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The Multicomponent Nature of Teratoma
Cell Adhesion Factor

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ABSTRACT

THE MULTICOMPONENT NATURE OF TERATOMA CELL ADHESION FACTOR

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The complex nature of the teratoma cell adhesion system has been demonstrated. Fractionation of crude ascites fluid on a DEAE cellulose ion exchange column made it possible to show that two or more components are involved in teratoma adhesion factor (TAF) activity. Glycoproteins in fractionated ascites fluid were localized on polyacrylamide gels. The possible role of these glycoproteins in teratoma cell adhesion and current hypotheses on the mechanism of carbohydrate involvement in intercellular adhesion are discussed.

INTRODUCTION

In recent years the role of the cell surface in morphogenesis and development has received an enormous amount of attention. One of the most important areas is the research that has been and is being done on cell adhesion, cell movement and cell recognition.

Insight into the problems of cell adhesion and cell recognition has been greatly facilitated by the discovery and use of cell adhesion or cell aggregating factors (AF). Generally, AFs are washed or chemically removed from the cells of a tissue. When AF is added back to a suspension of single cells, it causes the cells to aggregate. Most AFs show a specificity for the type of cells they will aggregate: i. e. , sponge AFs will preferentially aggregate cells from the same species of sponge; AFs obtained from organs or tissues (i. e. , embryonic chick liver or embryonic chick neural retina) will preferentially aggregate cells derived from that specific organ or tissue. On the basis of such findings AFs are referred to as species specific or tissue specific.

AFs have been isolated from a number of sources: Humphreys (1963) and Moscona (1963) have isolated a species specific AF from the sponge Microciona prolifera; Lilien and Moscona (1967) have isolated a tissue specific AF from embryonic chick neural retina; Kuroda (1968) has isolated a tissue specific AF from embryonic chick liver; Rosen et al. (1973) have isolated a species specific AF from the cellular slime mold, Dictyostelium discoideum, and

Oppenheimer and Humphreys (1971) have isolated a preferentially cell type specific AF from mouse teratoma. Of these AFs the neural retina AF (Mc Clay and Moscona, 1974), the M. parthena AF (Henkart, et al., 1973), the M. prolifera AF (Margoliash et al., 1965) and the D. discoideum AF (Simpson, et al., 1974) have been purified.

Purified or fractionated AF preparations will undoubtedly be of great help in this area of research. The purified AFs will suggest to us the nature of molecules which are involved in cell adhesion and recognition and hopefully they will also help in the elucidation of the mechanism by which these molecules mediate cell adhesion and recognition.

This report will be specifically concerned with an AF similar to that isolated by Oppenheimer and Humphreys (1971) from male 129/J mice bearing ascites teratoma tumors. This is a unique system because teratoma cell adhesion factor (TAF) is somewhat cell type specific and is the only AF that has been isolated from a cancerous tissue in vivo. TAF is also unique because it holds the potential of providing a greater understanding of tumor cell surfaces and abnormal tumor cell adhesion and recognition.

Previously, TAF has been obtainable only in the crude ascites fluid form from the mouse. A method will be described here for the fractionation of crude TAF using DEAE cellulose ion exchange chromatography.

Materials and Methods

Teratoma Cell Line. Teratoma cells (Oppenheimer et al.,

1969) were obtained in 1966 from Dr. Leroy Stevens of the Jackson Laboratory. The cell line has been maintained by intraperitoneal passage in young male 129/J mice. Teratoma cells were grown for 5-10 days, after which time the mice began to show abdominal bloating. Mice were sacrificed by cervical dislocation to obtain both teratoma cells and teratoma adhesion factor (TAF). The contents of the peritoneal cavity were removed, placed in 15ml conical glass centrifuge tubes and centrifuged for 5 minutes at 180xg in an International Clinical Centrifuge (rotor #221). Ascites fluid was removed and centrifuged at 3000xg for 10 minutes at 4C in a Sorvall RC2-B Superspeed Centrifuge (Type SS-34 rotor). The supernatant from this centrifugation constituted the crude TAF preparation used in these experiments.

Aggregation Assay. Teratoma cells were washed three times in Hepes-Saline buffer (HS) and suspended in HS. $1-2 \times 10^6$ cells/ml were incubated with TAF at 37C for 15 minutes in capped 1 dram vials on a gyratory shaker (66rpm, 4 5/8 inch diameter of rotation). Vials were diluted with 10ml of "Isoton" (isotonic buffered saline; Coulter Electronics, Hialeah, Florida), and the disappearance of single cells determined using an electronic particle counter (Model 112 LT Celloscope, Particle Data, Inc., Elmhurst, Ill.). Details of the electronic particle counter method used in this aggregation assay have been published by Oppenheimer and Odencrantz (1972). Aggregation at any given concentration (vol/vol) of TAF is recorded as the percent disappearance of single cells from suspension. Only TAF preparations that aggregated 70% or more single cells at 5%

(vol/vol) TAF were used in these experiments.

Chromatography. Diethylaminoethyl (DEAE) cellulose, capacity 1.0meq/gm medium mesh (Sigma), was prepared according to the procedure described by Peterson (1970). After final washing DEAE cellulose was suspended in 0.01M Tris-HCl buffer at pH 8.6. All column work was carried out in a refrigerator at 4C. A glass column, 0.9cm x 25cm, fitted with a porous glass support disc and Teflon stopcock (Fisher and Porter Co.) was filled to a height of 15cm. An excess of two column volumes of 0.01M Tris-HCl (pH 8.6) was used to equilibrate the column. A 1.0ml sample of crude TAF was layered onto the top of the cellulose bed and 1.0ml fractions were collected (Golden Retriever Pup Model 1100 fraction collector, Instrumentation Specialties Co., Lincoln, Nebraska). After the sample had entered the column, elution was started with a 10ml volume of 0.01M Tris-HCl, pH 8.6. The 0.01M buffer was followed by 10ml volumes of 0.1M, 0.3M, 0.5M, 0.6M, 0.7M and 0.9M Tris-HCl buffer, pH 8.6, unless otherwise stated. Elution was by gravity flow in all column runs. The flow rate was approximately 60ml/hour with a slight decline in rate at high buffer concentrations (0.7M - 0.9M). Protein elution profiles were determined by reading absorbance at 280nm using quartz spectrophotometer cells (Lightpath Cells, Inc., Saint Louis, Missouri) in a Beckman DB-G grating spectrophotometer. Aggregation promoting activity of all column fractions was tested at 50% (vol/vol), unless otherwise stated.

Polyacrylamide Gel Electrophoresis. Five percent polyacrylamide (Bio-Rad Laboratories) gels were prepared in 0.188M Tris

Glycine buffer (pH 8.9) using the procedure of Weber et al. (1970) with the following variations: 0.04ml N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories) and 1.0ml of ammonium persulfate (15mg/ml) (Bio-Rad Laboratories) were added instead of the suggested amounts (0.045ml and 1.5ml respectively). This was done in order to slow down the rate of polymerization of the acrylamide and provide more uniform gels. Gels were poured to a height of 13cm in glass tubes (7mm inside diameter, 15cm length). Electrophoresis was carried out in a refrigerator at 4°C with 0.094M Tris-0.094M Glycine pH 8.9 reservoir buffer. Current was regulated with a D.C. power supply (Buchler Instruments, Nuclear-Chicago; Chicago, Illinois). Gels were preelectrophoresed for one hour at 2ma/gel to remove excess catalyst and impurities in the gels. The upper tray reservoir buffer was poured off to allow direct application of the samples to the top of the gels. DEAE cellulose column fractions were dialysed against two 4-liter changes of 0.1M Tris-HCl, pH 8.6 buffer. Protein content of all samples was determined by the Lowry method (Lowry, et al., 1951) and adjusted to 10µg/50µl with 0.1M Tris-HCl buffer (pH 8.6). Ten micrograms of protein was loaded onto each gel in a 50µl volume unless otherwise stated. Buffer was carefully layered over the samples with a capillary pipet and the upper tray reservoir buffer was poured back. Samples were allowed to enter the gels under a loading current of 1ma/gel for 15 minutes. The current was increased to 2ma/gel and electrophoresis continued for 45 minutes to one hour. The end of each run was determined by emergence of a bromophenol blue tracking dye from the bottom of the

gels. Gels were removed from the tubes using a 12cc syringe filled with water and fitted with a 2-inch 22-gauge needle. Gels were stained for 20-60 minutes with Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad Laboratories): 1.25 gm dissolved in 227 ml methanol, 46ml glacial acetic acid and 227ml of water. Destaining was accomplished by diffusion in 7% acetic acid. To accelerate destaining, gels were put into perforated plastic tissue culture tubes emersed in a large beaker of 7% acetic acid and placed on a magnetic stirrer. Gels were also stained for carbohydrate using the periodic acid Schiff's (PAS) staining method as described by Kapitany and Zebrowski (1973). The gels were photographed using Kodak Plus-X pan film and a 35mm camera fitted with a close-up lens.

Ultrafiltration. Ultrafiltration experiments were carried out with an Amicon 8MC micro-ultrafiltration system (Amicon Corporation, Lexington, Mass.). Diaflo membranes with molecular weight cutoff ranges of 50,000, 100,000 and 300,000 daltons were used (XM50, XM100A and XM300, respectively).

Results

DEAE Cellulose Column Chromatography. Stepwise elution with increasing concentrations of Tris-HCl buffer (pH 8.6) produced similar protein elution profiles in eight DEAE cellulose column runs of the teratoma adhesion factor. Six protein peaks were consistently obtained (Figure 1). In the initial experiment protein peak #1 contained 37% aggregation promoting activity when assayed at 50% (vol/vol) fraction to cells. In six of seven subsequent experiments column fractions showed no significant amount of aggregation. The

Figure 1. Fractionation of crude ascites fluid using DEAE cellulose column chromatography and the aggregation promoting activity of individual column fractions. Protein (—) was determined by reading absorbance at 280nm; absorbance due to the eluting buffer was subtracted from each reading. Each fraction was assayed at 50% (vol/vol) cells for TAF activity (· · · · ·).

Column dimensions: 0.9cm x 15cm

Eluting buffer: 0.01M - 0.9M Tris-HCl, pH 8.6

Flow rate: 40-60ml/hr

only other instance of aggregation also involved protein peak #1. No other protein peak showed aggregation activity by itself. Therefore, it was assumed that molecules required for aggregation were present in peak #1. The lack of consistent activity in peak #1 suggested that components from other protein peaks might also be involved. To test this the fractions in protein peak #1 were pooled and combined 1:1 with the remaining column fractions and assayed for aggregation promoting activity. Figure 2 shows that combination of protein peak #1 with protein peak #5 or with peak #6 gave 60% aggregation when assayed at 50% (vol/vol) combined fractions to cells.

The aggregation promoting affect of different Tris-HCl buffers on cells alone and on protein peak #1 is shown in Figure 3. Eight percent aggregation can be attributed to the affect of buffer on cells and about 12% aggregation to the affect of buffer on the aggregation promoting activity of protein peak #1. 100-200 μ g/ml bovine serum albumin (BSA) was added to protein peak #1 to see if a non-specific protein interaction was responsible for the increased aggregation promoting activity found in recombined column fractions (peak #1 recombined with peaks #5 and #6). The level of aggregation was below that of controls even when up to 200 μ g/ml BSA was added.

To determine the relationship between recombined column fractions and the resulting increase in aggregation promoting activity, varying amounts of protein peaks #5 and #6 were added to peak #1 and assayed for activity. Figure 4 shows that when the ratio of peaks #1/#5 and #1/#6 is as high as 0.9, 90% aggregation is obtained. The aggregation promoting activity stays above 90%

Figure 2. Aggregation promoting activity in recombined DEAE cellulose column fractions. The fractions in protein peak #1 were pooled and mixed 1:1 with each column fraction. Recombined fractions were assayed for TAF activity at 50% (vol/vol) with cells.

Figure 3. The effect of buffer concentration on the aggregation promoting activity of protein peak #1 and on cells alone. Buffer plus protein peak #1 (o) and buffer alone (Δ) were assayed at 50% (vol/vol) with cells.

Figure 4. Aggregation promoting activity of recombined column fractions from protein peaks #1, #5 and #6. The ratio of peak #1/peaks #5 and #6 was decreased by starting with peak #1 alone and increasing the amount of peak #5 and #6. The various ratios of recombined fractions were assayed at 50% (vol/vol) with cells.

aggregation as the ratio of peaks #1/#5 and #1/#6 is lowered to 0.6. Any further decrease in the amount of protein peak #1 (ratios 0.5-0.1) results in a decline of aggregation promoting activity (Figure 4). The decline may be due to the dilution of peak #1 by peaks #5 and #6. Figure 4 suggests that protein peak #1 contains the primary component(s) responsible for activity, and that peaks #5 and #6 contain a component(s) that, at low concentrations (ratio 0.9), has a dramatic enhancing affect on the aggregation promoting activity of protein peak #1.

Units for aggregation promoting activity were defined to determine if an increase in specific activity was obtained with the DEAE cellulose column procedure. Unit activity is defined as the amount of protein that will aggregate 50% of the cells in a standard cell suspension in 15 minutes. The specific activity of crude ascites fluid is between 1.15 and 1.35 units/mg protein. In column runs having all the aggregation promoting activity in protein peak #1, the specific activity is 1.07 units/mg protein. The specific activity in recombined column fractions (peak #1 with peaks #5 and #6) is 2.50 units/mg protein. The relatively small increase in specific activity suggests that little purification of TAF is obtained with the DEAE cellulose column procedure. A true determination of the specific activity may be complicated by the fact that more than one molecule is involved in the activity of TAF. The existence of multiple components involved in TAF activity and the low specific activity obtained after the DEAE cellulose column procedure indicate that the teratoma cell adhesion system is complex.

Figure 5. Polyacrylamide gels showing the protein and glycoprotein bands from crude ascites fluid (d, e) and protein peaks #1 (c), #5 (b) and #6 (a). Gels a-d were stained for protein with the CBB staining method. The PAS staining method was used to stain gels for glycoprotein. The PAS positive bands in a-d are indicated by a dash (-).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out on crude ascites fluid and DEAE cellulose column fractions from protein peaks #1, #5 and #6. Two gels were run under identical conditions for each sample. One gel was stained for protein (CBB staining method) and the parallel gel stained for glycoprotein (PAS staining method). The protein and glycoprotein (PAS positive) bands are shown in Figure 5. Crude ascites fluid has 10-12 distinct protein bands and 4-5 PAS positive bands (Figure 5d and 5e). Protein peak #1 (Figure 5c) has 7-8 distinct protein bands; one of these bands which is located in a high molecular weight region of the gel is PAS positive. Protein peaks #5 and #6 both have five distinct protein bands; peak #5 has one PAS positive band in a low molecular weight region of the gel while peak #6 has no PAS positive bands. The relationship between TAF activity and these protein and glycoprotein bands is discussed below.

Micro-ultrafiltration. Micro-ultrafiltration experiments were carried out to obtain an estimate of the molecular weight of molecules involved in TAF activity. All aggregation promoting activity remained in the retentate using the XM300 membrane filter. The Amicon XM300 membrane filter is the largest presently available and will retain molecules above 300,000 molecular weight. This suggests that molecules involved in TAF activity have a molecular weight greater than 300,000 daltons.

Discussion

The complex nature of the teratoma cell adhesion system has been demonstrated. DEAE cellulose anion exchange column chroma-

tography suggests the involvement of more than one component in TAF activity (Figures 1, 2 and 4). One component, perhaps the primary component, is eluted from the DEAE cellulose column in the starting buffer. On that basis it can be inferred that this component (or components) is either neutral or cationic. Another component involved in TAF activity is eluted from the DEAE cellulose column only at high buffer concentrations, suggesting that this component is highly anionic. Whether or not one of these components is a subunit of the other has not been determined. These results suggest that more than one component may be involved in TAF activity.

Glycoproteins, glycolipids and polysaccharides have been postulated to be the mediators of intercellular adhesion (Oppenheimer et al., 1969; Oppenheimer and Humphreys, 1971; Oppenheimer, 1973 and Oppenheimer, 1975). The functional involvement of specific carbohydrate has been demonstrated in the teratoma cell adhesion system (Oppenheimer, 1975). In view of this, glycoproteins in different DEAE cellulose column fractions were localized in polyacrylamide gels. Protein peak #1 was found to have only one glycoprotein (PAS positive) band; furthermore, this PAS positive band was located in a high molecular weight region of the gel. These facts and the fact that protein peak #1 was found to have aggregation promoting activity by itself suggest that this glycoprotein band may contain one of the molecules involved in TAF activity. Protein peak #5 also contains a PAS positive band, but this band is located in a low molecular weight region of the gel. Whether or not this band

is a subunit of the large molecular weight glycoprotein band found in protein peak #1 is not known. Protein peak #6 does not contain any glycoprotein bands.

The most recent hypothesis concerning the mechanism by which complex carbohydrates are involved in intercellular adhesion has been published by Roseman (1970). This hypothesis has been referred to as the "Enzyme-Substrate" hypothesis; according to this model the binding of a carbohydrate substrate (glycoprotein, glycolipid or polysaccharide) on one cell surface is brought about by an enzyme (glycosyltransferase) on an adjacent cell surface. When the appropriate sugar nucleotide is present, the sugar residue is added to the end of the substrate carbohydrate chain and the bond between the cells is broken. It is possible that many different glycosyltransferases and carbohydrate substrates could be involved on the cell surface at any one time. One can readily see that the synthesis of new glycosyltransferases or the presence of the required sugar nucleotides could change the adhesive properties of a cell during development.

In some aggregation experiments with the teratoma cell adhesion factor, spontaneously dissociating cell aggregates have been observed. Dissociation of the aggregates could be due to glycosylation of TAF; once this glycosylation takes place the adhesive bonds between cells would be broken and the aggregates would dissociate. These results could be interpreted to suggest that TAF acts as a carbohydrate substrate for glycosyltransferases on the cell surface.

Another more general hypothesis dealing with the mechanism of differential cell adhesion and cell recognition is the "Cell-Ligand" hypothesis (Moscona, 1968). This hypothesis suggests that cell adhesion and recognition are mediated by the interaction of cell surface cementing components with cell surface receptor sites. Glycoprotein complexes are assumed to be the cell surface components involved and are referred to as cell ligands. According to the hypothesis, the degree of cell ligand and receptor site complementarity determines the degree of adhesiveness between two cells. TAF can be thought of as one such cell ligand which, after release from the cell surface, can be added back to a single cell suspension and cause aggregation.

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