Separation of Cells with Histochemically Demonstrable Acid Phosphatase Activity from Suspensions of Cells from Human Prostatic Carcinomas in an Isokinetic Gradient of Ficoll in Tissue Culture Medium

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SUMMARY

This report describes the separation of cells exhibiting histochemically demonstrable acid phosphatase from suspensions of cells obtained from human prostatic carcinomas by velocity sedimentation. In the unseparated suspensions of cells, 40.5 ± 7.7% of nucleated cells contained histochemically evident acid phosphatase. After cells were separated by velocity sedimentation, 86.4 ± 9.4% of the nucleated cells in the purest fractions exhibited histochemically demonstrable acid phosphatase activity. More than 95% of these cells excluded trypan blue. To our knowledge, this is the first report of a method for the separation of viable epithelial cells from human prostatic carcinomas.

INTRODUCTION

The purification of epithelial cells from carcinoma of the prostate is important since the stromal cells are frequently more numerous than epithelial cells in carcinomas (18). In a recent symposium, we have reviewed the application of gradient centrifugation to the purification of cells from cancers (21); we have also recently reviewed other methods for the purification of cells from tumors. We are not aware of any previously reported purifications of epithelial cells from human carcinomas. The biochemical and immunochemical comparison of malignant and benign prostatic epithelial cells would be facilitated if the malignant epithelial cells could be separated from other kinds of cells. Numerous investigators have encountered difficulties in the culture of malignant cells from tumors because of the overgrowth of cultures by stromal cells (6, 9). In cultures of benign human prostate, overgrowth of the cultures by fibroblasts has been an important problem (24). Overgrowth by fibroblasts might be circumvented if highly purified, malignant, epithelial cells (free of fibroblasts) were available for culture. We now report a method for the separation of viable epithelial cells from human prostatic carcinomas.

MATERIALS AND METHODS

Gradients and Centrifugation. Density gradients of Ficoll (polysucrose, average M. W. 400,000; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in tissue culture medium were constructed using the 2-chambered gradient generator (Lido Glass, Stirling, N. J.) described previously (19). The gradients were contained in 100-mI polycarbonate centrifuge tubes (Tube 2806; International Equipment Co., Needham Heights, Mass.) and centrifuged in the MSE Mistral 6L centrifuge (Measuring and Scientific Equipment, Ltd., London, England). During velocity cell separation experiments, the centrifuge speed was monitored continuously with an electronic stroboscope (General Radio Co., Concord, Mass.).

The 85-mI, isokinetic gradient that was used in velocity cell separation experiments has been described in detail (17); it varies linearly from 2.7% w/w Ficoll at the sample-gradient interface (13.7 cm from the center of revolution) to 5.5% w/w Ficoll at the gradient-cushion interface (26.7 cm from the center of revolution). Tissue culture medium and solutions of Ficoll for the isokinetic gradient were sterilized by filtration (Millipore Corp., Bedford, Mass.). Cells in the isokinetic gradient sediment with constant velocities; velocity sedimentation in the isokinetic gradient is most useful for the separation of cells that have different diameters (23).

Gradients for isopycnic sedimentation varied linearly from 4.1% w/w Ficoll at the sample-gradient interface (14.2 cm from the center of revolution) to 43.0% w/w Ficoll at the gradient-cushion interface (26.0 cm from the center of revolution). Solutions of Ficoll for the isopycnic gradient are too viscous to be sterilized by filtration and were autoclaved as described previously (19). Cells are separated according to differences in density by isopycnic sedimentation.

With methods previously reported (17, 23), we found that cells having histochemically demonstrable acid phosphatase activity were best separated from the other cells of...
prostatic carcinomas after centrifugation for 13 min at 4° using a centrifugal force of 74 x g measured at the sample-gradient interface. Isopycnic centrifugation was performed using a centrifugal force of 800 x g, measured at the sample-gradient interface for 90 min at 4°.

**Cells for Starting Sample Suspension.** Preliminary experiments included a comparison of 0.1% Pronase (EM Laboratories, Inc., Elmsford, N. Y.) (5, 14, 15), 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.) (3, 15), and 0.05% collagenase (Sigma Chemical Co., St. Louis, Mo.) (12, 13) to determine which enzyme gave the largest number of histochemically acid phosphatase-positive cells per g of tissue.

Prostate tissue was obtained from surgical specimens at the University Hospital and Veteran's Administration Hospital in Birmingham, Ala. For the 8 prostatic carcinomas studied in the separation experiments reported here, 5 were from transurethral prostatectomies and 3 were from open prostatectomies. These gave comparable results. Tissue obtained from surgery was placed immediately in cold tissue culture medium. After sections were taken for diagnostic purposes, the prostate were minced in cold tissue culture medium containing 10% fetal calf serum. The fragments were digested for 14-20 min periods in successive changes of enzyme solution. The cells in the solutions from each of the 20-min digestions were decanted and cooled in an ice bath for 5 min. The suspensions were then centrifuged at 97 x g for 7.5 min to sediment the cells. The cells were resuspended in 5 volumes of medium containing 10% fetal calf serum and kept in an ice bath until the tissue was exhaustively digested.

The 1st 4 digestions were discarded because they contained many red blood cells and cells that did not exclude trypan blue. The suspensions of cells from the 5th through the 14th digestions were filtered through a single layer of Nitex (Tobler, Ernst, and Traber, Inc., Elmsford, N. Y.) having a pore diameter of 100 μm. This resulted in the starting sample suspensions that contained 6.5 to 21.6 x 10^6 cells in 7-mi volumes that were layered over the gradients.

**Gradient Fractions.** After centrifugation, gradients were collected by displacement with a dense sucrose solution using the gradient tapping cap (Halpro, Inc., Rockville, Md.) which was described earlier (23). The gradients were collected in 4-mi fractions except for the 1st fraction of each gradient, which was the 7-mi starting sample volume layered over the gradients. Cell counts were performed on all gradient fractions with hemocytometer chambers. Refractive indices of all fractions were measured with an Abbe refractometer (Arthur H. Thomas Co., Philadelphia, Pa.). Slides for microscopic examination were prepared with the cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.). Duplicate slides from each fraction were stained in parallel with Wright stain, the Gomori (10) lead method for acid phosphatase, and the Burstone (4) naphthol method for acid phosphatase. Slides for the demonstration of acid phosphatase were fixed for 5 min in 10% formalin containing 1% calcium chloride. The slides stained with the Gomori method for acid phosphatase were counterstained with hematoxylin for 5 min. Those stained by the Burstone method were counterstained with 1% methyl green for 5 min. These 2 methods yielded comparable results as reported previously (7). Differential cell counts were done by counting 200 cells from each fraction and 500 cells from the starting sample suspensions of unseparated cells and from the modal fractions from the gradients.

**RESULTS**

**Starting Sample Suspension.** Preliminary experiments showed that disaggregation with 0.1% Pronase gave a higher number of cells with histochemically demonstrable acid phosphatase activity than disaggregation with 0.25% trypsin or 0.05% collagenase (Table 1). Pronase was used for the preparation of cell suspensions for all cell separation experiments. With 0.1% Pronase, we obtained an average of 1.51 x 10^6 cells/g of prostatic carcinoma. The standard deviation was ±0.74 x 10^6; the maximum, 2.73 x 10^6; the minimum, 0.67 x 10^6. The starting sample suspensions (Fig. 1) contained 38.1 ± 11.9% red blood cells, 25.5 ± 10.1% cells having histochemically demonstrable acid phosphatase activity, and 36.4 ± 7.6% nucleated cells lacking acid phosphatase activity. In the starting sample suspensions, 40.5 ± 7.7% of the nucleated cells contained histochemically demonstrable acid phosphatase. Over 95% of these cells excluded trypan blue.

**Velocity Sedimentation in the Isokinetic Gradient.** The cells from the prostatic carcinomas with histochemically demonstrable acid phosphatase activity were never widely separated from other cells of prostatic carcinomas. The best 1-step separation of these cells occurred after sedimentation for 13 min, using a centrifugal force of 74 x g (Chart 1). The purification obtained after isokinetic sedimentation is shown in Table 2. The purest fraction of acid phosphatase-positive cells was found adjacent to or on the gradient-cushion interface in Fractions 21 or 22 (Figs. 2 to 4). These fractions contained 14.3 ± 15.3% red blood cells 74.0 ± 16.4% cells having acid phosphatase activity, and 11.6 ± 13.5% nucleated cells. The standard deviation was ±0.74 x 10^6; the maximum, 2.73 x 10^6; the minimum, 0.67 x 10^6. The starting sample suspensions (Fig. 1) contained 38.1 ± 11.9% red blood cells, 25.5 ± 10.1% cells having histochemically demonstrable acid phosphatase activity, and 36.4 ± 7.6% nucleated cells lacking acid phosphatase activity. In the starting sample suspensions, 40.5 ± 7.7% of the nucleated cells contained histochemically demonstrable acid phosphatase. Over 95% of these cells excluded trypan blue.

**Comparison of Cells Obtained with Various Enzymes.** Because of the wide range of the data as the result of large differences between individual human prostatic carcinomas, the comparisons presented in this table are comparisons of collagenase, trypsin, and Pronase as agents for the dispersion of the same 3 prostatic carcinomas. The mean values obtained for these 3 prostatic carcinomas are different from the mean values obtained from all 8 prostatic carcinomas that we disaggregated with the aid of Pronase (see the values given in the text).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total cells</th>
<th>AP + cells</th>
<th>% viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>2.91 ± 0.45</td>
<td>0.58 ± 0.27</td>
<td>89.3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1.92 ± 0.25</td>
<td>0.98 ± 0.61</td>
<td>91.3</td>
</tr>
<tr>
<td>Pronase</td>
<td>3.32 ± 1.37</td>
<td>1.23 ± 0.81</td>
<td>94.0</td>
</tr>
</tbody>
</table>

* Cells with histochemically demonstrable acid phosphatase activity.  
* Viability, trypan blue dye exclusion.  
* Mean ± S.E.
Separation of Cells from Prostatic Carcinomas

Chart 1. Separation of prostatic carcinoma cells with velocity sedimentation in the isokinetic gradient. Arrow, sample-gradient interface on the density plot. Because of the large variability among patients, 3 representative profiles are shown. Despite the variability among patients, Fractions 21 and 22 from the isokinetic gradient consistently contained large proportions of cells that stained intensely for acid phosphatase and that contained lipofuscin granules.

Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Sample (%)</th>
<th>Fractionb</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood</td>
<td>38.1 ± 11.9</td>
<td>6</td>
<td>74.1 ± 15.7</td>
</tr>
<tr>
<td>AP+</td>
<td>25.5 ± 10.1</td>
<td>22</td>
<td>74.0 ± 16.4</td>
</tr>
<tr>
<td>AP−</td>
<td>36.4 ± 7.6</td>
<td>8</td>
<td>48.3 ± 15.4</td>
</tr>
</tbody>
</table>

* Percentage of cell type in starting sample suspension after disaggregation with Pronase.
* Modal fraction number.
* Percentage of cell type in modal fraction.
* Mean ± S.E.
* AP+, cells with histochemically demonstrable acid phosphatase activity; AP−, cells lacking histochemically demonstrable acid phosphatase activity.
* Numbers in parentheses, percentage of nucleated cells.

9.7% cells lacking histochemically demonstrable acid phosphatase activity. Thus, 86.4 ± 9.4% of the nucleated cells in Fractions 21 or 22 had histochemically demonstrable acid phosphatase activity. More than 95% of the cells in these fractions excluded trypan blue. Most of these cells contained lipofuscin granules. Lipofuscin granules have been described (2, 16) as being characteristic of epithelial cells from the prostatic carcinomas and from prostates without carcinoma. Fractions 8, 9, and 10 from the isokinetic gradients contained a modal population of cells having histochemically demonstrable acid phosphatase activity. This population consisted of 31.9 ± 12.3% red blood cells 10.0 ± 7.5% cells having no acid phosphatase activity, and 49.1 ± 13.7% cells having histochemically demonstrable acid phosphatase activity. In contrast to the intensely stained, acid phosphatase-positive cells in Fractions 21 and 22, the less rapidly sedimenting, acid phosphatase-positive cells stained less intensely for acid phosphatase and lacked lipofuscin granules. As evidenced by the Wright stain, many of these cells were granulocytes; very few had the morphological appearance of epithelial cells. The proportion of nucleated cells having no acid phosphatase activity varied. The modal population of cells without demonstrable acid phosphatase was located in Fraction 8 (±1 fraction). It contained 53.5 ± 25.3% red blood cells, 11.6 ± 12.3% cells having acid phosphatase activity, and 34.8 ± 15.4% cells lacking acid phosphatase activity. The modal population of red blood cells was found in Fraction 6; it contained 70.0 ± 15.7% red blood cells. Acid phosphatase-positive cells...
made up 9.8 ± 7.5% of the cells in Fraction 6. The remaining 20.1 ± 10.7% of the cells lacked acid phosphatase activity.

**Isopycnic Sedimentation.** Cells with histochemically demonstrable acid phosphatase activity were not separated from other cells of prostatic carcinomas by isopycnic sedimentation (Chart 2). After isopycnic sedimentation, 57.3 ± 7.5% of the cells recovered from the gradients were in only 1 4-ml fraction from the 22-fraction gradient; 79.3 ± 5.7% of the recovered cells were in 3 fractions. The cells were found in the gradient between the densities of 1.05 and 1.12 g/ml.

**Recovery of Cells from the Gradients.** Following separation in the isokinetic gradient, 84.7 ± 5.5% of the red blood cells, 73.5 ± 11.2% of the acid phosphatase-positive cells, and 79.6 ± 10.4% of the cells having no histochemically demonstrable acid phosphatase activity that were layered over the gradients were recovered from the gradients. Following isopycnic sedimentation, 53.1 ± 9.3% of the red blood cells, 63.0 ± 10.5% of the cells having histochemically demonstrable acid phosphatase activity, and 61.9 ± 5.9% of the cells lacking acid phosphatase activity that were layered over the gradients were recovered from the gradients. Much of the loss of cells probably resulted from the wall-effect artifact which is present in all centrifugation ovens the gradients were recovered from the gradients. Following isopycnic sedimentation, 53.1 ± 9.3% of the red blood cells, 63.0 ± 10.5% of the cells having histochemically demonstrable acid phosphatase activity, and 61.9 ± 5.9% of the cells lacking acid phosphatase activity that were layered over the gradients were recovered from the gradients. Much of the loss of cells probably resulted from the wall-effect artifact which is present in all centrifugation ovens the gradients were recovered from the gradients. Much of the loss of cells probably resulted from the wall-effect artifact which is present in all centrifugation ovens the gradients were recovered from the gradients. Much of the loss of cells probably resulted from the wall-effect artifact which is present in all centrifugation ovens the gradients were recovered from the gradients.

**DISCUSSION**

Previously, we have described the purification of blasts from the peripheral blood of patients with leukemia (1), the purification of individual kinds of cells from experimental tumors (20, 25, 26, 27), and the purification of lymphocytes and atypical histiocytes from the tumor of Hodgkin’s disease. Human prostatic carcinomas have differed from other neoplasms that we have studied in that our yield (cells/g) of cells from human prostatic carcinomas has been small and highly variable. In general, except for the human tonsil (26) and the tumor of Hodgkin’s disease (22), human tissues have been obtained as cell suspensions only with relatively low efficiency. In part, this observation may be the consequence of the fact that most of the commonly used techniques for obtaining cell suspensions have been developed with and applied primarily to the tissues of experimental animals.

While the purified cells with histochemically demonstrable acid phosphatase were less pure than those we obtained from hamster prostates (7), they were more highly purified than those we obtained from human prostates without carcinoma (11). As in the case of human prostates without carcinoma, the cells with histochemically demonstrable acid phosphatase in Fractions 21 and 22 from the isokinetic gradients contained the lipofuscin granules that have been described (2, 16) in epithelial cells of human prostates and prostatic carcinomas. We were not able to distinguish between benign and malignant epithelial cells in these fractions, and it seems likely that both benign and malignant epithelial cells were contained in these fractions in most experiments. While the cells from prostatic carcinomas differed considerably among the 8 patients studied (Chart 1), the observed differences in sedimentation profiles did not appear to be related to the proportions of the prostates that were replaced by carcinoma microscopically. As a condition for being included in this study, patients were required to have histologically diagnosed prostatic carcinoma from previous needle or transurethral biopsies. Perhaps for this reason, most of the prostates used for these cell separation experiments were more extensively replaced by carcinoma than the average prostates that are shown to have carcinoma following surgical resection. Since morphometric studies were not performed, we do not know what proportion of the cells in the original tissue contained histochemically demonstrable acid phosphatase, and we do not know what proportion of the acid phosphatase-positive cells may have been destroyed during the enzymic dissociation.

The method described in this report permits the purification of viable epithelial cells from human prostatic carcinomas. Franks et al. (8) reported that prostatic epithelial cells separated mechanically exhibited “a failure to survive and proliferate.” We hope that further study will disclose more efficient methods by which the cells from prostatic carcinomas can be obtained in suspension. In reviewing methods by which cells may be obtained in suspension (23), we pointed out that the selection of efficient methods appears to be highly dependent upon the particular tissue and the particular species to be studied. Even with the suspensions of cells that we obtained, the purification might be im-

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**Discussion Chart 2.** Separation of cells from prostatic carcinoma with isopycnic sedimentation. Arrow, sample-gradient interface on the density plot.
proved by the use of a 2nd purification step. Many qualita-
tively different methods for the separation of cells are availa-
ble (23) and should be tested.

REFERENCES


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