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ISOLATION OF PLASMA MEMBRANE FRAGMENTS FROM CULTURED MURINE MELANOMA CELLS[†]

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The various manipulations involved in the isolation of membrane fragments from culture fluids of murine melanoma cells were examined to discern their effect on membrane fragment structure. Ultracentrifugation and gel chromatography were compared using the presence of marker enzymes and the sensitivity to a non-ionic detergent (Triton X-100). Fractionation of media by gel chromatography resulted in only one major form of membrane particles, while ultracentrifugation, followed by resuspension, produced at least two major populations from the identical material. These results indicate that the optimal procedure for membrane fragment isolation is fractionation by an agarose-containing gel, followed by concentration using PEG 20,000.

Cancer cells may release tumor-associated products, such as degradative enzymes, cell adhesion components, angiogenesis factors, and tumor-associated antigens (1,2,3). These tumor-associated components appear to be released more readily from highly metastatic tumors than from similar tumors of lower metastatic potential (4), suggesting that shedding of tumor products may enable tumor cells to escape host immune surveillance (5,6). These components can be shed from the cell membrane in soluble or in macromolecular form (as plasma membrane fragments) into the peripheral circulation of the host. The presence of certain tumor markers, plasma membrane-specific marker enzymes, and glycoprotein receptors, in the absence of cytosol components, suggests that membrane shedding occurs from viable cells (7). These plasma membrane fragments have been demonstrated in sera, cyst fluids and ascites fluids from patients with ovarian cancer, secretion fluids of villous adenoma of the rectum, and culture media of human fetal cells, HeLa cells, and various murine tumor cell lines (3,8-11).

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Although the relationship of membrane fragments to metastasis and escape from immune destruction has been considered (12,13), little emphasis has been directed toward their intact isolation. Since previous work was limited to biochemical and immunochemical characterization of these components after disruption of the membrane unit, alterations of the membrane fragment-structure by manipulations associated with separation (such as centrifugation and resuspension) were not considered (3,4,7,8). For studies on the possible functional aspects of membrane fragments (such as their interactions with immune cells), it is important to isolate the components with minimum alteration of their structure. Since such alterations might modify their apparent functions, this study examines the manipulations and conditions involved in membrane fragment isolation to discern the optimal procedure.

MATERIALS AND METHODS

Cell Culture. A murine melanoma line (B16-F10), which was kindly provided by Dr. I.J. Fidler (Fredrick Cancer Research Facility - NCI, Fredrick, Maryland), was used in this study. The cells were maintained in complete minimum essential medium (CMEM), consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (heat-inactivated), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 200 mM L-glutamine, 2XMEM vitamin solution, 100 μg/ml streptomycin and 100 IU/ml penicillin.

For study of serum-free conditions, the cells were added to dishes in the presence of 10% fetal bovine serum. After 48 to 72 hours, the cells were washed and incubated overnight in serum-free media.

Radiolabeling of Membrane Components. The melanoma cells were metabolically labeled with [^{3}H]galactose (11.5 Ci/mmol). Cells were incubated with media containing 1 μCi/ml for six hours at 37°C. After 6 hours, medium containing [^{3}H]galactose was removed and replaced with CMEM, except for serum-free cells to which CMEM without serum was added, for 24 hours. After 24 hours, the media were removed, centrifuged at 27,000 xg, and utilized for the separation techniques. Under serum-free conditions, the media from multiple dishes were pooled to normalize the shed material to equivalent cell numbers. The pooled media were concentrated by PEG 20,000 to achieve an equivalent volume.

Gel Chromatography. The medium, containing radiolabeled components, was chromatographed on either a Bio-Gel A-50m or a Bio-Gel A-150m (Bio-Rad Laboratories, Richmond, Calif.) column (1.5 x 50 cm), equilibrated with 10 mM phosphate-buffered saline (PBS), pH 7.4. Fractions (1.5 ml) were collected and 0.28 ml aliquots were counted for the presence of tritium.

Enzyme assays. All enzyme reactions were carried out at 37°C and cell homogenates were used as positive controls.

5'-Nucleotidase activity was examined by the procedure described by Frick and Lowenstein (14). Protein was denatured by 5% TCA (final concentration) and aliquots of the protein-free solution were removed for colorimetric determination of inorganic phosphate (P_i). The determination of P_i concentration utilized the reduction of ammonium molybdate with ascorbic acid (15). The (Na^+-K^+)-ATPase activity was assayed as previously described (16). Colorimetric determination of P_i was performed as above. Succinate-

cytochrome c reductase activity was determined by the method described by Tisdale (17). The molar extinction coefficient of 18.5 x 10^3 $M^{-1}cm^{-1}$ was utilized to calculate the activity. Acid phosphatase was determined by the procedure of Walter and Schutt (18), using a reaction mixture consisting of 10 mM p-nitrophenyl phosphate. The molar extinction coefficient of 18.3 x 10^3 $M^{-1}cm^{-1}$ was utilized to calculate the activity.

Protein determination. The level of protein was determined by the method of Lowry et al (19), utilizing a Sigma protein standard.

RESULTS AND DISCUSSION

Aliquots of media (1.5 ml), containing radiolabeled components, were applied to either Bio-Gel A-50m or Bio-Gel A-150m columns (1.5 x 50 cm) and 1.5 ml fractions were collected and examined for the presence of the radiolabel. The resulting profiles are presented in Figure 1. Using the Bio-Gel A-50m column, three peaks are demonstrated (the peak containing materials smaller than the lower exclusion limit, is not shown in any figure). Peak A is present in the void volume, while peaks B and C are retained by the gel. The presence of peak A in the void volume indicates a molecular weight exceeding 5 x 10^7 daltons. The profile, using Bio-Gel A-150m, is somewhat altered in that peak A is slightly retained from the calculated void volume and peaks B and C are not resolved as separate peaks. The retention of peak A suggests a molecular weight less than 1.5 x 10^8 daltons.

Various membrane marker enzymes were assayed to determine the source of the shed material. 5'-Nucleotidase and (Na^+-K^+)-ATPase were present only in peak A, while acid phosphatase and succinate-cytochrome c reductase activities were not detectable in the three peaks. 5'-Nucleotidase was examined throughout these studies to discern changes in the composition of the peaks.

Since some serum components may form complexes with membrane components (20,21), the presence of serum may produce artifacts. To insure that the "high molecular weight" material was not the result of a serum-mediated artifact, the release of materials from cells was examined in the absence of serum (Figure 2). Peak A appears unchanged in position, while peak B-C appears to be slightly shifted and diminished. These results indicate that peak A is not the result of complexes with serum components; however, the B-C peak may consist of some aggregates with serum components. Although the

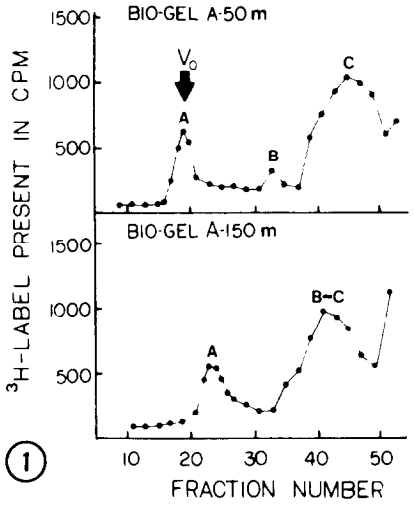
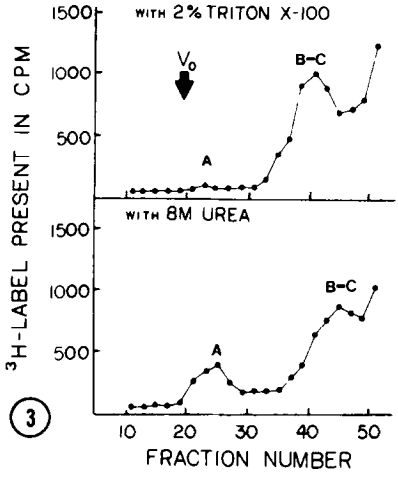


FIGURE 1: Profile obtained by fractionation of culture fluid (1.5 ml) applied to Bio-Gel A-50m (top) and Bio-Gel A-150m (bottom) columns. 1.5 ml fractions were collected and examined for ^{3}H-label.

FIGURE 2: Profile obtained by fractionation of serum-free media (1.5 ml) by a Bio-Gel A-50m column (top) and a Bio-Gel A-150m column (bottom). 1.5 ml fractions were collected and examined for the ^{3}H-label.

presence of serum does not affect the nature of released "membrane-like" material, it is critical to the cell's ability to proliferate and synthesize membrane constituents, both of which are correlated with membrane shedding (22,23).

To examine the nature of the materials in these peaks, disruptive agents were used to study the hydrophobic and ionic interactions of components within the shed material. The two agents used were urea (8 M) and Triton X-100 (2%) to examine ionic and hydrophobic interactions, respectively. Figure 3 presents the profiles resulting after fractionation of media treated by these agents as described in the figure legend. Treatment with Triton X-100 resulted in the elimination of peak A, but did not appreciably alter peak B-C. Previous studies demonstrated that plasma membrane fragments from ascites and cyst fluids of patients with ovarian cancer were characterized by their dissociation by Triton X-100 (3). This sensitivity is likely to be due to lipid-protein interactions and indicates that peak A also consists of lipid-protein associated material. In contrast to the limited Triton X-100 sensitivity, all peaks exhibited some sensitivity to urea treatment, indicating the presence of ionic interactions (such as protein-protein) in all peaks.



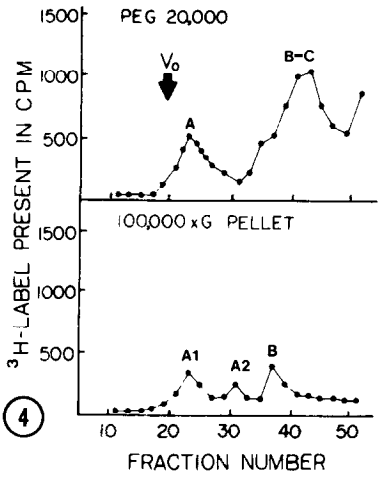


FIGURE 3: Fractionation profile of culture fluid treated with Triton X-100 (top) and urea (bottom) applied to Bio-Gel A-150m columns. Samples (1.5 ml) were either incubated with 2% Triton X-100 for 2 hours at 37°C or 8 M urea for 30 minutes at 37°C and applied to the columns.

FIGURE 4: Fractionation profile of media which has been concentrated by PEG 20,000 (top) and sedimented by centrifugation at 100,000xg (bottom) applied to Bio-Gel A-150m columns.

The suggestion that these membrane fragments are associated by hydrophobic interactions would indicate that this material is fragile, at least with respect to various manipulations such as high speed centrifugation, agitation, or sonication. The general approach used to isolate membrane fragments is pelleting by ultracentrifugation. This technique enables the concentration of shed material which appears in a dilute form in media from cultured cells to be studied. We have utilized PEG 20,000 concentration (combined with gel chromatography) as an alternate procedure. An aliquot of media was placed in dialysis tubing, with a molecular weight cutoff of 6,000 daltons and the sealed tubing was placed into a container of PEG 20,000. This concentration continued overnight at 4°C and the concentrated material was removed (in this case, the material was resuspended to its original volume with PBS). This sample (1.5 ml) was applied to a Bio-Gel A-150m column, as before. The resulting profile (Figure 4, top) is not significantly different from the one obtained with the sample applied directly without further treatment (Figure 1). A second aliquot of the original medium was centrifuged at 100,000xg for 1 hour and the pellet was washed with PBS and resuspended to its original volume by agitation with a Vortex mixer. This sample (1.5 ml)

was applied to a Bio-Gel A-150m column and the resulting profile (Figure 4, bottom) indicates the presence of 3 peaks: a peak in the peak A position (designated A1); a peak between peak A and B-C positions (designated A2, due to presence of 5'-nucleotidase); and a peak at the leading edge of the B-C peak (designated B, due to the absence of 5'-nucleotidase). The combined quantity of material in peaks A1 and A2 was similar to the amount usually seen in peak A, suggesting that, in addition to sedimenting material from peak B-C, centrifugation alone or in combination with resuspension causes the breakage of some membrane fragments. In a similar study, ultracentrifugation of isolated peak A material resulted in peaks A1 and A2, as well as a minor peak in the B-C position (data not shown). Due to this alteration in membrane fragment structure, any effect in a functional assay (for example, lymphocyte mitogenesis) cannot be adequately ascertained, since interpretation may be complicated by factors introduced in isolation procedures.

In summary, the membrane-like material, isolated from the void volume fraction of Bio-Gel A-50m columns and from a peak which appears to be slightly retained by Bio-Gel A-150m columns, is sensitive to solubilization by Triton X-100, in contrast to the material in the other peaks, and possesses plasma membrane marker enzymes. Ultracentrifugation and resuspension of membrane fragments produced at least two major forms of membrane particles, indicating that the centrifugation approach to membrane fragment isolation is inappropriate for functional studies. Since concentration by PEG 20,000 does not appreciably alter the membrane fragments, the optimal procedure for their isolation appears to be fractionation by either Bio-Gel A-50m (or Sepharose 2B) or Bio-Gel A-150m, followed by concentration using PEG 20,000.

REFERENCES

1. Black, P.H. (1980) Adv. Cancer Res. 32:75-199.
2. Fishman, W.H., Inglis, N.R., Vaitukaites, J., Stolbach, L.L. (1975) Natl. Cancer Inst. Monograph 42:63-73.
3. Taylor, D.D., Homesley, H.D., and Dollgast, G.J. (1983) Amer. J. Reprod. Immunol. (in press).
4. Wang, B.S., McLoughlin, G.A., Richie, J.P., and Mannick, J.A. (1980) Cancer Res. 40:288-292.
5. Nicolson, G. and Poste, G. (1976) New Engl. J. Med. 295:253-258.

6. Davey, G.C., Currie, G.A., and Alexander, P. (1976) Br. J. Cancer 33:9-14.
7. Nordquist, R.E., Anglin, J.H., and Lerner, M.P. (1978) Br. J. Cancer 37:776-779.
8. Taylor, D.D., Homesley, H.D., and Dollgast, G.J. (1980) Cancer Res. 40:4064-4069.
9. DeBroe, M.E., Wieme, R.J., Logghe, G.N., and Roels, F. (1977) Clin. Chim. Acta 81:237-245.
10. Singer, R.M., White, L.J., Perry, J.E., and Dollgast, G.J. (1975) Cancer Res. 35:3048-3050.
11. Poste, G. and Nicolson, G.L. (1980) Proc. Natl. Acad. Sci. USA 77:399-403.
12. Kim, U., Baumler, A., Carruthers, C., and Bielat, K. (1975) Proc. Natl. Acad. Sci. USA 72:1012-1016.
13. Poste, G. and Fidler, I.J. (1980) Nature 283:139-146.
14. Frick, P.G. and Lowenstein, J.W. (1976) J. Biol. Chem. 251:6372-6378.
15. Butcher, R.W. and Sutherland, E.W. (1962) J. Biol. Chem. 237:1244-1250.
16. Mansier, P. and Lelievre, L.G. (1982) Nature 300:535-537.
17. Tisdale, H.D. (1967) Methods in Enzymology, vol. 10, pp. 213-215, Academic Press, New York.
18. Walter, K. and Schutt, C. (1974) Methods of Enzymatic Analysis, pp. 856-860, Academic Press, New York.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.
20. Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133:709-724.
21. Low, D.A., Baker, J.B., Koonce, W.C., Cunningham, D.D. (1981) Proc. Natl. Acad. Sci. USA 78:2340-2344.
22. Liepins, A. (1982) Techniques in Somatic Cell Genetics, pp. 1-20, Plenum Press, New York.
23. Neumann, C. and Sorg, C. (1983) Eur. J. Immunol. 13:143-147.