Articles

Angiogenic Activity of Human Tumor Plasma Membrane Components†

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ABSTRACT: Plasma membranes from the human colon adenocarcinoma cell line HT-29 have been isolated and examined for the presence of angiogenic activity. Membrane-associated macromolecules extracted with Triton X-100 were fractionated on immobilized wheat germ agglutinin. The fraction which bound specifically (about 200 ng of protein/mL packed cells) was highly angiogenic when assayed on the chick embryo chorioallantoic membrane. As little as 0.2 ng of this human tumor derived material consistently induced neovascularization. Similarly, 1–2 ng of this material implanted into the rabbit cornea induced new vessel growth (5–8 mm) within 10 days. The plasma membranes of eight other human tumor lines were examined for angiogenic activity. For each, the wheat germ agglutinin bound material induced neovascularization at the low nanogram level. In contrast, the wheat germ agglutinin bound material derived from purified plasma membranes of two normal human diploid fibroblast cell lines failed to induce an angiogenic response on the chick chorioallantoic membrane, even at microgram levels.

The de novo development of a host-derived vascular network in the immediate environment of solid tumors and the importance of this neovascularization to tumor progression are now well established (Folkman, 1974; Folkman & Cotran, 1976; Auerbach, 1981; Gullino, 1981; Vallee et al., 1985). Such tumor-induced angiogenesis has been attributed to the secretion of tumor angiogenesis factors, TAFs,[1] by viable tumor cells (Folkman & Cotran, 1976). We have isolated, purified, and characterized a protein, angiogenin, from medium conditioned by the human colon adenocarcinoma cell line HT-29 (Fett et al., 1985a). Angiogenin is a basic protein of molecular weight 14 500 which shares sequence homology with certain ribonucleases (Strydom et al., 1985). Moreover, we have demonstrated that a lower molecular weight fraction in medium conditioned by the same cell line possesses angiogenic activity (Fett et al., 1985b).

Although a wide variety of starting materials have been used for attempts at such purification of angiogenic activity, including solid tumors, tumor cell homogenates, and medium conditioned by tumor cells grown in vitro, the plasma membranes of these cells have not previously been specifically examined. However, many of the distinguishing characteristics of transformed cells are expressed at the cell surface (Yogeeswaran, 1980; Hakomori & Kannagi, 1983), and there is ample evidence for direct cell–cell contact between tumor and capillary endothelial cells within solid tumor masses (Folkman & Cotran, 1976). Therefore, in parallel with these studies on tumor-secreted molecules, the tumor cell surface has been examined for the presence of such activity, both to evaluate the role of the tumor cell plasma membrane in angiogenesis and to provide possible alternative starting materials for purification. This report demonstrates that the products isolated from the plasma membranes of nine human tumor cell lines, including HT-29, contain potent angiogenesis-inducing factors while those derived from two human diploid fibroblast cell lines do not.

Table I: Cell Lines

name	designation	description
HT-29		primary colon adenocarcinoma
WiDr	CCL-218[a]	primary colon adenocarcinoma
COLO 320 DM	CCL-220	undifferentiated colon adenocarcinoma
COLO 320 HSR	CCL-220.1	substrain of COLO 320 DM
COLO 205	CCL-222	colorectal adenocarcinoma
COLO 201	CCL-224	substrain of COLO 205
SW 480	CCL-228	colorectal adenocarcinoma
A549	CCL-185	adult lung carcinoma
RD	CCL-136	embryonal rhabdomyosarcoma
WI-38	CCL-75	normal embryonic lung fibroblast
WI-26	CCL-95	normal embryonic lung fibroblast

[a] American Type Culture Collection catalog number, Rockville, MD.

Experimental Procedures

Cells and Culture Conditions. Table I lists the human cell lines studied. They include two normal diploid fibroblasts, a rhabdomyosarcoma, a lung adenocarcinoma, and seven colonic adenocarcinoma lines. These were grown as monolayer cultures in 75 and 150 cm^2 flasks (Costar, Cambridge, MA) in DME containing 4.5 g/L glucose (MA Bioproducts, Walkersville, MD). It was supplemented further with 250 μg/L amphotericin B (Gibco Laboratories, Grand Island, NY), 50 mg/L gentamicin (MA Bioproducts), 2 mM L-glutamine, and

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[1] Abbreviations: TAF(s), tumor angiogenesis factor(s); DME, Dulbecco's modified Eagle's medium; WGA, wheat germ agglutinin; CAM, chorioallantoic membrane; MDH, malate dehydrogenase; G6Pase, glucose-6-phosphatase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; psi, pounds per square inch; DME/5, DME supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

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5% (v/v) fetal bovine serum (DME/5).

The HT-29 cell line (Fogh & Trempe, 1975) was also grown as a large-scale suspension culture at the Monsanto Co., St. Louis, MO, according to methods detailed elsewhere (Tolbert et al., 1981; Feder & Tolbert, 1983). Cells from large-scale suspension cultures were harvested by continuous-flow centrifugation and prepared as 30% suspensions (w/v) in DME/5 supplemented with 8% (v/v) dimethyl sulfoxide, 3% (w/v) sucrose, and 15% (v/v) fetal bovine serum. The cell suspension was frozen (1–3 °C/min) to −70 °C prior to further processing.

Purification of Plasma Membrane Fractions. (*A*) *Monolayer Cultures.* Five confluent 150 cm^2 flasks from each of the cell lines (Table I) were harvested at confluence with a cell scraper; the cells were washed 3–5 times by centrifugation (400*g*, 5 min, 4 °C) in 500 mL of Dulbecco's calcium- and magnesium-free phosphate-buffered saline, pH 7.4 (buffer A), then resuspended in 30 mL of ice-cold 5 mM sodium phosphate buffer, pH 7.0 (buffer B), containing 0.03% aprotinin (Sigma Chemical Co., St. Louis, MO), and incubated with gentle stirring at 4 °C for 60 min. After incubation, cells were disrupted mechanically with 10–250 strokes of a 40-mL Dounce homogenizer with a tight ("A") pestle. The resulting cell homogenates were clarified by centrifugation (18000*g*, 20 min), and the pelleted material was discarded. The crude membrane fractions were then pelleted from the clarified homogenates by centrifugation (78000*g*, 120 min) and washed in 30 mL of buffer B in a Beckman Model L5-65 centrifuge with an SW 27.1 rotor. The final pellet containing the crude membrane fraction was resuspended in 10 mL of buffer B.

The plasma membrane fraction was purified further by isopycnic centrifugation on discontinuous sucrose gradients using a modification of established procedures (Warren & Glick, 1969; Neville, 1975). Ten-milliliter aliquots of crude membrane fractions were made 45% (w/v) in sucrose and transferred into 38-mL polyallomer centrifuge tubes. The membrane fractions were then overlaid with 5 mL each of 35%, 30%, 25%, and 20% (w/v) sucrose in buffer B and then with 5 mL of sucrose-free buffer B. The tubes were centrifuged in an SW 27.1 rotor (78000*g*, 24 h). The upper two layers from each tube (0–20% sucrose) were pooled as gradient fraction 1. The next layer (20–25% sucrose) was discarded. The fourth and fifth layers (30–45% sucrose) were pooled as gradient fraction 2. The materials which pelleted through the 45% sucrose layer were pooled as gradient fraction 3. The gradient fractions were diluted 10-fold with buffer B, pelleted by centrifugation (100000*g*, 60 min), and then resuspended in 5 mL of buffer B.

(*B*) *Large-Scale Suspension Cultures.* Frozen HT-29 cell suspensions (approximately 500 mL of packed cells/lot) were thawed, suspended 3–5 times in 10 volumes of buffer A, pelleted by centrifugation (2000*g*, 30 min, 4 °C), and then resuspended in 3 volumes of ice-cold buffer B containing 0.03% aprotinin. The cell suspension was transferred to a nitrogen cavitation chamber (Paar Instruments, Moline, IL), pressurized to 1100 psi, and incubated with gentle stirring for 1 h at 4 °C. Pressure was reduced gradually to atmospheric over a period of about 10 min, and the resulting homogenate was clarified by centrifugation (pellet discarded). The clarified material was then made 45% in sucrose and subjected to isopycnic centrifugation as described above. Gradient fractions 1–3 were collected and washed as described above for the preparations of monolayer cultures. Fractions 1 and 2 were dialyzed against distilled water and lyophilized. Fraction 3 was resuspended in 5 mL of buffer B and extracted with Triton X-100 (see below).

Radiolabeling and Enzyme Markers. One milliliter of packed HT-29 cells from monolayer cultures (approximately 1×10^8 cells) was reacted with ^{125}I by the soluble lactoperoxidase/glucose oxidase method to radiolabel cell-surface molecules (Hubbard & Cohn, 1972). The labeled cells were processed as indicated above for monolayer cultures and aliquots held for analysis at each step of the plasma membrane purification. After radioactivity counting, the samples were assayed for the presence of the established intracellular enzyme markers (Solyom & Trams, 1972) G6Pase (endoplasmic reticulum) [assayed according to Baginski et al. (1968)] and MDH (cytosol and mitochondrial membranes) by standard methods using a commercially available kit (Sigma Chemical Co.). β-Glucuronidase was assayed according to Talalay et al. (1946).

Membrane Solubilization and WGA–Agarose Affinity Chromatography. The 5-mL plasma membrane fractions obtained from each cell line were made 0.5% (v/v) with Triton X-100 and extracted for 18–24 h at 4 °C. Insoluble plasma membrane components were pelleted by centrifugation (100000*g*, 60 min, 4 °C). The soluble plasma membrane components were diluted with an equal volume of buffer B containing 0.5% Triton X-100 (4 °C) and applied to a column containing 5 mL of agarose-immobilized WGA (Vector Laboratories, Burlingame, CA). After loading and elution of the unbound fraction (WGA-1) with buffer A containing 0.5% Triton X-100, the column was washed extensively with buffer A containing 3.0 M NaCl and 0.5% Triton X-100 to remove nonspecifically or weakly bound material (WGA-2). The specifically bound, *N*-acetylglucosamine-enriched fraction (WGA-3) was eluted with buffer A containing 0.5% Triton X-100 and 0.2 M *N*-acetylglucosamine. Excess detergent was removed from all samples by treatment with SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, CA) as described by Holloway (1973), and each fraction was then dialyzed exhaustively in 6000–8000 molecular weight cutoff dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) against distilled, deionized water and lyophilized.

Gel Electrophoresis Procedures. SDS–PAGE was carried out on 15% slabs according to Laemmli (1970) but with the stacking gel omitted. After electrophoresis, the gels were washed twice for 5 min in 10% ethanol, fixed overnight in 5% formaldehyde/25% ethanol (Steck et al., 1980), and silver stained as described by Switzer et al. (1979). Staining was stopped with Kodak rapid fixer, and the gels were washed thoroughly with distilled water.

Protein Concentrations. Protein concentrations were determined by the dye binding assay described by Bradford (1976).

Angiogenesis Assays. Samples were assessed routinely for angiogenic activity by implantation on the chick embryo chorioallantoic membrane (CAM assay). Selected samples were also assayed by implantation into the rabbit cornea. In the standard CAM assay used in our laboratory, 5-μL volumes of aqueous sample containing up to 50 μg of protein were applied to a minimum of five sterile Thermanox 15-mm disks (Flow Laboratories Inc., Rockville, MD) and allowed to dry under laminar flow conditions. These disks were inverted and applied to the CAM surface of 9-day-old chick embryos (Spafas, Inc., Norwich, CT) through 1–2 cm^2 "windows" cut through the shell on day 4. Eggs were viewed at 24-h intervals for 3 days through a Nikon stereoscope and scored as "positive" or "negative" on the basis of the presence or absence of blood vessel infiltration into the sample area.

Table II: Plasma Membrane Isolation from Monolayer HT-29 Cells[a]

cell fraction	protein (mg)	cpm ($\times 10^{-3}$)	G6Pase[b] (milliunits)	MDH[c] (milliunits)
homogenate	227	ND[d]	ND[d]	ND[d]
clarified homogenate	39	ND[d]	10800	3140
100000*g* pellet	8.2	370	6375	474
gradient fraction				
1	1.2	2	21	1
2	0.9	34	4950	73
3	1.0	390	30	0.2

[a] Starting material was 3 mL of packed cells. [b] Marker for endoplasmic reticulum. [c] Marker for cytosol and mitochondria. [d] Not determined.

The statistical significance of the CAM results obtained 54 ± 2 h after implantation was assessed by methods detailed elsewhere (Fett et al., 1985a). A significance level of ≤5% was required for a sample to be considered active.

Selected samples, judged to be positive in the CAM assay, were also tested in the rabbit corneal implant assay using a modification of established procedures (Langer & Folkman, 1976). Methylcellulose (4000 cP; Fisher Chemical Co., Pittsburg, PA) was autoclaved dry, then dissolved (2% w/v) in sterile distilled water, and mixed by stirring overnight at 4 °C. Fifty-microliter volumes of 2% methylcellulose were spotted onto a clean, dry mylar sheet (LKB, Gaithersburg, MD). The viscous hemispherical gels formed were then injected with 10-μL volumes of aqueous sample or control material, allowed to dry under laminar flow conditions to form clear pellets, and peeled from the mylar sheet. New Zealand white rabbits were anesthetized by intravenous administration of sodium nembutal (40 mg/kg). Local anesthesia in the area of the eyelids was attained by subcutaneous injection of 0.5–1.0 mL of 2% lidocaine hydrochloride. A 3–5-mm slit was made at the apex of the cornea, and a "pocket" extending to within 2 mm of the corneal limbus was produced with an iris spatula. Sample- or control-loaded 2% methylcellulose pellets, which rehydrate almost instantaneously upon contact with the corneal surface, were gently introduced into the corneal pockets by using an iris spatula, and then the eye was extensively irrigated with normal saline. Chloromycetin opthalmic ointment (1%) was applied to each eye, and the eyes were taped shut loosely to avoid drying and infection prior to the dissipation of anesthetic effects. The eyes were examined daily over a period of 14 days for the presence of infiltrating vessels, free of inflammation, that extend from the corneal limbus toward the gel implant.

RESULTS

Purification of HT-29 Plasma Membranes from Monolayer Cultures. The HT-29 cells were quite refractory to mechanical disruption by Dounce homogenization. Even after incubation in a hypotonic "swelling" buffer, more than 150 strokes with a tight-fitting pestle were required in order to approach 90% cell lysis, as monitored by phase-contrast microscopy. Nevertheless, this means of homogenization has been continued to be employed. It maintains organelle integrity during cell lysis, thus minimizing contamination of the desired plasma membranes with mitochondrial and nuclear membrane components.

Differential centrifugation then clarified cell homogenates and effectively eliminated intact cells, nuclei, and other organelles from the crude membrane pool. The total membrane fraction was then pelleted from the clarified homogenate by ultracentrifugation at higher *g* values. Highly purified plasma membranes were obtained by further centrifugation using a sucrose step gradient, based upon the high specific density displayed by plasma membranes (Warren & Glick, 1969; Neville, 1975). Purity and yield of the plasma membranes were assessed by monitoring both the distribution of cell-surface-labeled ^{125}I and the specific activities of the established intracellular marker enzymes G6Pase (endoplasmic reticulum) and MDH (cytosol and mitochondria) during isolation (Solyom & Trams, 1972). As shown in Table II, gradient fraction 3, the densest material, which pelleted through 45% sucrose, contained 92% of the ^{125}I with <0.6% of the total G6Pase and <0.3% of the total MDH, an indication of the high purity of the plasma membranes.

Table III: Comparison of Monolayer- and Suspension-Grown HT-29 Cells

plasma membrane fraction	monolayer[a]		suspension[b]	
	5% dose[c] (ng)	protein (μg/mL cells)	5% dose[c] (ng)	protein (μg/mL cells)
Triton X-100 extracted plasma membrane	385	53	360	76
WGA-1	9200	49	7850	66
WGA-2	ND[d]	4	ND[d]	9
WGA-3	0.2	0.2	0.2	0.4

[a] Starting material was 3 mL of packed cells. [b] Starting material was 500 mL of packed cells. [c] Minimal quantity required for positive CAM response ($p \leq 0.05$). [d] Not determined.

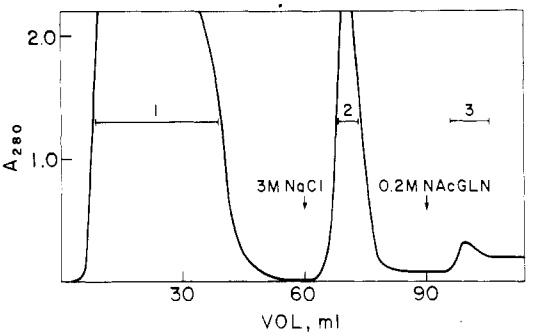

FIGURE 1: Affinity chromatography on agarose-immobilized WGA. The 100000*g* supernatant of Triton X-100 solubilized plasma membranes from HT-29 cells grown as monolayer cultures (150 μg of protein in 3 mL of buffer A with 0.5% Triton X-100) was applied at 15 mL/h to a 5-mL bed volume of gel. Three-milliliter fractions were collected. After a stable base line was reestablished, the column was washed with 50 mL of buffer A containing 3.0 M NaCl and 0.5% Triton X-100 (WGA-2). Buffer A containing 0.5% Triton X-100 and 0.2 M *N*-acetylglucosamine was then applied to the column to elute fraction WGA-3.

Solubilization and Lectin Fractionation of Angiogenic Activity. HT-29 plasma membranes from several preparations were pooled and extracted with 0.5% Triton X-100 in buffer B for 18–24 h at 4 °C, after which insoluble material was pelleted by ultracentrifugation at 100000*g* and discarded. After detergent extraction, the solubilized plasma membrane fraction obtained was tested for angiogenic activity by employing the chick CAM. Protein concentrations ≥10 μg/egg were lethal, due to an apparent toxic effect of the sample on the developing embryos. After dilution to protein concentrations below 1–2 μg/egg, however, this effect was not apparent, and the material was active (at the 5% significance level) to concentrations as low as 300–400 ng/egg (Table III, columns 2 and 3). The solubilized plasma membrane components were then applied to a WGA column to isolate glycosylated molecules containing *N*-acetylglucosamine (Figure

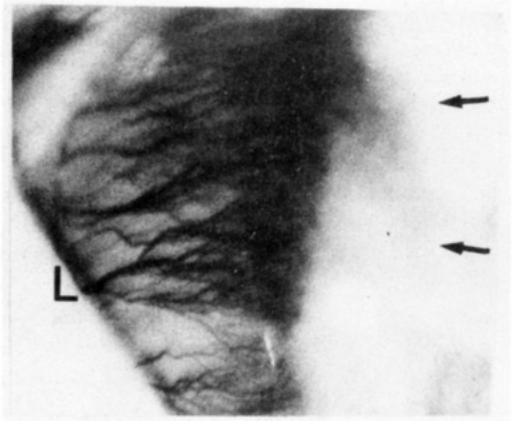

FIGURE 2: Angiogenic response produced by ~2 ng of WGA-3 in the avascular stroma of the rabbit cornea after 9 days. Newly formed vessels extend from the corneal limbus (L) toward the sample implantation site (arrows).

1). The unbound (WGA-1) fraction, representing 95% of the starting material, contained measurable though low TAF activity. Approximately 10 μg of this material was required to elicit a significant positive response (Table III, columns 2 and 3). Nonspecifically bound material was washed from the column with 10 volumes of buffer A containing 3.0 M NaCl and 0.5% Triton X-100. This material (WGA-2) was not active consistently on the chick CAM and, hence, was discarded. The bound material (WGA-3) which was eluted specifically with 0.2 M *N*-acetylglucosamine was reproducibly highly potent, including a neovascular response at or below the 5% significance level on the chick CAM at concentrations as low as 0.2 ng/egg (Table III, columns 2 and 3). The strong angiogenic response produced by 2 ng of WGA-3 in the avascular stroma of the rabbit cornea after 9 days is shown in Figure 2. WGA-3 was heterogeneous when examined by SDS–PAGE and silver staining.

Membrane-Associated Angiogenesis in Other Human Cell Lines. Six additional colon tumor lines, two noncolon tumor lines, and two lines of normal human embryonic lung fibroblasts were grown in monolayer culture, processed as described above, and assessed for the presence of plasma membrane associated angiogenic activity. Unlike the HT-29 line, these cells were all disrupted easily by Dounce homogenization, none requiring more than 50 strokes to attain ≥90% cell lysis. For each cell line, 100 μL–1.0 mL aliquots of materials generated at each step of the purification procedure were held for assessment of angiogenic activity in the chick CAM. The amount of material necessary for a positive angiogenic response at the 5% significance level was determined by limiting dilution from a maximum of 50 μg/egg. Indeed, angiogenic activity can be detected on and partially purified from the plasma membranes of each of the tumor lines investigated (Table IV). In all cases, the lectin-bound fraction requires a lower dose to achieve significance, and the lectin-unbound fraction a higher dose, than does the starting material, indicating that immobilized WGA provides an effective affinity matrix for the purification of such angiogenic entities from human tumors. In contrast, no activity could be detected in any of the plasma membrane-derived and lectin affinity-purified fractions prepared from the human diploid fibroblast cell lines WI-38 and WI-26, used as normal controls, even at microgram levels of protein.

Angiogenic Activity of Plasma Membranes Purified from HT-29 Suspension Cultures. For large-scale preparations, nitrogen cavitation replaced Dounce homogenization for the mechanical disruption of intact cells. Up to 2 L of cell suspensions (5×10^7 cells/mL) is equilibrated in a 100% nitrogen atmosphere under pressures up to 1500 psi. After about 1 h, the cells are saturated with nitrogen. When the pressure is rapidly released through a narrow (200-μm) aperture, the nitrogen gas expands and forms intracellular bubbles which rupture the relatively inflexible plasma membrane. The degree of disruption of cells and organelles is related both to the pressure attained and to the period over which the pressure is released. An optimal scheme for the HT-29 cell line required exposure to 1100 psi of nitrogen for 60 min at 4 °C and release of pressure over 10 min. This method proved to be effective for efficient and reproducible disruption of large quantities of cells which maintained nuclear/organelle integrity while keeping volumes within practical limits. Table III compares the protein recovered and the CAM activity of fractions derived from the purified plasma membranes of HT-29 cells grown in monolayer and suspension cultures. For the two sources, both the yield of plasma membrane per milliliter of packed cells and the minimum dose required to attain a 5% significance level of all fractions examined were similar.

Table IV: Angiogenic Activity of Human Cell Lines on the Chick CAM[a]

cell line	5% doses[b] (ng)		
	Triton X-100 extract	WGA-1	WGA-3
HT-29	385	9220	0.2
WiDr	370	7000	0.5
COLO 320 DM	2000	8600	220
COLO 320 HSR	1850	6000	250
COLO 205	500	3000	1
COLO 201	585	5200	12
SW 480	525	2400	26
A 549	400	1200	10
RD	385	4500	80
WI-38	NA[c]	NA[c]	NA[d]
WI-26	NA[c]	NA[c]	NA[d]

[a] Membrane fractions were prepared from cells grown in monolayer culture by using procedures described under Experimental Procedures. [b] Minimum quantity required for positive CAM response ($p \leq 0.05$). [c] Not active at 25 μg/egg or lower. [d] Not active at 1 μg/egg or lower.

Discussion

There is now ample clinical and experimental evidence linking the rapid growth of solid neoplasms to their ability to induce angiogenesis (Folkman & Cotran, 1976; Auerbach, 1981; Gullino, 1981). Containment of neoplasia through specific control of tumor angiogenesis is a therapeutic concept that has led to a number of efforts to identify and isolate the factor(s) involved in tumor neovascularization [see Vallee et al. (1985) and references cited therein]. The starting materials in which angiogenic activity has been detected and, in some cases, reported to be isolated have varied from homogenates of solid tumors and tumor cell suspensions maintained as ascites or in tissue culture to tumor-conditioned medium. The tumor cell plasma membrane has not been examined specifically for the presence of molecules exhibiting angiogenic activity, although many of the characteristics by which transformed and normal cells can be distinguished are reflected in differences exhibited by the cell surfaces (Yogeeswaran, 1980; Hakomori & Kannagi, 1983). Moreover, within solid tumors, direct cell–cell contact between capillary endothelial and tumor cells is well established (Folkman & Cotran, 1976). Thus, it seemed reasonable that certain angiogenic activity might be expressed on the plasma membranes of tumor cells.

In establishing the cellular disruption techniques, special attention was paid to the maintenance of nuclear and lysosomal

integrity. When the harshest procedure for HT-29 cells, i.e., Dounce homogenization, was used, greater than 91% of the nuclei were recovered in the 18000*g* homogenate pellet as monitored by phase-contrast microscopy. In addition, β-glucuronidase activity, a marker of lysosomal enzymes, was not detected either in the 18000*g* supernatant following homogenization or in the purified membranes (gradient fraction 3) obtained after isopycnic centrifugation (data not shown). Activity was, however, recovered in the 18000*g* homogenate pellet, indicating the presence of intact lysosomes in this fraction separated from the crude membranes.

Highly purified plasma membranes were obtained from the human colonic carcinoma cell line HT-29. This cell line was chosen for our initial studies since colon tumors constitute a major fraction of cancer deaths and have been particularly refractory to all forms of treatment except surgery (Carter, 1976; Silverberg, 1979). Purity of the plasma membranes was established by the absence of specific enzyme activities which served as markers for cytosol, endoplasmic reticulum, and mitochondrial membranes. The yield of plasma membranes was estimated on the basis of the recovery of cell-surface-iodinated material in the plasma membrane pellet. The highly purified plasma membrane fraction was extracted with the nonionic detergent Triton X-100 to solubilize membrane-associated macromolecules. Affinity chromatography on immobilized WGA resulted in material enriched in *N*-acetylglucosamine-containing macromolecules which are probably expressed on the cell exterior surface. This material, which contained numerous bands visible by silver staining after SDS–PAGE, was highly angiogenic when assayed on the chick CAM. Dose response studies indicated that as little as 0.2 ng of protein consistently elicited significant angiogenic activity. Similarly, after the implantation of 1–2 ng of this material, invasion of new vessels into the rabbit cornea was observed (Figure 2).

The plasma membranes from eight other human tumor lines including colon and lung carcinomas, and a rhabdomyosarcoma, were isolated in order to evaluate the general validity of this observation. Membrane-associated macromolecules from each tumor source solubilized with detergent gave similar patterns of activity (at the 5% significance level) when assayed on the chick CAM at concentrations of 0.37–2.0 μg/egg (Table IV). More importantly, the corresponding WGA-bound material consistently induced significant angiogenesis when applied at substantially lower concentrations ranging from 0.2 to 250 ng/egg. In contrast, the purified plasma membranes and lectin-bound material isolated from a limited number of normal diploid fibroblast lines by using identical protocols failed to elicit any angiogenic activity, even when assayed at microgram levels (Table IV). Thus, WGA chromatography provides an effective affinity step for the isolation of membrane-associated angiogenesis activity from human tumor cells.

The low yields of membrane-associated angiogenic material isolated from the quantities of tumor cells obtainable by conventional in vitro techniques necessitated a substantial increase in the quantity of available starting materials before further purification could be attempted. This was achieved by the development of large-scale (100–1000-L) suspension cultures of mammalian cells (Feder & Tolbert, 1983). Such cell culture facilities have proven invaluable in our efforts to isolate secreted angiogenic molecular species from both rat and human sources (Vallee et al., 1985; Fett et al., 1985b).

Large-scale isolation of purified HT-29 plasma membranes should now be possible. Nitrogen cavitation procedures permit the disruption of hecto- to kilogram quantities of cells while retaining nuclear integrity and keeping volumes within the limits acceptable for further processing. Indeed, cells can be disrupted efficiently at only a 1:4 dilution of packed cells, allowing up to 0.5 kg of cells to be disrupted in a single pass through the 2-L capacity nitrogen cavitation chamber. This effectively increases the yield of plasma membranes several hundredfold. Importantly, the angiogenic activity of the plasma membranes obtained in this manner is comparable to that of small-scale monolayer cultures (Table III). Thus, it is now possible to generate microgram rather than nanogram quantities of angiogenic WGA-purified material, allowing further biochemical characterization to proceed.

The clear demonstration of potent angiogenesis activity associated with the tumor cell plasma membrane has a number of mechanistic implications. Almost since its existence was first postulated, TAF was suggested to be a diffusible factor based on the observations that tumor cels implanted within microfiltration chambers, which do not allow direct cell–cell contact, still induce host neovascularization (Ehrmann & Knoth, 1968; Greenblatt & Shubik, 1968; Gitterman & Luell, 1969). Moreover, tumor tissues implanted in the rabbit eye up to 2 mm from the corneal limbus and up to 5 mm from the iris still induce ingrowth of vessels from these vascular beds, consistent with induction by diffusion (Folkman & Cotran, 1976). In addition, molecules exhibiting angiogenic activity have been purified partially from cell-free conditioned medium of a number of tumor cell lines (Vallee et al., 1985), and more specifically from HT-29 conditioned medium (Fett et al., 1985a,b).

However, one characteristic of tumor cells, but not of normal cells, is their ability to continually release membranous material into the local environment, either directly as soluble products or indirectly in the form of microvesicles, a phenomenon termed "shedding" (Black, 1980). Consequently, certain extracellular angiogenic activity may represent plasma membrane-derived products. Such membrane-associated molecules may serve a role different from and perhaps complementary to those that are secreted. For example, maintenance of the tumor vasculature at the periphery of or perhaps within the substance of large, established tumors may depend more upon direct tumor cell–endothelial cell contact.

Although the angiogenic factors under study are not yet fully characterized, their association with the plasma membrane may provide clues as to their nature. A large body of research now implicates abnormal glycolipids in the maintenance of the tumorigenic state [for a review, see Hakomori & Kannagi (1983)]. It is conceivable that one class of angiogenically active molecules is a family of membrane-derived glycolipids, and this may account for a number of the unusual chromatographic properties that have been observed by others, while explaining the difficulties which have been experienced in their characterization (Weiss et al., 1979; Brown et al., 1980; Fenselau et al., 1981).

The presence of a membrane-associated angiogenic activity also has therapeutic implications. It is clear that an antibody or other antagonist to a membrane-associated molecule that mediates such activity might not only inhibit tumor growth at the level of the developing vasculature but also bind to the tumor cell surface and promote antibody-mediated cytotoxicity. Such a bifunctional antibody might prove particularly effective as an inhibitor of tumor angiogenesis.

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Properties of Red Cell Membrane Proteins: Mechanism of Spectrin and Band 4.1 Interaction†

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ABSTRACT: Interactions between human red cell's band 4.1 and spectrin were studied by fluorescence resonance energy transfer and batch microcalorimetry techniques. The association constant (K_a = 8.6 × 10^7 M^{-1}), the stoichiometry (one molecule of band 4.1 to one molecule of spectrin), the reversibility, and the enthalpy (ΔH = −6 kcal/mol) were determined. A proton uptake was observed to take place as a result of the spectrin–band 4.1 complex formation. In addition to the protonation of the reaction products, the entropic contribution ($-T\Delta S$) has been observed to be responsible for approximately 50% of the binding free energy. We concluded that the environment plays a significant role in the stabilization of the complex. Since band 4.1 has been required for the maintenance of the cytoskeletal stability, small alterations of the binding energies or the degree of interaction could have a pronounced effect on the structure of the erythrocyte membrane.

Although the molecular organization of the human red cell membrane has been a center of intensive research, several fundamental gaps in the understanding of what makes cells both elastic and durable still remain unfilled. In the process of studying structure and function of mammalian erythrocytes, it has become clear that the membranes' shape and mechanical properties are defined by a network of cytoplasmic proteins, named a cytoskeleton [see reviews by Steck (1974), Marchesi et al. (1976), Lux (1979), and Branton (1981)]. Since then, attempts to elucidate the structure of the erythrocyte mem-

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