Velocity sedimentation of tumor cells: a comparison of methods[1]

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Summary. Tumor cells isolated from a murine fibrosarcoma were grown in primary culture for two days and then separated on a basis of size by velocity sedimentation. Centrifugal elutriation and STAPUT methods were compared for their ability to isolate biophysically unique tumor subpopulations. The isolated cell fractions were assayed for cell number, incorporation of triatiated thymidine and Coulter volume. Both methods were comparable with regard to ability to separate tumor cells on a basis of size. Elutriation had the advantage of speed but required sophisticated equipment. The STAPUT method was less expensive but required somewhat longer times for separation.

A solid tumor cannot be considered a mass of homogeneous cells each experiencing identical environmental stress. Rather it should be considered a composite of subpopulations differing in physiological conditions such as availability of nutrients, oxygen tension, presence of metabolic waste products, and pH. These differences present a rationale for investigating morphologically similar but physiologically and biophysically distinct tumor cell subpopulations. Several cell separation techniques have been used to isolate these unique cell populations.

Velocity sedimentation has proven to be an extremely powerful tool in the study of differentiation[3], metastasis[4], and treatment effects[5-7]. Velocity sedimentation, which separates cells on the basis of size, has been accomplished by several methods.

Centrifugal elutriation methods have recently been reviewed by Pretlow[8]. Briefly, tumor cells are balanced between centrifugal and centripetal forces in a modified centrifuge with a specialized rotor. As the centripetal forces are increased in a step fashion, cells are removed from the rotor on the basis of size. The smallest cells are obtained in the early fractions while the larger cells predominate in the later fractions.

A 2nd method used for size separation of cells is the STAPUT method as described by Miller and Phillips[9]. This velocity sedimentation method has been used to isolate tumor subpopulations grown in plateau cultures or as multicell spheroids[10]. The method allows cells to sediment at unit gravity through a shallow gradient in a cylindrical chamber with a conical base. The larger cells sediment faster than smaller cells which serves as a basis for the separation.

The present communication compares centrifugal elutriation and STAPUT methods for isolating subpopulations of murine fibrosarcoma tumor cells.

Methods. Fibrosarcoma (FSa) tumor cells were grown in the hind leg of C3H/KAM specific pathogen-free mice. The tumor was removed aseptically and made into a single cell suspension using enzymatic disaggregation[11]. The tumor cells were transferred to 32-ounce glass bottles (10^6/bottle) with 10 ml of modified McCoy's 5A media with 20% fetal calf serum. The cells were incubated at 37 °C in 5% CO_2 for 24 h when the media was changed to remove any unattached tumor cells. The cells were then incubated for an additional 24 h at which time they were treated with tritiated thymidine (sp. act. 2.0 Ci/mM), 0.5 μCi/ml for 10 min. Cold thymidine was added to prevent further uptake of the label. The cells were then trypsinized and washed twice with solution A (8 g NaCl, 0.4 g KCl, 1 g glucose and 0.35 g $NaHCO_3$ in 1 l of water). The cells were resuspended and separated by either centrifugal elutriation or by the STAPUT method.

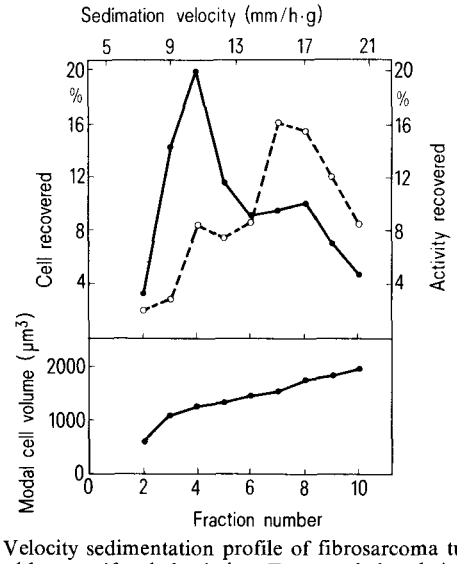

Fig. 1. Velocity sedimentation profile of fibrosarcoma tumor cells separated by centrifugal elutriation. Top panel closed circles represent percent cell recovery, open circles percent ^{3}HTdR recovery vs fraction number or sedimentation velocity. Lower panel modal cell volume of the isolated tumor fractions.

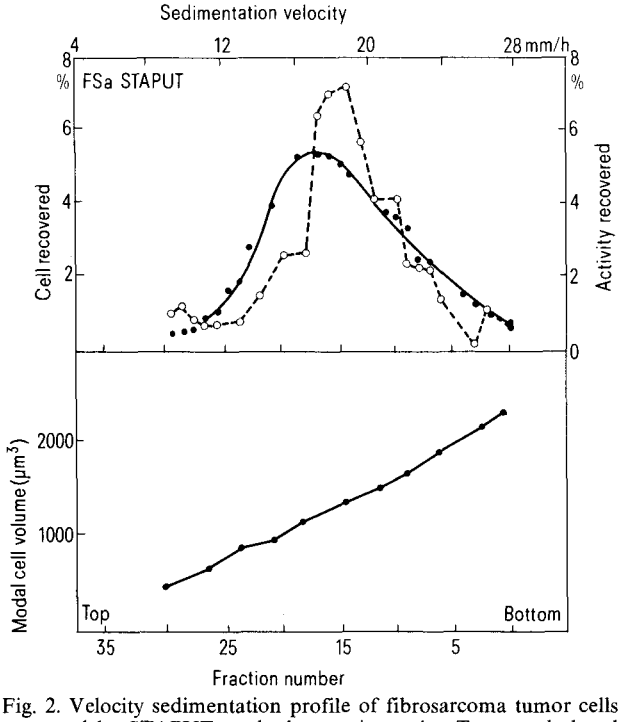

Fig. 2. Velocity sedimentation profile of fibrosarcoma tumor cells separated by STAPUT methods at unit gravity. Top panel closed circles represent percent cell recovery, open circles percent ^{3}HTdR recovery vs fraction number or sedimentation velocity. Lower panel modal cell volume of the isolated tumor fractions.

Fibrosarcoma tumor cells (2×10^8) to be elutriated were resuspended in 20 ml media with 5% FCS. The elutriator was operated at a constant 1510 rpm while varying the flow rate of media through the rotor from about 5 to 29 ml/min. 12 50-ml fractions were obtained and analyzed.

The tumor cells to be separated by the STAPUT method were loaded (6×10^7 in 20 ml of 0.5% BSA) through the bottom of the 884 ml chamber followed by a 2-4% bovine serum albumin (BSA) gradient. The cells were allowed to sediment for 2.66 h in a cold room at 4 °C. After discarding the volume of the cone, 33 fractions were collected of 20 ml each. All fractions obtained from either elutriation or STAPUT were analyzed for cells per fraction, cell volume and ^{3}HTdR incorporation.

Figure 1 presents the results obtained in separating FSa tumor cells by centrifugal elutriation. The top panel shows the percent cell recovery as a function of sedimentation velocity (SV) or fraction number. The majority of the cells are removed in fraction 4 which relates to a SV of about 11 mm/h. Subsequent fractions have between 10 and 4% recovery of tumor cells. The open circles in this panel show where pulse labeled (^{3}HTdR) tumor cells are removed from the rotor. It is apparent that these labeled cells are larger and sediment at a more rapid rate than the smaller cells which predominate in fractions 3-5. The bottom panel shows the modal volume of cells obtained from the various elutriator fractions. The cells varied in a linear fashion from about 700 to 2000 μm^3 with the DNA synthesizing cells approximating 1800 μm^3.

Figure 2 shows similar results using the STAPUT method at unit gravity. Because the cells separated by the STAPUT method were collected in 33 fractions as opposed to 10 fractions in the elutriation experiments, the percent cell recovery per fraction was significantly lower in the STAPUT experiments. The peak in the ^{3}HTdR labeled cells was noted in fractions representing cells with cell volumes larger than average. This effect was not as apparent using the STAPUT as it was in cells separated by elutriation. The bottom panel of figure 2 shows the relationship of modal cell volume to sedimentation velocity. The cell volumes varied from 585 to 2800 μm^3 in the fractions collected. The line was linear with a correlation coefficient equal to 0.998.

Both centrifugal elutriation and the STAPUT methods are extremely effective in separating tumor cells on a basis of size. Cells experiencing uniform conditions of exponential growth increase steadily in size with age during the division cycle[12]. Since the SV of a cell is proportional to two-thirds power of its volume, velocity sedimentation methods can be used to separate and isolate subpopulations of cells having similar sizes and DNA contents from asynchronous populations. The enrichment of tumor cells on a basis of size allows the cell cycle phase effects to be studied in relation to many disciplines such as tumor biology, experimental chemotherapy and experimental radiation therapy[5-7].

Both separation methods can be accomplished under sterile conditions. Elutriation, although faster, requires an expensive and sophisticated centrifuge; while the STAPUT method can be accomplished cheaper but requires additional time for separation.

1 Supported in part by NIH-NCI grants No. CA-06294 and CA-18628.
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Ciliary structures in the branchial unicellular glands of the grass shrimp, *Palaemonetes pugio*[1]

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Summary. A unicellular exocrine gland possessing an epicuticular ductule occurs in grass shrimp gills. This gland displays ultrastructural changes in relation to the molt cycle. These changes include an increase in the quantity of secretory granules during late premolt, and the development of ciliary axonemes in relation to ductule formation.

Both unicellular[2,3] and multicellular[4] exocrine glands of Crustacea resemble the class III dermal glands of insects[5] in having a cuticular ductule. The process of ductule formation has thus far been elucidated in the case of a variety of insect dermal glands[6-11] and a tricellular dermal gland in a crustacean[4], but not in the case of unicellular dermal glands of Crustacea. To bridge this gap in the literature, we examined the ultrastructure of unicellular glands in the branchial epithelium of the grass shrimp, *Palaemonetes pugio*.

Materials and methods. Gills from grass shrimp at various stages of the molt cycle[12,13] were fixed and processed for electron microscopy[4,14]. Sections were stained with lead citrate and uranyl acetate and viewed with a Philips 201 electron microscope.

Results. Unicellular exocrine glands with a short ductule sparsely populate the subcuticular regions of the pleurobranchiate gill axes on the sides facing the cephalothorax (figure 1). The gland cell (30-45 µm) is surrounded by epithelial cells except for a narrow (about 3 µm) neck which contacts the branchial cuticle (figures 1 and 2). A septate desmosome (about 1 µm) joins the basal portion of the cytoplasmic neck to the adjacent epithelium. The cytoplasm contains a relatively dense nucleus, numerous mitochondria and cisternae of rough endoplasmic reticulum, as well as occasional golgi and multivesicular bodies. Additionally, the cytoplasm contains secretory granules (0.2-1.8 µm diameter) with extremely dense or moderately lucent contents (figure 1). The secretory granules are most abundant during late premolt and nearly absent during