., 1970; Robson et al., 1977; Dvorak et al., 1979; Zacharski et al., 1983). Furthermore, fibrin deposition has been documented on blood-borne malignant cells (Wood, 1964; Chew et al., 1976), and it has been suggested that it may participate in the metastatic process (Wood, 1974; Chew & Wallace, 1976). These observations have led investigators to look for an abnormal procoagulant substance in malignant tissue (Gordon et al., 1975; Boggust et al., 1967; Pineo et al., 1973; Sakuragawa et al., 1976; Khato et al., 1974; Dvorak et al., 1981). The original efforts by O'Meara and his associates to isolate and characterize a tumor procoagulant (Boggust et al., 1967; O'Meara & Thornes, 1961) concluded that the heat-labile lipoprotein substance that they had partially purified consisted of free fatty acids associated with serum proteins such as serum albumin (Boggust et al., 1968). More recently, a substance was partially purified from bronchial and ovarian cyst mucous and saliva (Pineo et al., 1973) that initiated coagulation by directly activating factor X. It was a heat-stable (100 °C for 15 min) procoagulant with no apparent esterase activity that was suggested to participate in the hypercoagulability associated with mucous-secreting adenocarcinomas (Pineo et al., 1973). In addition, crude extracts of leukemic cells contain a high molecular weight (200 000) factor X activating procoagulant (Sakuragawa et al., 1976).

Initial studies performed in this laboratory demonstrated a procoagulant in partially purified extracts of rabbit V2 carcinoma and two human tumors that was inhibited by diisopropyl fluorophosphate, was active in factor VII depleted bovine plasma, and directly activated purified factor X (Gordon et al., 1975). Recent work in this laboratory on a purified preparation of cancer procoagulant demonstrated inhibition of the activity by cysteine proteinase inhibitors, such as mercury and iodoacetamide (Gordon & Cross, 1981). This procoagulant activity was identified in serum-free medium from transformed fibroblasts (Gordon & Lewis, 1978), in extracts from malignant human tissue (Gordon et al., 1979), and in malignant cell suspension (Gordon et al., 1982) but not in medium from normal fibroblasts or in normal tissue extracts. These observations have been confirmed by others (Curatolo et al., 1979; Hilgard & Whur, 1980; Colucci et al., 1980).

In the present study, we describe two methods to obtain pure cancer procoagulant (CP)[1] from rabbit V2 carcinoma extracts. We first isolated the protein by sequential application of classical purification techniques. The final product was used to produce specific antibodies which were utilized to prepare an immunoadsorbent column. This column allowed the rapid preparation of homogeneous CP for physical and chemical characterization studies. The properties of CP were compared to those of other coagulation factors. This study provides data which suggest that CP may be a coagulation factor unique to malignant tissue.

Experimental Procedures

Materials

Rabbit brain thromboplastin (Difco, Detroit, MI) and purified Russell's viper venom (RVV-XAE, Sigma, St. Louis,

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[1] Abbreviations: CP, cancer procoagulant; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PAGIF, polyacrylamide gel isoelectric focusing; VB, veronal buffer; DTT, dithiothreitol; RVV, Russell's viper venom; RBT, rabbit brain thromboplastin; *pI*, isoelectric point; PCMB, *p*-(chloromercuri)-benzoate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; DFP, diisopropyl fluorophosphate; GSH, glutathione.

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MO) were used as standards in the coagulation assay and as representative procoagulants for comparison of the enzymatic properties with cancer procoagulant. Human factor VII deficient plasma was purchased from George King (Overland Park, KS). Fresh citrated human plasma was collected and processed as described previously (Gordon et al., 1975). Diisopropyl fluorophosphate was purchased from Schwarz/Mann (Orangeburg, NY). Iodoacetamide and dithiothreitol were purchased from Sigma (St. Louis, MO). Bis-Tris/propane buffer was obtained from Research Organic Inc. (Cleveland, OH) and veronal buffer from Sigma.

Cyanogen bromide activated Sepharose and phenyl-Sepharose resins were from Pharmacia Fine Chemicals (Psicataway, NJ); 1.5m agarose (Bio-Gel A-1.5m) and *p*-(chloromercuri)benzoate–agarose (Affi-gel 501) were obtained from Bio-Rad Laboratories (Richmond, CA). All chemicals for analytical SDS–PAGE were from Bio-Rad. Servalyt Precotes (pH range 3–10) for isoelectric focusing were purchased from Serva (Heidelberg, FRG). Freund's adjuvant (complete and incomplete) was from Colorado Serum Co. Laboratories (Denver, CO). All other chemicals were of reagent grade.

Methods

Source of Cancer Procoagulant. V2 carcinoma cells were injected into the thigh muscle of young (2 kg) New Zealand white rabbits, and the animal's weight and tumor size were monitored twice per week until the animal's weight began to decline and the tumor was large. The tumors ranged in size from 50 to 200 g. Tumor tissue was cut into 0.5–1-cm-thick slices to increase the surface area and extracted in three changes of 300 mL of 20 mM VB (pH 7.8) for 3 h each at 4 °C. The three extracts were pooled, centrifuged at 1200*g* for 15 min to remove cellular debris, and concentrated about 10–20-fold over a PM-10 ultrafiltration membrane (Amicon, Lexington, MA). We have homogenized tumor samples or extracted them in other buffer systems and found lower recovery of activity. The concentrated sample was either carried through the purification sequence or adjusted to contain 5 mM benzamidine and stored at −70 °C for later use. We found the activity stable for several months under these conditions. Frozen samples were thawed and dialyzed against 2 L of 20 mM VB (pH 7.8) for 12 h with two changes of the buffer to remove benzamidine for use in the purification sequence.

Measurement of Protein. The column chromatography elutions were monitored by measuring the absorbance at 280 nm. The protein content of aliquots of pooled fraction samples from each step of the purification was determined with a modified Lowry method (Bensadoun & Weinstein, 1976).

Assay for Procoagulant Activity. The procoagulant activity of samples from each step of the purification was assayed by measuring the single-stage coagulation time of recalcified citrated normal human plasma, as described previously (Gordon et al., 1975, 1979; Gordon & Lewis, 1978; Gordon & Cross, 1981). Russell's viper venom (RVV) was used as a coagulation standard and as a factor X activating serine proteinase control. Procoagulant activity of the samples or standard controls was expressed as the percent decrease in the clotting time compared to the corresponding buffer blank (blank time − clotting time/blank time). There was a linear relationship between the percentage decrease in clotting time of the single-stage recalcification assay and the concentration of RVV from 10^{-1} to 10^{-4} mequiv/mL (Gordon & Lewis, 1978).

As a criteria to distinguish cancer procoagulant from tissue factor, factor VII deficient human plasma was used in the assay to determine the dependence of procoagulant activity on factor VII (Nemerson, 1966). RVV and rabbit brain thromboplastin (RBT) were used as positive and negative controls, respectively.

Inhibition and Activation of Procoagulant Activity. To determine whether or not the procoagulant activity had the cysteine proteinase characteristics described previously (Gordon & Cross, 1981), two aliquots of pure, active CP were made 0.1 mM with $HgCl_2$ or 1 mM with iodoacetamide, respectively. Each aliquot was incubated with the inhibitor for 30 min at 37 °C and reassayed for procoagulant activity.

To determine the sensitivity of CP to thiol group activator compounds, pure samples inhibited with mercury were made 10 mM with KCN, 2 mM with DTT, and 2 mM with EDTA. They were incubated for 30 min at 4 °C, dialyzed overnight against 0.02 M Bis-Tris/propane buffer (pH 6.5), and then assayed for procoagulant activity. This reactivation procedure was mandatory for measuring activity when mercury was employed in steps of the purification scheme.

Gel Electrophoresis. Analytical gel electrophoresis was performed to check the purity of the protein preparation and to determine CP molecular weight. SDS–polyacrylamide slab gel electrophoresis was carried out in a 10% gel at pH 8.9 by the modified Laemmli method (Laemmli, 1970). Samples (4 parts) were mixed with 1 part of sample buffer solution containing 10% β-mercaptoethanol, 10% SDS, 40% glycerol, and 0.01% pyronin in 0.125 M Tris base (pH 6.9) and heated for 2 min at 90 °C. Nonreduced samples were prepared in the same buffer with β-mercaptoethanol omitted. The molecular weight of pure procoagulant was estimated by determining the electrophoretic migration of reduced proteins with known molecular weight. Gels were stained for protein with Coomassie brilliant blue.

Non-SDS–polyacrylamide disc gel electrophoresis was performed in a 7.5% gel at pH 8.9 according to the method of Maurer (1971). A sample containing 40 μg of CP was inhibited with 0.1 mM $HgCl_2$, and equal portions were applied to identical disc gels in 0.3 M NaCl and run at 4 °C with a current of 3 mA per gel. One gel was stained for protein with Coomassie blue. The other gel was cut into 2-mm slices, and each slice was eluted separately in 0.2 mL of 0.02 M Bis-Tris/propane buffer (pH 6.5) containing 0.15 M NaCl at 4 °C for 24 h on a shaking platform. Aliquots (0.1 mL) of each eluate were activated with 10 mM KCN, 2 mM DTT, and 2 mM EDTA by the method described above and assayed in the single-stage clotting assay using factor VII deficient human plasma. The remaining 0.1 mL of the eluate from gel slice 24 was applied to SDS–PAGE in sample buffer (pH 6.9) and electrophoresed to confirm the purity and apparent molecular weight of the procoagulant protein.

Carbohydrate Staining. Carbohydrate staining was performed on an SDS–polyacrylamide (10%) gel with periodic acid–Schiff's reagent (Zacharius & Zell, 1969) to determine the sialic acid and the hexose content of CP. To calibrate the carbohydrate staining of glycoproteins, a standard curve was constructed by using from 0.5 to 20 μg of iron-free human transferrin (Sigma, St. Louis, MO), which contains 4% hexose and sialic acid (Jamison, 1965), or from 0.02 to 20 μg of carbohydrate. After electrophoresis, the gel was stained for carbohydrate. The gel was scanned on a densitometer, and the weights of the peak areas under the tracing were plotted against the corresponding amount of carbohydrate. Linear regression analysis of the standard curve a gave correlation coefficient of 0.994 and indicated that as little as 0.02 μg of carbohydrate could be detected with this method.

A sample of partially purified CP (about 90% pure) was equally divided and loaded on two halves of an SDS slab polyacrylamide gel. Each aliquot of the sample contained 75 μg of protein (or 68 μg of CP), about 5 times the usual amount of protein for an SDS–PAGE sample; we intentionally overloaded the gel so as to increase the lower limit of detection of carbohydrate groups in the sample. As standard controls, iron-free human transferrin, which contains carbohydrate (Jamieson, 1965), and bovine serum albumin, which does not contain carbohydrate (Peters & Reed, 1977), were applied to both halves of the gel. After the gel was electrophoresed, it was cut in two halves, and half was stained for protein with Coomassie blue and the other half was stained for carbohydrate with Schiff's reagent. The gels were scanned on a densitometer as described above, and the weights of the tracing of the peak were compared with the calibration curve of standard human transferrin.

Isoelectric Focusing. Analytical isoelectric focusing was performed to check the purity of the protein preparation and to determine the isoelectric point (p*I*) of CP. Lyophilized pure CP (10 μg) was dissolved in 10 μL of 2% ampholine (pH range 3–10) and was applied to precast 5% polyacrylamide gels (Servalyt Precotes). Focusing was carried out on an LKB FBE 3000 flatbed apparatus (LKB Produkter, Bromma, Sweden) for 16 h at 4 °C at a constant 300 V. The gel was stained with Coomassie brilliant blue and destained with methanol/acetic acid/water (40:10:50). The p*I* was determined by the location of standard proteins with known isoelectric points, including cytochrome *c* (p*I* = 9.6), hemoglobin (p*I* = 6.8), human apotransferrin (p*I* = 6.1), ovalbumin (p*I* = 4.7), and soybean trypsin inhibitor (p*I* = 4.5). In addition, one edge of the gel was cut in 0.5-cm pieces, and each piece was placed in 0.5 mL of 0.01 M KCl for 3 h to elute the ampholines. The pH values of the 0.01 M KCl solutions corresponded to the pH gradient of the gel (Righetti & Drysdale, 1976).

Amino Acid Analysis. A sample of the pure CP preparation was dialyzed 24 h against three changes of 0.5 M acetic acid (pH 3.0) to eliminate contaminating free amino acids. Aliquots containing 50–60 μg of protein were lyophilized and then hydrolyzed in 6 N HCl containing 1% phenol at 110 °C for 22 and 72 h. Amino acid analysis was performed with a Beckman 121 M amino acid analyzer. An unhydrolyzed aliquot of the sample was analyzed for free contaminant amino acids, and this background was subtracted from the analysis. Cysteine was determined as cysteic acid after performic acid oxidation by the method of Hirs (1967). Isoleucine and valine values were calculated from the 72-h hydrolysis. No correction was made for amino acid destruction after hydrolysis.

Antiserum Production and Characterization. Cancer procoagulant (200 μg/0.5 mL) purified by method 1 (see below) was emulsified with an equal volume of complete Freund's adjuvant and injected in three sites along the midback region of a goat. Booster immunizations were made at 14, 21, and 28 days, and at monthly intervals thereafter by using 50 μg of pure protein emulsified in incomplete Freund's adjuvant. Blood was obtained form the jugular vein of the goat at monthly intervals after the initial immunization.

The IgG goat antiserum was purified following standard procedures, including ammonium sulfate precipitation (Axelson et al., 1973) and DEAE-cellulose ion-exchange chromatography (Fahey & Terry, 1978).

Since the goat antibody was reactive to normal (non-tumor-bearing) rabbit serum protein(s), a "normal rabbit serum" affinity column was prepared as described below. The concentrated partially purified antibody sample was then applied to this column. The column was washed through with 0.05 M potassium phosphate buffer, pH 7.4, and unbound IgG was collected. The column was regenerated by running 3 M NaSCN and reequilibrating in 0.05 M potassium phosphate buffer, pH 7.4.

The antibody titer was tested against crude and purified cancer procoagulant by crossed immunodiffusion. The immunodiffusion plate was 1% agarose in low-salt veronal buffer (ionic strength = 0.025, pH 8.2) coated on gel bond film (FMC Corp., Rockland, ME). The samples were allowed to immunoreact for 48 h. The gel was then repeatedly washed (using sodium phosphate buffer, pH 7.4) and pressed to remove nonimmunoprecipitated protein. After the gel was washed, it was stained with crocein scarlet and Coomassie brilliant blue and destained in 0.3% acetic acid (Crowle & Cline, 1977).

Chromatography Column Preparation. (*A*) *Benzamidine Affinity Column.* A benzamidine–Sepharose affinity resin was prepared by coupling ε-aminohexanoic acid to cyanogen bromide activated Sepharose (Cuatrecasas, 1970). Hexanoyl-Sepharose (2 g) was coupled with *p*-aminobenzamidine (100 mg) with soluble carbodiimide, while maintaining the pH at 4.75. After thorough washing of the resin with distilled water, a 1 × 11 cm column was packed and equilibrated with 10 mM VB (pH 7.8) containing 50 mM NaCl and 1 mM EDTA.

(*B*) *Gel Filtration Column.* A gel filtration chromatography column (1.5 × 100 cm) was packed with 1.5m agarose gel. It was equilibrated with 10 mM VB (pH 7.8) containing 0.5 mg/mL crude phospholipid and then washed free of excess phospholipid with 10 mM VB (pH 7.8). The flow rate of this column was about 1 mL/min.

(*C*) *Phenyl-Sepharose Column.* A phenyl-Sepharose hydrophobic chromatography column (1 × 5 cm) was equilibrated in 0.01 M VB (pH 7.8) containing 0.5 mg/mL crude phospholipid, washed free of phospholipid with the VB, then washed with 10% dimethyl sulfoxide (Me_2SO) in VB, and finally equilibrated with 10 mM VB (pH 7.8).

(*D*) *Immunoaffinity and Normal Rabbit Serum Affinity Column.* An immunoadsorbent affinity column was prepared from the goat antiserum to CP. IgG antiserum fraction was purified as described above, lyophilized, and reconstituted in 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl. It was mixed with CNBr-activated Sepharose resin (5–10 mg of protein/mL of bed volume) overnight at 4 °C. After unreacted sites were blocked with 1 M ethanolamine (pH 8), the resin was washed with 0.1 M $NaHCO_3$ buffer (pH 8.3) and 0.5 M NaCl in 0.1 M sodium acetate buffer (pH 4) and then equilibrated in 20 mM VB (pH 7.8).

Serum from a normal, non-tumor-bearing, rabbit was diluted 1:7 with 0.05 M potassium phosphate buffer (pH 7.4) and was coupled to CNBr-activated Sepharose resin in a procedure identical with that described for goat IgG.

(*E*) *p-(Chloromercuri)benzoate–Agarose Column.* PCMB affinity resin coupled to agarose (Affi-gel 501, Bio-Rad Corp.) was equilibrated in 0.02 M Bis-Tris/propane buffer (pH 6.5) and packed in a 1 × 10 cm column. The resin was regenerated by running 100 mL of 0.01 M $HgCl_2$ and 0.02 M EDTA in 0.05 M sodium acetate buffer (pH 4.8) and 100 mL of 0.2 M NaCl and 0.001 M EDTA in 0.05 M sodium acetate buffer (pH 4.8) and by reequilibrating with 0.02 M Bis-Tris/propane buffer (pH 6.5).

Purification. (*A*) *Method 1.* This purification procedure has been described previously (Gordon, 1981; Gordon & Cross, 1981). Briefly, concentrated crude tumor extract (6 mL) was applied to a benzamidine affinity chromatography column, and unbound protein was removed with 10 mM VB (pH 7.8)

Table I: Purification of CP

method	steps	total protein (mg)	clotting act.[a] (units)	sp act. (units/mg)	yield[b] (%)	x-fold purification
1	crude extract	2.6×10^3	788	0.303	100	
	benzamidine affinity chromatography 1	247	838	3.4	106	11.2
	1.5m agarose chromatography	25.6	485	18.9	61	62.3
	benzamidine affinity chromatography II	1.3	248	190.7	31	627.4
	phenyl-Sepharose chromatography	0.16	108	675	14	2228
2	crude extract	2.1×10^5	3229.5	0.1538	100	
	AS precipitate	213.8	2911.7	13.6	90	88
	immunoaffinity chromatography	5.2	1829	351.7	57	2287
	PCMB affinity chromatography					
	1 mM $HgCl_2$ elution	1.2	243.3	202.7	7	1318
	2 mM $HgCl_2$ elution[c]	0.9	654.2	726.8	20	4726
	50 mM GSH elution	1.5	170	113	5	734

[a] The activity was calculated from a calibration curve with different concentrations of Russell's viper venom (RVV): 1 unit = activity of 1 mequiv/mL of RVV in the single-stage clotting assay. [b] Percentage of the total units present in the original extract. [c] Fraction containing the pure protein.

containing 50 mM NaCl and 1 mM EDTA. The bound protein was eluted with 1.0 M propionic acid. The acid eluate was neutralized, concentrated about 20-fold, dialyzed against 10 mM VB (pH 7.8), applied to the 1.5m agarose gel filtration column, and eluted with 10 mM VB (pH 7.8). The high molecular weight peak, containing 60–70% of the procoagulant activity, was applied to the benzamidine affinity resin. Unbound protein was removed with 10 mM VB (pH 7.8) containing 50 mM NaCl and 1 mM EDTA and then with 0.1% Triton X-100 in 10 mM VB (pH 7.8). Proteases were eluted with 0.05 and 0.5 M propionic acid. The acid eluates were neutralized, dialyzed against 20 mM VB, and concentrated. The acid eluate was applied to a phenyl-Sepharose hydrophobic affinity column; unbound protein was eluted with 10 mM VB (pH 7.8). Procoagulant was eluted with 10% dimethyl sulfoxide (Me_2SO) in 20 mM VB, concentrated and dialyzed against 20 mM VB, and reconstituted with 0.1 volume of 2 mg/mL rabbit brain cephalin (Sigma) to stabilize the procoagulant protein. Although approximately 20% of the applied procoagulant activity was recovered in the veronal buffer, the Me_2SO eluate generally contained 40–60% of the remaining activity and was a single protein band by SDS–PAGE.

(*B*) *Method 2.* Solid ammonium sulfate was added to 50 mL of concentrated crude extract to 40% saturation. After being stirred for 20 min at 4 °C, the sample was centrifuged at 5000*g* for 30 min at 4 °C. The ammonium sulfate precipitate was dissolved in 10 mL of 20 mM VB (pH 7.8) and dialyzed for 16 h against 20 mM VB. The sample was applied to the immunoadsorbent column. The column was washed with 20 mM VB (pH 7.8) (wash I) until the A_{280} of the wash was the same as that of the buffer. A second wash (wash II) using 100 mL of 1 M urea and 1% Tween-20 in 20 mM VB was performed to wash off nonspecifically bound proteins. Cancer procoagulant was eluted from the column by 100 mL of 3 M NaSCN in 40 mM VB. The eluate was immediately dialyzed against 0.02 M Bis-Tris/propane buffer (pH 6.5), concentrated 10 times on an Amicon PM-10 ultrafiltration membrane, and assayed for activity. An aliquot of the sample analyzed by analytical SDS–PAGE showed a single major band and other minor contaminants. To eliminate those contaminants, the immunoaffinity eluate was applied to a PCMB affinity column. The column was first washed with 0.02 M Bis-Tris/propane (pH 6.5) and then with 1 M urea and 1% Tween-20 in buffer until the A_{280} of the wash was the same as that of the buffer. Specifically bound protein was eluted with 100 mL of 1 mM $HgCl_2$ and 100 mL of 2 mM $HgCl_2$ followed by 50 mL of 50 mM glutathione to strip the column. The fractions corresponding to each eluate were pooled and concentrated 20–30-fold on an Amicon PM-10 ultrafiltration membrane. The pooled sample was analyzed for purity on SDS–PAGE and on PAGIF.

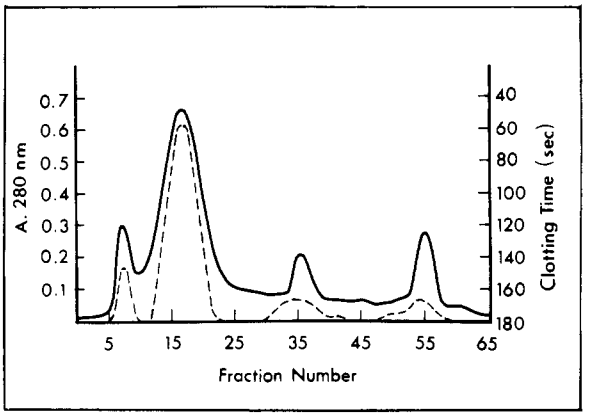

FIGURE 1: Agarose 1.5m gel filtration column chromatography elution profile. The acid eluate from the affinity chromatography step was fractionated on the 1.5m agarose gel filtration column in 10 mM veronal buffer (pH 7.8). The procoagulant activity [clotting time (---)] and protein [absorbance at 280 nm (—)] were measured.

It was necessary to reactivate the pooled samples eluted with mercury from the PCMB column in order to assay the procoagulant activity. The reactivation was performed with KCN, DTT, and EDTA, as described above, and they were assayed for activity by the one-stage recalcification time of normal and factor VII deficient human plasma.

Results

Tumor Extract. The concentrated extracts of rabbit V2 carcinoma were assayed for activity by the one-stage clotting assay. The extract's activity, expressed as RVV units (see Methods), was used to calculate the yield and purification factor of each purification step. Table I shows a representative scheme of the purification by methods 1 and 2.

Purification. Method 1. Cancer procoagulant (CP) was first purified from V2 carcinoma extract by a 4-step purification process. In the first benzamidine affinity chromatography step, the quantitative recovery of the procoagulant activity in the 1 M propionic acid eluate was greater than 100%, probably due to the removal of protease inhibitors present in the tumor extract. The active fractions were carried to the second purification step on an agarose gel filtration column with an exclusion limit of about 15×10^6 daltons. The elution profile from this column (Figure 1) gave four major protein peaks; most of the procoagulant activity (about 60%) was in the high molecular weight included peak. The void volume

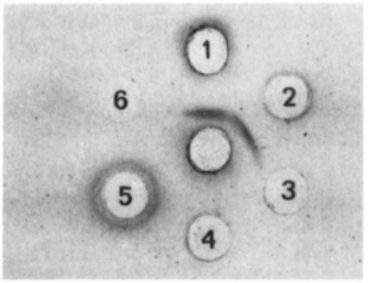

FIGURE 2: Ouchterlony double immunodiffusion. The immunodiffusion plate was 1% agarose in veronal buffer (ionic strength = 0.025, pH 8.2). The center well was loaded with 20 μL of purified goat antiserum against CP (2 mg of IgG/mL of buffer). The outer wells were filled with 20 μL of the following samples: (1) V2 carcinoma crude extract; (2) purified CP; (4) BSA; (5) normal (non-tumor-bearing) rabbit serum; wells 3 and 6 were empty. After the gel was washed and dried, it was stained with crocein scarlet and Coomassie blue, as described.

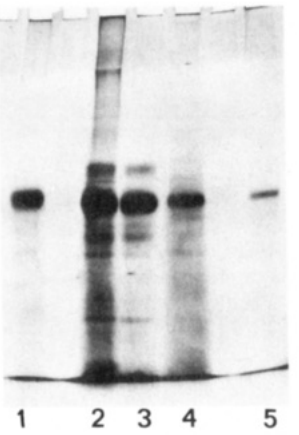

FIGURE 3: SDS–polyacrylamide (10%) slab gel electrophoresis of samples from each step of the purification of CP by method 2. (1) BSA standard; (2) crude extract; (3) redissolved ammonium sulfate precipitate of crude extract; (4) 3 M NaSCN eluate from immunoaffinity chromatography; (5) 2 mM $HgCl_2$ eluate from PCMB affinity chromatography column (pure CP).

contained about 15% of the activity, and the remaining activity was eluted with the low molecular weight proteins. The major procoagulant peak was applied to the second benzamidine affinity chromatography gel. The column was washed with a nonionic detergent (0.1% Triton X-100) and dilute propionic acid (0.05 M) to eliminate adsorbed and weakly bound contaminants. No procoagulant activity was recovered in the protein sample eluted by Triton X-100, and little activity was recovered with the protein eluted by 0.05 M propionic acid. About 30% of the original procoagulant activity was recovered in the 0.5 M acid eluate. This eluate was applied to a phenyl-Sepharose hydrophobic affinity column. The Me_2SO eluate from this affinity column generally contained 40–60% of the applied procoagulant activity and showed a single protein band on SDS–PAGE. By this purification scheme (Table I), CP was purified about 2200-fold, and the total recovery of the activity was 14%. The preparation was homogeneous on SDS–PAGE (Gordon, 1981) with an apparent molecular weight of 68 000. A goat was immunized with the CP purified by this method.

Antiserum Production and Characterization. The goat IgG serum fraction was purified and was characterized by Ouchterlony double immunodiffusion. The specificity of the antiserum is demonstrated in Figure 2. A single precipitin arc was present on the immunodiffusion plate against the crude extract (well 1) and the purified protein (well 2), with a line of identity between the two wells. The antibody was not immunoreactive with normal (non-tumor-bearing) rabbit serum proteins (well 5), particularly serum albumin and other proteins with a molecular weight similar to CP. BSA was also chosen as a control protein for this study because it has similar molecular weight and p*I* as CP.

Purification. Method 2. Crude V2 extract was brought to 40% saturation with crystalline ammonium sulfate. The precipitate contained 90% of the original activity and was carried through the immunoaffinity chromatography procedure. Neither wash I nor wash II from the immunoaffinity column contained measurable procoagulant activity in either normal or factor VII deficient plasma in the clotting assay. Only the 3 M NaSCN eluate contained factor VII independent procoagulant activity (57% of the original), and CP was purified more than 2000-fold (Table I).

Despite the apparent specificity of the goat antibody to CP, other proteins bound to the column were present in the immunoaffinity eluate (Figure 3) and appeared as minor protein bands on SDS–PAGE. In order to obtain a homogeneous preparation, we used an organomercurial agarose column to bind CP through the mercaptide group followed by elution using competing sulfhydryl compounds. In a series of preliminary experiments, we tried several of these compounds, including glutathione (2, 5, and 10 mM), DTT (0.1, 10, and 100 mM), and mercury (1 and 2 mM). We found mercury gave the best result. For this reason, we used $HgCl_2$ to elute CP, but the eluted protein had no procoagulant activity in the presence of mercury. Treatment of the samples with 10 mM KCN, 2 mM DTT, and 2 mM EDTA restored the procoagulant. The 2 mM $HgCl_2$ eluate contained procoagulant activity, both in normal and in factor VII deficient plasma, and showed one single protein band on the SDS–PAGE and analytical PAGIF. The 1 mM $HgCl_2$ and the 50 mM glutathione eluates contained 7% and 5% of the total activity, respectively, and included some other protein contaminants. As shown in Table I, CP was purified about 4700-fold by this procedure, with a 20% recovery of procoagulant activity.

When samples of immunoaffinity-purified CP (20 μg) were run at pH 8.9 on 7.5% polyacrylamide disc gels, the results shown in Figure 4 were obtained. The single-stage clotting assay in factor VII deficient plasma of an aliquot (0.1 mL) of individual eluted gel slices, previously reactivated as described under Methods, revealed a single peak of procoagulant activity that coincided with the protein-stained band. About 65% of the procoagulant activity in the original sample applied to the gel was recovered in the three eluted fractions. Analysis by SDS–PAGE of the remaining aliquot from gel slice 24 (0.1 mL) demonstrated that the samples containing procoagulant activity also contained a single protein band with an apparent molecular weight of 68 000.

The pure protein obtained by method 2 had the same enzymatic characteristics, molecular weight, and isoelectric point as the protein purified by method 1, but the recovery was greater and the time required to execute the procedure was about 5 times faster.

Properties of CP. The final preparation of CP both by method 1 and by method 2 appeared to be homogeneous by analytical SDS–PAGE and PAGIF. A single protein was seen with and without reduction of β-mercaptoethanol (Figure 5). In the Laemmli system (Laemmli, 1970), the reduced protein showed an apparent molecular weight of 68 000. Analytical PAGIF of pure CP on a Servalyt Precote gel in the pH range 3–10 showed a single protein band with a p*I* of 4.8.

As shown in Table II, in the one-stage clotting assay the purified samples showed equal activity in normal and factor

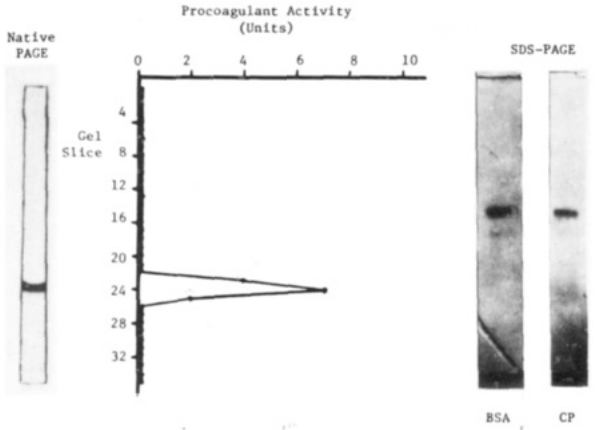

FIGURE 4: Correlation of procoagulant activity and electrophoretic migration of pure CP. Two samples containing 20 μg of purified cancer procoagulant were electrophoresed on identical non-SDS (native) (7.5%)–polyacrylamide disc gels. After electrophoresis, one native gel was stained for protein (left side), and the second gel was sliced into 2-mm segments. Each segment was eluted into 0.2 mL of Bis-Tris/propane buffer (pH 6.5) as described under Methods and assayed in the single-stage clotting assay, utilizing factor VII deficient human plasma. The procoagulant activity (expressed as RVV units) of each 2-mm slice eluate was plotted to show that the activity profile matches the protein stain profile of the native gel. To confirm the molecular weight of the procoagulant protein eluted from the native gel, 0.1 mL of the eluate from gel slice 24 was mixed with the SDS–PAGE sample buffer and electrophoresed on an SDS–polyacrylamide slab gel with BSA standard and stained for protein (right side).

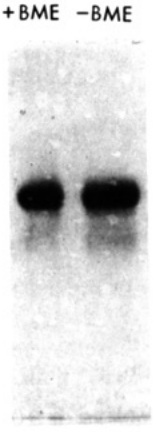

FIGURE 5: SDS–polyacrylamide slab gel electrophoresis of 20 μg of purified CP that was either reduced, with β-mercaptoethanol (+BME), or not reduced (−BME). An aliquot (4 parts) of the purified procoagulant was mixed with 1 part of sample buffer with or without 10% β-mercaptoethanol, heated at 100 °C for 2 min, and electrophoresed on a 10% polyacrylamide slab gel.

VII deficient human plasma. The procoagulant activity was inhibited by 1 mM iodoacetamide and 0.1 mM mercury and was restored by treatment with 10 mM KCN, 2 mM DTT, and 2 mM EDTA. In Table III, inactive samples obtained in the last step of purification method 2 by eluting with mercury were reactivated by KCN, DTT, and EDTA. Aliquots of the same samples were inhibited again by making them 1 mM with iodoacetamide or 0.1 mM with $HgCl_2$. Previous studies demonstrated that CP purified by method 1 directly activated pure bovine factor X (Gordon & Cross, 1981; Gordon, 1981).

Amino Acid and Carbohydrate Composition of CP. The amino acid composition of CP is shown in Table IV. The results are the average of four determinations on a Beckman amino acid analyzer from four separate pure CP samples that were purified by method 2. The prevalent amino acids are serine with 125 residues per mole, glycine with 123 residues per mole, glutamic acid with 82 residues per mole, lysine with 53 residues per mole, and aspartic acid with 46 residues per mole. These five amino acids account for more than 50% of the whole molecule. Basic amino acids (lysine and arginine) comprise about 10% of the total, whereas glutamic acid and aspartic acid are about 19% of the total. Our analysis shows

Table II: Dependence of Cancer Procoagulant Activity on Factor VII[a]

	normal plasma		factor VII deficient plasma	
	clotting time (s)	% act.	clotting time (s)	% act.
rabbit brain thromboplastin (n = 3)	107 ± 5	47 ± 2	198 ± 6	0 ± 3
Russell's viper venom (n = 3)	101 ± 6	49 ± 3	104 ± 4	47 ± 2
cancer procoagulant (n = 4)	140 ± 5	30 ± 3	145 ± 7	28 ± 3

[a] To deteremine the dependence of procoagulant activity on factor VII, samples containing from 2 to 10 μg of pure cancer procoagulant in 100 μL of 0.02 M Bis-Tris/propane buffer (pH 6.5) were assayed in the one-stage clotting assay described under Methods, utilization normal human plasma or factor VII deficient human plasma. Russell's viper venom (2 ng in 100 μL of 0.15 M NaCl), was used as a positive coagulation control in the assay in both normal and factor VII deficient plasma. Rabbit brain thromboplastin (40 μg in 100 μL of 0.15 M NaCl) was used as a positive coagulation control in the assay in normal plasma and as a negative coagulation control in the assay in factor VII deficient plasma. The clotting times were normalized to a blank of 200 s and were expressed as a percent activity which was computed as a percent decrease of clotting time compared to the corresponding buffer blank (blank time − clotting time/blank time). The mean ± standard error of the mean of n clotting time measurements and the computed percent activity are presented.

Table III: Effect of Cysteine Proteinase Activators and Inhibitors on Cancer Procoagulant Activity

		clotting assay		
treatment	n	s	%	P
	(A) Reactivation[a]			
$HgCl_2$				
untreated	16	203 ± 1	0	<0.005
treated	16	160 ± 2	21	
	(B) Inhibition[b]			
iodoacetamide				
untreated	4	155 ± 4	23	<0.005
treated	4	202 ± 3	0	
$HgCl_2$				
untreated	4	163 ± 3	20	<0.005
treated	4	205 ± 2	0	

[a] Samples obtained by the immunoaffinity chromatography step (method 2) were applied to a PCMB affinity column. No activity was recovered in the fractions eluted with $HgCl_2$ from this column. To measure the clotting activity, the fractions (40 μg of protein/mL) were made 10 mM KCN, 2 mM DTT, and 2 mM EDTA, incubated at 4 °C for 30 min, and dialyzed overnight against 0.02 M Bis-Tris/propane (pH 6.5). [b] For inhibition studies, aliquots of the reactivated fractions (A) were made 1 mM iodoacetamide or 0.1 mM $HgCl_2$, respectively, and incubated at 37 °C for 30 min. Untreated samples, reactivated samples, and inhibitor-treated samples were assayed for activity by measuring the recalcified clotting time of normal citrated human plasma. The results are expressed as the mean clotting time of n untreated and treated samples ± SEM normalized to a blank clotting time of 200 s or as the percent activity which was calculated as the percent decrease in the clotting time compared to the corresponding buffer blank. The mean difference ± SEM of the difference between the activities of the untreated sample and its reactivated and inhibited counterpart was compared with a paired t test.

Table IV: CP Amino Acid Composition

amino acid	% of total residues	residues/mol of protein[a]
lysine	8.1	53
histidine	3.9	26
arginine	2.3	15
aspartic acid	7.1	46
threonine	5.1	33
serine	19.1	125
glutamic acid	12.5	82
proline	3.6	24
glycine	18.7	123
alanine	6.4	42
cysteine[b]	1.2	8
valine	3.1	20
methionine	1.7	11
isoleucine	2.6	17
leucine	3.4	22
tyrosine	2.0	13
phenylalanine	2.2	14
tryptophan	ND[c]	ND[c]
	total 100	674

[a] Based on the molecular weight of 68 000 as determined by SDS–polyacrylamide electrophoresis. [b] Determined as cysteic acid according to Hirs (1967). [c] ND, not determined.

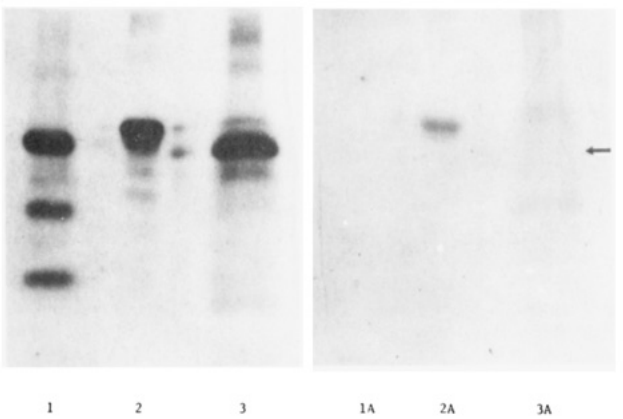

FIGURE 6: Coomassie blue staining and periodic acid–Schiff's reagent staining of CP and standard controls on an SDS–polyacrylamide (10%) slab gel. (Left panel) Samples stained with Coomassie blue. (Right panel) Samples stained with Schiff's reagent. (1 and 1A) Standard BSA (20 μg); (2 and 2A) standard transferrin (20 μg); (3 and 3a) cancer procoagulant (68 μg).

eight cysteine (or half-cystine) residues per mole. Contaminant amino acids in the unhydrolyzed protein were lysine, histidine, aspartic acid, threonine, serine, glycine, alanine, and leucine. Each of the four hydrolysates was corrected for these contaminants by subtracting the nanomoles of the contaminant from the nanomoles of each respective amino acid in the hydrolyzed sample before calculating the amino acid composition. No attempt was made to determine how many of these residues are present as glutamine and asparagine.

The carbohydrate content of CP was estimated by periodic acid–Schiff's staining of CP on SDS–PAGE. Figure 6 shows a slab SDS–PAGE that contained human transferrin, BSA, and a 90% pure CP sample and was stained for protein with Coomassie blue and for carbohydrate with Schiff's reagent. As expected, standard transferrin (20 μg) showed positive staining by both staining techniques, and standard BSA (20 μg) was positive by only the protein staining. The CP sample (68 μg of pure CP) showed a prominent band of molecular weight 68 000 stained for protein (Figure 6, left panel), but the same band was undetectable on the half of the gel stained for carbohydrate (Figure 6, right panel). Therefore, 1 nmol of pure CP contained less than 0.11 nmol of hexose or 0.06 nmol of sialic acid (<1 mol/mol of protein).

Discussion

The increased activation of the coagulation system and the numerous experimental data suggesting a role for fibrin in malignant disease led us to look for a procoagulant from neoplastic tissue. Early studies (Gordon et al., 1975, 1979, 1982; Gordon & Cross, 1981; Gordon & Lewis, 1978; Gordon, 1981) suggested that we had found an enzyme that was derived from tumor tissue that may not be derived from normal tissue or a component of the normal coagulation cascade. However, more conclusive evidence for the unique properties of CP required the purification and physical and chemical characterization of this enzyme.

Alterations in the blood coagulation system, including changes in clotting factors and platelet activation, of rabbits bearing the V2 carcinoma have been documented (Wood, 1974). Moreover, fibrin has been found on potentially metastatic blood-borne V2 carcinoma cells (Wood, 1964). Thus, the V2 carcinoma was a convenient malignant cell line for this study because it produced large (50–200 g) reproducible tumors. From one tumor of approximately 100 g, we obtained about 50 mL of concentrated extract containing very high procoagulant activity. We have described two procedures for the purification of CP from these extracts.

The first purification procedure for cancer procoagulant utilized classical protein purification techniques and attempted to take advantage of the active-site characteristics and hydrophobic properties of this protein to separate it from other proteins. Since cancer procoagulant is inhibited by 5 mM diisopropyl fluorophosphate (Gordon et al., 1975), it seemed probable that a benzamidine affinity chromatography step would be an efficient purification method. The second step of the purification procedure utilized the observation that CP aggregated into high molecular weight complexes (approximately 10^6) when concentrated to more than 2 mg of protein per milliliter of sample; molecular sieve chromatography permitted CP to be resolved from other proteins with molecular weights less than 150 000, a molecular weight cut-off that is common for most serine proteinases (Perlman & Lorand, 1970a,b). Following gel filtration column chromatography, the benzamidine affinity chromatography step was repeated. A final hydrophobic affinity chromatography step resulted in relatively poor yield but highly purified protein. Equilibration of the 1.5m agarose resin and the phenyl-Sepharose resin with phospholipid substantially improved both the resolution of proteins in the column eluates and the recovery of CP activity from the columns.

The purified procoagulant was used to immunize a goat, and the goat anti-CP antiserum was used to prepare an immunoadsorbent column. The second purification procedure purified CP by immunoaffinity chromatography. However, the overall purification efficiency and recovery of activity for both methods were impossible to calculate accurately probably because inhibitors present in the crude extract masked the procoagulant activity, so the calculated purification data (Table I) are probably an underestimate of the true level of purification. However, the final product of the two purification sequences appeared to be a homogeneous cancer procoagulant enzyme. Evidence that we have purified CP to homogeneity is derived from three independent criteria. The final protein preparation, both by method 1 and by method 2, was a single band on SDS–PAGE and focused to a single protein band when analyzed on PAGIF. In addition, immunoaffinity-purified CP was electrophoresed on a native (non-SDS) polyacrylamide gel (Figure 4), and gel slice eluates were reelec-

Table V: Comparison of Some Properties of Cancer Procoagulant with Those of Other Factor X Activators and of Cathepsin B

	cancer procoagulant	human factor VIIa	human factor IXa	bovine tissue factor	human cathepsin B
mol wt	68000	40000	44000	43000	25000
p*I*	4.8	5.6	4.3–4.45		4.5–5.5
no. of polypeptides	1	2	2	1	1
carbohydrate content	<1 mol/mol	13%	20%	present	<1 mol/mol
	residues/mol				
amino acid	cancer procoagulant	human factor VIIa	human factor IXa	bovine tissue factor	human cathepsin B
lysine	53	17	22	17	10
histidine	26	10	9	5	8
arginine	15	20	14	10	8
aspartic acid	46	32	37	34	23
threonine	33	20	22	35	10
serine	125	31	22	35	17
glutamic acid	82	44	45	29	23
proline	24	21	13	22	17
glycine	123	37	42	36	29
alanine	42	21	19	28	13
cysteine	8	20	13	5	12
valine	20	19	27	31	15
methionine	11	2	3	3	4
isoleucine	17	12	15	14	13
leucine	22	32	19	28	10
tyrosine	13	10	12	9	13
phenylalanine	14	11	14	11	7
tryptophan	ND	ND	12	1	7

[a] Properties of factor VII or VIIa are from Broze & Majerus (1980), Gladhaug & Prydz (1970), and Bajaj et al. (1981). [b] Properties of factor IX or IXa are from Osterud & Flengsrud (1975) and Osterud et al. (1978). [c] Properties of tissue factor are from Bach et al. (1981). [d] Properties of cathepsin B are from Barrett (1973, 1977b).

trophoresed on SDS–PAGE and analyzed for procoagulant activity in factor VII depleted plasma; SDS–PAGE showed a single protein band with an apparent molecular weight of 68 000, and the staining profile of the single band on the native gel matched the procoagulant activity profile.

Characterization of the physical and chemical properties of CP revealed that it was a single-chain polypeptide, with an apparent molecular weight of 68 000, in both the presence and absence of reduction by β-mercaptoethanol. It had a p*I* of 4.8. The amino acid composition showed a predominance of serine, glycine, and glutamic acid in the 674 amino acid residues. There was a prevalence of the acidic amino acids over the basic amino acids which would explain the low p*I*. There were eight residues of cysteine per mole of CP. Estimation of carbohydrate content by periodic acid–Schiff's staining suggested that the hexose and sialic acid content was less than 1 mol/mol of protein. Furthermore, there was no evidence of glucosamine and galactosamine on the amino acid analysis of CP, which provides further support for the lack of carbohydrate in the protein.

The enzymatic properties of CP have been described previously and were confirmed in the present study. CP initiated blood coagulation in the absence of factor VII (Gordon et al., 1975; Gordon & Cross, 1981) by directly activating pure bovine factor X. SDS–PAGE analysis of the proteolytic conversion of factor X to factor Xa by CP appears to have similar activation peptides and to proceed by a mechanism similar to that for RVV (Gordon, 1981). However, this activation of factor X is inhibited by cysteine proteinase inhibitors such as iodoacetamide and Hg^{2+} (Gordon & Cross, 1981). Furthermore, CP has other cysteine proteinase characteristics including binding to *p*-(chloromercuri)benzoate and optimum activity at about pH 6.5 (Gordon & Cross, 1981). In the present study, we also demonstrated that CP was sensitive to chemical activators of cysteine proteinases including DTT, EDTA, and KCN (Barrett, 1977a,b; Perlman & Lorand, 1970a,b; Gould & Liener, 1965). Thus, several lines of evidence support the cysteine proteinase nature of cancer procoagulant. There was some confusion by early evidence that CP was inhibited by DFP, suggesting it was a serine proteinase (Gordon et al., 1975, 1978; Gordon & Lewis, 1979). This was clarified by demonstrating that CP was probably being inhibited by an impurity in the commercial preparation of DFP rather than by the DFP itself (Gordon & Cross, 1981), results that are consistent with the cysteine proteinase nature of CP. All of the known coagulation enzymes, including thrombin, factors Xa, IXa, VIIa, and XIa, and RVV, are serine proteinases; we are not aware of reports showing other mammalian cysteine proteinases that initiate coagulation.

To further delineate the unique properties of CP, we compared the physical and chemical characteristics of CP (Table V) to those of factors VIIa and IXa (the known coagulation factors that activate factor X), of tissue factor (the procoagulant present in normal tissues), and of cathepsin B (a common mammalian cysteine proteinase). Virtually all of the properties listed in Table V reveal differences between CP and these other proteins. These data, together with the evidence that this cysteine proteinase directly activates factor X (Gordon & Cross, 1981; Gordon, 1981), suggests that CP is different than previously described coagulation factors.

Comparison of CP properties with those of tissue factor shows that tissue factor is a glycoprotein which has an amino acid composition and a molecular weight different from CP. To initiate coagulation, tissue factor requires the presence of factor VII (Nemerson & Bach, 1982). Finally, there is no evidence that tissue factor is sensitive to iodoacetamide and mercury. Therefore, CP is probably not tissue factor.

Since CP is a cysteine proteinase with cathepsin B like properties, we compared the physicochemical characteristics of CP to those of cathepsin B. This comparison is relevant in light of the recent reports on the role of cysteine proteinases in tumor malignancy and invasiveness (Recklies et al., 1980; Liotta et al., 1979; Sloane et al., 1981) and in tumor cell induced platelet aggregation (Honn et al., 1982). Similarities

between CP and cathepsin B are the following: (1) the two enzymes have a similar acidic isoelectric point, a property which distinguishes both of them from plant cysteine proteinases, like papain, ficin, and bromelain, whose p*I*'s are about pH 9 (Barrett, 1977a,b; Vavreinova & Turkova, 1975); (2) both CP and cathepsin B have no carbohydrate, a property common to papain but not to ficin and bromelain (Barrett, 1977a,b; Vavreinova & Turkova, 1975). Major differences between CP and cathepsin B include the molecular weight (CP is about 3 times higher in molecular weight than cathepsin B) and amino acid composition (Table V).

Although the species from which cancer procoagulant was purified is not the same as those from which the other proteins were obtained, it seemed unlikely that this would account for the major differences in these physical, chemical, and enzymatic properties. This hypothesis is supported by our recent studies which showed that the physical and enzymatic characteristics of a proteolytic procoagulant from human amnion–chorion were the same as those of CP from rabbit V2 carcinoma (Gordon et al., 1985; Falanga & Gordon, 1985).

The properties of cancer procoagulant were also compared to other procoagulants that have been found in malignant cells. Tissue factor is the other major procoagulant found in a high percentage of malignant tissues, and comparison to tissue factor is described above. The procoagulant identified by O'Meara and his associates or Pineo et al. was not adequately characterized to make an effective comparison. Although some of the properties of CP seem different than those of procoagulants described by previous investigators, it seems possible that both groups may have been working with cancer procoagulant. Recent reports by Dvorak have described a factor V like cofactor on malignant cells, and it is quite clear that this activity is not cancer procoagulant (Dvorak, 1979, 1981).

Thus, comparison of the propeties of cancer procoagulant with those of known serine and cysteine proteinases suggests that this proteolytic procoagulant associated with malignant tissue may represent a new coagulation factor and supports the concept that cancer procoagulant may be a unique proteinase associated with dedifferentiated malignant tissue (Gordon & Cross, 1981; Gordon & Lewis, 1978; Gordon et al., 1979, 1982) or undifferentiated fetal tissue (Gordon et al., 1985; Falanga & Gordon, 1985). It seems possible that malignant cells may produce cancer procoagulant and this procoagulant may be responsible for the abnormal fibrinogen metabolism and local fibrin deposition associated with cancer. The availability of purified cancer procoagulant will enable us to answer these and other questions about the pathophysiology of fibrinogen/fibrin metabolism in malignant disease.

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DNA Tris-Intercalation: First Acridine Trimer with DNA Affinity in the Range of DNA Regulatory Proteins. Kinetic Studies†

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ABSTRACT: A trimer made up of three acridine chromophores linked by a positively charged poly(aminoalkyl) chain was synthesized as a potential tris-intercalating agent. The length of the linking chain was selected to allow intercalation of each chromophore according to the excluded site model. ^{1}H NMR studies have shown that, at 5 mM sodium, pH 5, the acridine trimer occurred under a folded conformation stabilized by stacking interactions between the three aromatic rings. DNA tris-intercalation of the dye at a low dye/base pair ratio was shown by measurements of both the unwinding of PM2 DNA and the lengthening of sonicated rodlike DNA. The trimer exhibits a high DNA affinity for poly[d(A-T)] ($K_{app} = 8 \times 10^8$ M^{-1}, 1 M sodium) as shown by competition experiments with ethidium dimer. Kinetic studies of both the association with poly[d(A-T)] and the exchange between poly[d(A-T)] and sonicated calf thymus DNA have been performed as a function of the ionic strength. In 0.3 M sodium the on-rate constant ($k_1 = 2.6 \times 10^7$ M^{-1} s^{-1}) is similar to that reported for other monoacridines or bis(acridines), whereas the off-rate constant is much smaller ($k_{-1} = 1.2 \times 10^{-4}$ s^{-1}), leading to an equilibrium binding constant as large as $K_{app} = 2.2 \times 10^{11}$ M^{-1}. A plot of log (k_1/k_{-1}) as a function of log [Na$^+$] yielded a straight line whose slope shows that 5.7 ion pairs (out of 7 potential) are formed upon the interaction with DNA. From this linear relationship a K_{app} value of 10^{14} M^{-1} in 0.1 M sodium can be estimated. Such a value reaches and even goes beyond that of some DNA regulatory proteins. This acridine trimer appears to be the first synthetic ligand with such a high DNA affinity.

Gene expression mainly depends upon interactions between nucleic acids and regulatory proteins. Such interactions are characterized by both high affinity and high specificity (Riggs et al., 1970; Lin & Riggs, 1972). Therefore, compounds that aim to interfere with genetic expression should ideally present these two properties.

On one hand, it has been shown that base specificity can be achieved by means of hydrogen bonding between nucleic acid bases and donor or acceptor groups borne by the ligand (Seeman et al., 1976; Hélène, 1977; Ohlendorf et al., 1982).

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