Separation and Characterization of Neoplastic Cell Subpopulations of a Transplantable Rat Pancreatic Acinar Carcinoma

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ABSTRACT

The transplantable pancreatic acinar carcinoma established in F344 rats demonstrates heterogeneity of cytodifferentiation with cell types ranging from those containing no zymogen granules to cells with mature zymogen differentiation. Two relatively homogeneous subpopulations of cells have been isolated by isopyknic Percoll gradient centrifugation from the heterogeneous cell population of this tumor. The subpopulation obtained at a density of 1.0987 g/ml was designated the granule-enriched fraction (GEF) and contained morphologically differentiated cells with abundant zymogen granules. The other subpopulation, obtained at a density of 1.0789 g/ml, consisted of poorly differentiated cells lacking zymogen maturation and was thus termed the granule-deficient fraction (GDF). In both fractions, greater than 97% of the cells were viable and maintained linear rates of [3H]leucine and [3H]thymidine incorporation into protein and DNA for up to 3 hr following isolation. High-resolution autoradiographic analysis of [3H]thymidine incorporation revealed that 20% of the cells in GDF subpopulation and 12% of the cells in GEF subpopulation synthesize DNA. Morphological and morphometric analyses of the isolated GEF and GDF subpopulations of the acinar carcinoma revealed distinct differences in nuclear:cytoplasmic ratio, secretory granule content, and degree of polarity. The GEF subpopulation contained significant amounts of stored zymogen, as evidenced by higher levels of amylase and lipase activities. In contrast, the GDF subpopulation displayed very low levels of these enzymes. Both GDF and GEF subpopulations produced tumors when injected s.c. into F344 rats.

Characterization of concanavalin A (Con A)-binding sites on the plasmalemma of the GDF and GEF subpopulations by the Con A:peroxidase method indicated the presence of Con A receptors on pancreatic carcinoma cells regardless of the extent of cytodifferentiation in contrast to normal pancreatic embryogenesis, in which Con A receptors are discerned only in acinar cells containing mature secretory granules. The presence of Con A receptors on all cells of the GDF and GEF acinar carcinoma cell subpopulations suggests either that neoplastic transformation results in altered genetic expression whereby progenitor "stem" cells, which lack Con A receptors during normal pancreatic embryogenesis, acquire such receptors or that the pancreatic carcinoma arises from dedifferentiation of mature acinar cell which normally possesses Con A receptors.

INTRODUCTION

In recent years, several studies have dealt with the isolation and identification of purified populations of neoplastic cells from several experimental and human solid tumors using density gradient centrifugation. For the most part, these investigations were aimed at isolating various nontumor cells from the tumor cells (1, 12, 30-35, 45, 52) but not at separating the generally heterogeneous tumor cell population into subpopulations that depict differentiative and metastasizing potential (3, 13, 19). Highly purified subpopulations of neoplastic cells that are morphologically distinct and exhibit identifiable markers of gene expression would greatly facilitate investigations of cellular and biochemical events controlling not only differentiation in neoplastic cell population but also maintenance of the malignant phenotype. Furthermore, comparative studies of tumor cell subpopulations with normal cellular components of the tissue of origin of a given tumor may provide important clues about initiation and progression of neoplasia.

The distinct morphological and enzymatic parameters of differentiation in cells of the transplantable pancreatic acinar carcinomas of rats (22, 36, 39) provide a suitable model system to study tumor heterogeneity and cytodifferentiation (21, 48). Morphological analysis of the transplantable pancreatic acinar carcinoma established in the F344 strain of rats by Reddy and Rao (39) has revealed 4 cell subtypes, designated types 1 to 4, that display varying degree of cytodifferentiation (41). Type 4 neoplastic acinar cells possess all the characteristics of mature pancreatic acinar cells including abundant, well-formed secretory (zymogen) granules. Type 1 cells are undifferentiated and lack secretory granules and the Golgi apparatus. Tumor cell types 2 and 3 are considered as intermediate stages in this spectra of heterogeneous pancreatic acinar tumor cell populations. Biochemical and immunocytochemical studies have demonstrated the presence of at least 19 exocrine pancreatic enzymes in the secretory granules of these neoplastic cells (17, 42). Whether these cell types represent well-defined stages of differentiation or dedifferentiation sequence remains unclear (41). We now report the separation of 2 major subpopulations of cells from this pancreatic acinar carcinoma by isopyknic gradient centrifugation: one subpopulation, termed GEF, consists predominantly of type 3 and 4 (secretory granule-containing) cells described previously (41). The second subpopulation, termed GDF, consists of type 1 and 2 cells that lack granules. In this report, we describe the separation technique with Percoll as a gradient medium (29, 31) and present...
data on the functional, morphological, and membrane surface properties of the 2 isolated subpopulations.

**MATERIALS AND METHODS**

**Chemicals**

[methyl-3^H]Thymidine (specific activity, 20 Ci/mmole) and L-[3,4,5-^H]leucine (specific activity, 60 Ci/mmole) were obtained from New England Nuclear, Boston, Mass. Class IV collagenase (172 units/mg) and STI were obtained from Worthington Biochemical Corp., Freehold, N. J. Con A purified by chromatography on Sephadex and Percoll were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. BSA (Fraction V), 3,3'-diaminobenzidine, and horseradish peroxidase were obtained from Sigma Chemical Co., St. Louis, Mo. IIId L4 nuclear research emulsion was purchased from Polysciences, Inc., Warrington, Pa. All other chemicals were reagent grade.

**Transplantation of Pancreatic Acinar Carcinoma**

Small 1- to 2-mm fragments of pancreatic acinar carcinoma (39) were transplanted i.p. into the abdominal mesentery of weanling male F344 rats as described previously (36). These implants grew for the most part as single, large encapsulated tumors between 3 to 5 weeks. The tumor-bearing animals were starved for 24 hr prior to sacrifice, and the tumors were removed aseptically under light ether anesthesia.

**Preparation of Single-Cell Suspensions**

The tumors were dissociated into single cells by a technique involving a combination of mechanical fragmentation (47, 50) and EDTA:collagenase dissociation (2) as described below. All steps of the mechanical fragmentation were performed at room temperature in prewarmed KRB buffer containing 14 mM glucose, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 0.1 mg STI per ml, a complete L-amino acid supplement, 100 units penicillin per ml, and 10 µg streptomycin per ml. The supplemented KRB buffer was maintained at pH 7.4 with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Excised tumor was sectioned into 10- to 15-mm pieces with a scalpel and placed onto the surface of a 10-mesh stainless steel sieve (putative sieve opening of 1.7 mm) (U. S. Standard Sieve; Fisher Scientific Co., Pittsburgh, Pa.). The 10-mesh sieve was placed in tandem with a collecting pan, and the tumor was pressed gently through the sieve with the rubber end of a disposable 20-ml syringe. Tumor fragments were rinsed through to the collecting pan with prewarmed KRB and collected in 50-ml polypropylene centrifuge tubes. The fragments were then washed by centrifugation (200 x g, 5 min) in KRB buffer, 4% (w/v) BSA in complete KRB, and buffer in that sequence. Each 1 ml of washed carcinoma fragments was then placed in 6 ml of supplemented KRB buffer containing 200 units collagenase per ml and 0.5 mg STI per ml. Fragments were maintained in suspension by a shaker bath at 37° and were incubated for 15 min under 5% CO_2:95% O_2. This mixture was then centrifuged (200 x g, 5 min), the collagenase-containing supernatant was discarded, and the pellet was suspended 1.8 (v/v) in Ca^{2+}, Mg^{2+}-free KRB buffer containing 2 mM EDTA in addition to glucose, amino acids, and 0.1 mg STI per ml. After 2 successive 5-min incubations with EDTA at 37°, the mixture was centrifuged (200 x g, 5 min), and the pellet was suspended in supplemented KRB buffer containing 4% (w/v) BSA, strained through 20-µm nylon gauze, and sheared gently by 5 passages through a serological pipet with a 1.5-mm tip diameter. Viability of the preparation of dissociated acinar carcinoma cells resulting from the combined mechanical collagenase:EDTA treatment was monitored by the trypan blue exclusion test.

**Percoll Gradient Preparation and Cell Separation**

The density gradient was prepared by diluting Percoll 9:1 in 10X HSS containing Ca^{2+} and Mg^{2+} (Grand Island Biological Co., Grand Island, N. Y.). A 51% Percoll:HSS was prepared by diluting the 9:1 Percoll:HSS with 1 × HSS (no Ca^{2+} or Mg^{2+}) and adjusted to pH 7.35 with 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid after addition of 0.1 mg STI per ml. The gradient (28 ml) was then placed in 1- x 3.5-inch cellulose nitrate tubes. Cells prepared as above were suspended in Ca^{2+}- and Mg^{2+}-free KRB buffer and diluted to 5 × 10^7 cells/ml, and 6 ml of this suspension were carefully overlaid on the Percoll gradient. Tubes fitted with a special jacket were spun for 30 min at 13° in a Beckman JA-20 rotor in a Beckman J-21C centrifuge at 10,000 × g. In addition to the self-generated (isopyknic) centrifugation schema outlined above, 2 alternate methods of cell separation were tested for comparison. One method used 6 ml of 1.13 g Percoll per ml as a cushion, followed by 6 ml each of 1.2 g/ml, 1.08 g/ml, and 1.04 g/ml. Six ml of 5 × 10^7 cells/ml were layered over this discontinuous gradient and spun at 400 × g for 30 min in a Beckman TH-4 rotor in a Beckman TJ-6 centrifuge. The second method used a preformed Percoll gradient. Twenty-eight ml of 51% Percoll:HSS were prepared as described above. This was then spun at 400 × g for 30 min in a Beckman TH-4 rotor in a Beckman TJ-6 centrifuge. After centrifugation, tubes were fractionated immediately with a fractionation apparatus (MRA Corp., Boston, Mass.) mounted on a Beckman fraction collection apparatus. Tubes were fitted with a stopper and vent and filled with corn oil, and 1-ml fractions were recovered after puncturing each tube at the bottom. Twenty-eight fractions (1 ml each) were collected and washed free of Percoll by centrifugation in excess amounts of KRB buffer for 5 min at 200 × g.

Cell counts were performed with a standard blood-diluting pipet and hemocytometer. Viability of cells in the gradient fractions was monitored by trypsin blue exclusion. To determine the type of cells in each fraction, an aliquot of cells from each fraction was processed for electron microscopy.

**Density of fractions** was performed on all 28 fractions run with or without cells using an Abbe-3L refractometer. Refractive indices were then converted to gradient density using information on Percoll supplied by the manufacturer (Technical Booklet; Pharmacia). Density marker beads (Pharmacia) were also used for additional confirmation.

**Measurement of Protein and DNA Synthesis**

**Incorporation of [^3H]leucine into Protein.** The subpopulations of neoplastic acinar cells (5 × 10^6 cells/ml) isolated upon fractionation were preincubated in supplemented KRB buffer with 0.2% BSA for 15 min prior to the addition of [^3H]leucine (20 µCi/ml). The cells were incubated in a shaker bath at 37° for up to 3 hr intermittently gassing with 95% O_2:5% CO_2. At 0, 15, 30, 60, 120, and 180 min after the addition of the isotope, a sample was obtained and washed 3 times with chilled complete KRB and processed for DNA extraction, and the radioactivity incorporated into protein was measured by liquid scintillation spectrometry in a Beckman LS-9000 spectrometer (10). For light- and electron-microscopic autoradiography, cells were pulse labeled for 60 min with 50 µCi [^3H]leucine per ml and processed as described previously (44).

**Incorporation of [^3H]thymidine in DNA.** To assess the ability of isolated tumor cell subpopulations to synthesize DNA, fractionated cells were diluted to 5 × 10^6 cells/ml and preincubated for 15 min at 37°. [^3H]Thymidine (20 µCi/ml) was added to the siliconized flask containing the cells, and samples were removed at 0, 15, 30, 60, 120, and 180 min after the addition of the isotope, a sample was obtained and washed 3 times with chilled complete KRB and processed for DNA extraction and estimation (10), and the radiolabel incorporated into DNA was measured by liquid scintillation spectrometry.

To determine the number of cells incorporating [^3H]thymidine into DNA in the subpopulations, isolated cells were incubated for 60 min with 20 µCi isotope per ml at 37°. At the end of the pulse period, cells were washed 3 times with chilled KRB and processed for electron microscopy. Material was then sectioned, coated with photographic emulsion, and processed for light- and electron-microscopic autoradiography (36). A total of 800 to 1050 cells was counted for each isolated subpopulation to obtain the percentage of labeled cells.
Morphological and Morphometric Analyses

Cells were fixed in 96% ethanol at 4°C after pelleting with a Beckman microfuge according to the method of Brandtzæg (9) as described previously (17) and stained with hematoxylin and eosin for general morphological evaluation. In addition, unfixed cells were examined in a Zeiss Ultraphot III microscope using phase optics.

For light and electron microscopy, both tissue pieces and cells were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4, and were postfixed in 1% osmium tetroxide buffered with S-collidine at pH 7.4. After fixation, they were dehydrated and embedded in Epon 812 (24). For light-microscopic evaluation, 0.5-μm-thick sections were taken on a Porter Blum MT-2 microscope, mounted on glass slides and stained with toluidine blue:pyronin B, and were examined in a Zeiss Ultraphot microscope using Nomarski optics.

The isolated cells were classified into 4 cell types according to the criteria outlined in Table 1. The type 1 and 2 cells are referred to as GDF, and type 3 and 4 cells are referred to as GEF. For quantitative analysis of subfraction purity, the GDF and GEF from 4 experiments were embedded, and 3 blocks from each of the experimental groups were sampled. Silver:gold sections were collected on Formvar-coated 100-mesh copper grids, stained with uranyl acetate for 20 min and with lead citrate for 5 min. The sections were examined on a Phillips 200 electron microscope, and 4 random photographs were taken per block at a primary magnification of ×1860. Five hundred seventy-nine cells from GEF, 580 cells from GDF, and 559 nonfractionated cells whose cell diameter was greater than one-half of the maximum profile diameter (~5 μm) were analyzed to determine fraction purity.

Morphometric analysis was performed on 2 levels similar to the method of Black and Russo (6). For Level 1, the volume densities of cells, cytoplasm, and nuclei were determined from light-power electron micrographs of thin sections. For Level 2, the volume densities of cell compartments were determined from high-power electron micrographs of individual cells.

**Level 1.** At Level 1 (×4900), the volume densities (Vv) of the cytoplasm (Vcy), nuclei (Vn), and cell (Vcell) were determined from silver:gold Epon sections mounted on 50-mesh carbon-coated copper grids stained with uranyl acetate and lead citrate. Thirty-six low-power electron microscope grids were collected on Formvar-coated 100-mesh copper grids, stained with uranyl acetate for 20 min and with lead citrate for 5 min. The sections were examined on a Phillips 200 electron microscope, and 4 random photographs were taken per block at a primary magnification of ×1860. Five hundred seventy-nine cells from GEF, 580 cells from GDF, and 559 nonfractionated cells whose cell diameter was greater than one-half of the maximum profile diameter (~5 μm) were analyzed to determine fraction purity.

**Level 2.** For electron-microscopic observation at Level 2 (×17,000), silver:gold sections were cut of pellet fractions from 4 representative experiments. Samples were taken from 2 blocks of each experimental group used in Level 1. Micrographs were taken at ×5300 of 3 random cells/block, 2 blocks/experimental group, 4 individual experiments/fraction analyzed, yielding 24 micrographs with one cell included in each final print. Two subpopulations of pancreatic acinar carcinoma cells were analyzed in addition to nonfractionated and fractionated control dissociated cells. To serve as an additional comparison group, at least 14 of each of 4 cell types (types 1 to 2) described earlier (41) were also subject to Level 2 analysis.

Points of intersection overlying the various subcellular compartments were counted using a lattice where d equaled 5 mm. Compartments were divided into rough endoplasmic reticulum (RERC), the Golgi complex (G2), zymogen granules (ZG), loose (LCT) and tight (TCT), condensing vacuoles, autophagic vacuoles (AV), crystallloid bodies (CB), and cytoplasm (Cy). Volume densities were determined by the formulae:

\[ V_{\text{orgcy}} = P_{\text{org}}/P_{\text{cy}} \]
\[ V_{\text{cell}} = (P_{\text{cy}} + P_n)/P_t \]
\[ V_n = V_{\text{cell}} - V_{\text{cy}} = (P_{\text{cy}} + P_n)/P_t - (P_{\text{cy}}/P_t) \]

where \( P_{\text{cy}} \) = points overlying cytoplasm, \( P_n \) = points overlying nuclei, and \( P_t \) = points overlying total area studied.

**Enzyme Activities of Fractions**

To complement the morphological and morphometric analysis, biochemical assays of 2 enzymes to the pancreatic acinar cells were performed. Dissociated and fractionated acinar carcinoma cells were homogenized by 10 passes in a Potter-Elvehjem homogenizer and then sonicated with an ultrasonic cell disruptor (Model 350; Heat Systems-Ultrasonics, Inc. Plainview, N. Y.) in a 0.2 M bicarbonate buffer, pH 7.8, to extract all of the secretory proteins from the zymogen granules. The sonicate was then spun at 35,000 rpm for 60 min in a Beckman L5-65 ultracentrifuge using a Beckman Ti-50 rotor to obtain the secretory protein extract free of cellular debris. The enzyme activities were determined under conditions that showed linear relationships between activity and protein concentration: amylase by the method of Caraway (11) and lipase by the titrimetric method to determine the extent of

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**Table 1**

Characteristics and frequency of distribution of granule-deficient and granule-enriched tumor cells in the dissociated pancreatic acinar carcinoma cell suspension

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Frequency in dissociated cell population</th>
<th>Nuclear characteristics</th>
<th>Cytoplasmic characteristics and degree of specialization</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF (42%)</td>
<td></td>
<td></td>
<td>Narrow rim of cytoplasm, few strands of rough endoplasmic reticulum, occasional mitochondria</td>
</tr>
<tr>
<td>Type 1</td>
<td>16</td>
<td>Central, irregular</td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>26</td>
<td>Irregular, slightly eccentric</td>
<td>Increase in volume and evident polarity, abundant stacked rough endoplasmic reticulum, several mitochondria, rudimentary Golgi apparatus</td>
</tr>
<tr>
<td>Type 3</td>
<td>34</td>
<td>Eccentric</td>
<td>Apical-basal polarity evident, Golgi apparatus well formed and prominent, secretory granule small and immature</td>
</tr>
<tr>
<td>Type 4</td>
<td>24</td>
<td>Eccentric, crescent shaped</td>
<td>Cell fully differentiated and polar, Golgi apparatus fully formed, mature secretory granules occupy majority of apical cytoplasm</td>
</tr>
</tbody>
</table>

where \( V_{\text{orgcy}} \) = volume density of organelle in the cytoplasm studied, \( P_{\text{org}} \) = points overlying particular organelle, and \( P_{\text{cy}} \) = points overlying the cytoplasmic matrix.

Statistical analysis for both Levels 1 and 2 took into consideration factors outlined by Weibel et al. (51). Within experimental groups, there was no significant difference in the analysis of variance for the intra-group volume density data. Comparison between experimental groups was done using analysis of variance for volume density data and Student’s t test for all other data levels.
Localization of Con A-binding Sites by Con A-Peroxidase Method

Fractionated and washed subpopulations of GEF and GDF cells were fixed for 1 hr with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After fixation, the cells were rinsed in buffer and incubated with Con A (100 μg/ml) in cacodylate buffer for 30 min at room temperature. The cells were then rinsed in cacodylate buffer and suspended in buffer containing 200 μg horseradish peroxidase per ml for 1 hr at room temperature (5). After incubation in the peroxidase solution, the cells were rinsed in buffer and transferred to 3,3′-diaminobenzidine reaction mixture (15) and incubated for 1 hr at room temperature. Cells were then washed with cacodylate buffer, postfixed in OsO4, and processed for electron microscopy. The control samples were processed in the same way, but the Con A incubation step was omitted or methyl-α-D-mannopyranoside was added (0.2 M) to the Con A solution.

Tumorigenicity of GEF and GDF Subpopulations

To assess the ability of GEF and GDF neoplastic cells separated on Percoll gradient to induce tumors, fractionated subpopulations of tumor cells (5 x 10⁶ cells) were injected s.c. in 0.2 ml of KRB buffer into the flank of weanling male F344 rats. The latency period (i.e., the number of days between injection and the appearance of a palpable, growing tumor) was recorded.

RESULTS

Isolation of Neoplastic Acinar Cells

The highly vascularized i.p. transplants of the pancreatic acinar carcinoma are encapsulated and contain fluid-filled cystic spaces. The method used in the dissociation protocol is designed to minimize contamination of the isolated neoplastic acinar cells from stromal cells (e.g., connective tissue and blood elements) and takes advantage of the poorly formed intercellular junctions (20, 28) that exist between cords of blood elements) and takes advantage of the poorly formed intercellular junctions (20, 28) that exist between cords of tumor cells. Isolation of highly purified neoplastic acinar cells is facilitated by the following steps. (a) The solid tumor is minced and pressed through the 10-mesh sieve (the majority of connective tissue does not pass through the sieve). (b) Carcinoma fragments so obtained are washed to remove RBC and damaged cells by centrifugation in 4% BSA. And (c) after treatment of the fragments with collagenase and EDTA, the cell suspension is filtered and washed again in 4% BSA to remove RBC, endothelium, and nonviable cells. The resultant cell suspension contains >97% neoplastic pancreatic acinar cells. The combined mechanical and collagenase-EDTA disaggregation of solid tumor yielded on the average 5 x 10⁷ cells/g tumor (n = 7) and 97 ± 2% (S.E.) of these cells excluded trypan blue. The dissociated neoplastic pancreatic acinar cells are classified into 2 subpopulations. In an earlier study, we tentatively identified 4 types of neoplastic cells in this transplantable pancreatic acinar carcinoma (41). For the purposes of this study, we have identified types 1 and 2 as a subpopulation of cells termed GDF and types 3 and 4 as a subpopulation of cells termed GEF. A description and frequency of each cell subpopulation in the starting suspension is presented in Table 1.

Density Centrifugation

During the initial stages of cell separation, comparison of the self-generated (isopyknic) centrifugation gradients in Percoll to discontinuous or preformed gradients was made with morphologic criteria and fraction density analysis (data not presented). The self-generated Percoll gradient method provided optimum resolution of the 2 fractions of interest when compared to the discontinuous or preformed gradients. Accordingly, the results presented in this paper deal with GEF and GDF obtained by the self-generated gradient method.

A tube containing cells obtained from self-generated gradients of Percoll is shown in Chart 1. At Fractions 3 and 4, a peak in enzyme activity and morphological criteria for GEF cells was found; this coincided with a gradient density of 1.0967 (g/ml). At Fractions 13 and 14, a peak in GDF cells was determined by morphological criteria and coincided with a gradient density of 1.0789 (g/ml). Types 1 and 2 pancreatic acinar carcinoma cells were found to comprise ~77% of the GDF cells. Types 3 and 4 cells comprised ~88% of the GEF cells. Fractions 1 and 2 contained relatively small numbers of GEF cells. Fractions 5 to 12 contained mixtures of GDF and GEF cells and were less homogeneous in purity when compared to GEF cells in Fractions 3 and 4 or GDF cells in 13 and 14. Fractions 15 to 28 contained relatively small numbers of GDF cells and clumps of nonviable cells.

Morphological Identification

Morphological analysis of isolated subpopulations of pancreatic acinar carcinoma cells demonstrated distinct differences in the GDF and GEF cells. Phase-contrast microscopy of fractionated cells demonstrated striking differences in granule content and degree of polarity. The GEF cells contained abundant granules. In the GDF cells, granules were less evident or absent with the nucleus centrally located. Examination of 0.25-μm-thick Epon-embedded tissue sections by the Nomarski method revealed differences in nuclear:cytoplasmic ratios between the 2 isolated subpopulations. GDF cells have a large nuclear:cytoplasmic ratio with a scant cytoplasm (Fig. 1B), whereas the GEF cells have a small ratio and abundant secre-
Subpopulations of Pancreatic Acinar Carcinoma

tory granules (Fig. 1A). Electron microscopy of the isolated subpopulations is illustrated in Figs. 2 and 3.

Electron-Microscopic Analysis of Fraction Purity

Analysis of the purity of the isolated subpopulations was carried out on ultra-thin sections of 579 GEF, 580 GDF, and 559 nonfractionated pelleted cells embedded in Epon. First, as shown in Chart 2, cells were analyzed according to the number of secretory granules in the cytoplasmic compartment. Approximately 73% of the cells in GEF contained more than 20 granules, whereas 75% of the cells in GDF had less than 4 granules. In Chart 3, cells are classified into subpopulation with respect to their cytodifferentiation. As described earlier, Types 1 and 2 are grouped into GDF and Types 3 and 4 are grouped into GEF. When one assesses the ratio of GDF:GEF cells in the control dissociated cell population not subjected to gradient centrifugation, the value is 0.73. In GEF cells, this ratio is 0.14, a 5-fold decrease from controls. In GDF cells, this ratio is 3.27 or greater than a 4-fold increase from controls.

Morphometric Analysis of Isolated Tumor Cell Subpopulations

Level 1. Analysis of volume density of cytoplasm, nucleus, and cytoplasm:nuclear ratio is shown in Table 2. Clearly illustrated are the differences in cytoplasmic apportioning in the 3 groups studied. The control group (dissociated cells, un fractionated) is intermediate (0.5937) to the GDF cells (0.5164) and the GEF cells (0.6537) in terms of cytoplasmic volume density. This is also seen for the nuclear volume densities. Level 2. Volume densities of rough endoplasmic reticulum, mitochondria, cytoplasmic matrix, nucleus, the Golgi complex, zymogen granules, and all other cytoplasmic components (including loose and tight condensing vacuoles, autophagic vacuoles, crystalloid bodies, and lipid droplets) are depicted in Table 3. Values for the volume densities of the various organelles in Type 1 through 4 cells are included for comparison.

Chart 2. Distribution of tumor cells in the GDF (△) and GEF (▲) according to the number of secretory granules in the cytoplasm. Ultrathin sections of cells, those with cell diameter greater than one-half of the maximum profile diameter of ~5 μm, in GDF and GEF were analyzed according to the number of secretory granules, and the percentage of cells with 0 to 4, 5 to 9, 10 to 19, and >20 granules were recorded.

Chart 3. Distribution of granule-deficient (Types 1 and 2) cells and granule-enriched cells (Types 3 and 4) in the GDF and GEF separated on Percoll. The distribution of granule-deficient and granule-enriched cells in the dissociated tumor cell population (unseparated) is shown for comparison.

<table>
<thead>
<tr>
<th>Cells studied</th>
<th>Points overlying</th>
<th>Volume density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Unseparated control</td>
<td>1518</td>
<td>1039</td>
</tr>
<tr>
<td>GDF</td>
<td>1324</td>
<td>1240</td>
</tr>
<tr>
<td>GEF</td>
<td>1866</td>
<td>989</td>
</tr>
</tbody>
</table>

\(V_r/n:V_cy\), nuclear:cytoplasmic volume ratio.
Dissociated pancreatic acinar carcinoma cells were isolated on Percoll gradients as described in “Materials and Methods.” A total of 24 high-power electron micrographs (× 17,000) were analyzed from 4 separate experiments for each cell group. The data are given as a percentage of total cell volume rather than the relative cytoplasmic volume because of the different cytoplasmic volumes that occur due to the heterogeneous nature of the pancreatic acinar carcinoma cells.

<table>
<thead>
<tr>
<th>Cell group studied</th>
<th>Rough endoplasmic reticulum</th>
<th>Golgi apparatus</th>
<th>Zymogen granules</th>
<th>Mitochondria</th>
<th>Other components</th>
<th>Cytoplasmic matrix</th>
<th>Total cytoplasm</th>
<th>Nucleus</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>14.9</td>
<td>3.6</td>
<td>5.7</td>
<td>9.2</td>
<td>1.6</td>
<td>26.9</td>
<td>61.9</td>
<td>38.1</td>
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<tr>
<td>GEF</td>
<td>12.5</td>
<td>6.2</td>
<td>19.2</td>
<td>7.3</td>
<td>0.8</td>
<td>22.5</td>
<td>68.1</td>
<td>31.9</td>
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<td>GDF</td>
<td>13.5</td>
<td>2.6</td>
<td>1.3</td>
<td>6.4</td>
<td>0.4</td>
<td>24.9</td>
<td>49.1</td>
<td>50.9</td>
</tr>
<tr>
<td>Type 1</td>
<td>12.0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.2</td>
<td>0.2</td>
<td>23.0</td>
<td>39.4</td>
<td>60.6</td>
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<tr>
<td>Type 2</td>
<td>14.4</td>
<td>1.1</td>
<td>0.2</td>
<td>8.1</td>
<td>0.4</td>
<td>26.4</td>
<td>50.6</td>
<td>49.4</td>
</tr>
<tr>
<td>Type 3</td>
<td>15.7</td>
<td>6.8</td>
<td>3.0</td>
<td>10.2</td>
<td>1.9</td>
<td>28.0</td>
<td>65.6</td>
<td>34.4</td>
</tr>
<tr>
<td>Type 4</td>
<td>12.6</td>
<td>6.2</td>
<td>21.8</td>
<td>7.3</td>
<td>1.0</td>
<td>22.2</td>
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</tbody>
</table>

Zymogen granule content of the 3 fractions studied shows that the GEF cells have approximately 4 times as much secretory product as the controls. GDF cells contain less than one-fourth of the amount of zymogen granules as compared to the control group. In addition, there are distinct differences in the volume densities of the Golgi complex when the 2 isolated fractions are compared. Comparison of the organelle volume density distributions of the isolated subpopulations to Type 1 to 4 cells yields the following observations. (a) The GEF volume density data best approximate those of type 4 cells. And (b) the GDF volume density data are somewhat intermediate to those of Type 1 and 2 cells.

**Enzyme Activities of Fractions**

The enzyme activities of amylase in the Percoll-isolated pancreatic acinar carcinoma cell subpopulations is presented in Chart 1. The peak activity of both enzymes assayed (lipase not shown) coincided with the fractions isolated routinely as GEF cells. The level of enzyme activity in GEF cells was 4 to 6 times higher than that of GDF cells. These values correlate well with the secretory granule data derived from the morphometric analyses (Table 3).

**Functional Characterization of Isolated Subpopulations**

**[3H]Leucine Incorporation into Protein.** The GEF and GDF incorporated [3H]leucine at linear rates up to 3 hr (Chart 4). Leucine incorporation of the 2 isolated fractions was compared to a mixed population (designated control) of cells reconstituted from a single gradient tube treated similarly to the isolated subpopulations. No significant differences in the rates of incorporation were observed in the 3 populations studied. Light-microscopic autoradiographic experiments of the 3 fractions following continuous labeling with [3H]leucine for 1 hr demonstrated that nearly 100% of the cells incorporate the precursor.

**[3H]Thymidine Incorporation into DNA.** The GEF and GDF incorporated [3H]thymidine at linear rates up to 3 hr (Chart 5). No significant differences in the rates of incorporation were observed in the GDF or GEF as compared to controls over the period studied. [3H]Thymidine nuclear labeling, as studied by autoradiography of semithin sections of the GEF, GDF, and control cells, showed labeling indices of 10, 16, and 13%, respectively. No significant differences (p > 0.05) were detected between these 3 groups.

**Electron-Microscopic Autoradiography Analysis of DNA Synthesis**

To ascertain the specific phenotype of cells in each fraction capable of incorporating [3H]thymidine into their DNA, isolated subpopulations were subjected to electron-microscopic autoradiography. A total of 165, 244, or 135 cells was counted for the mixed control population, the GEF cells, and the GDF cells, respectively. The control population showed a 17% labeling index, which is comparable to previous work in our laboratory for dissociated cells. The GDF cells demonstrated a 29% labeling index and the GEF cells 12%.

**Con A-binding Properties of the Acinar Carcinoma Cell Plasmalemma**

Con A-binding sites on the plasmalemma of both GDF and GEF cells were visualized by the Con A: peroxidase method (5,

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**Table 3**

**Level 2 morphometric analysis of subcellular compartments of granule-enriched and granule-deficient tumor cells isolated from Percoll gradients**

<table>
<thead>
<tr>
<th>% of cell volume</th>
<th>Cell group studied</th>
<th>Rough endoplasmic reticulum</th>
<th>Golgi apparatus</th>
<th>Zymogen granules</th>
<th>Mitochondria</th>
<th>Other components</th>
<th>Cytoplasmic matrix</th>
<th>Total cytoplasm</th>
<th>Nucleus</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>14.9</td>
<td>3.6</td>
<td>5.7</td>
<td>9.2</td>
<td>1.6</td>
<td>26.9</td>
<td>61.9</td>
<td>38.1</td>
<td></td>
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<tr>
<td>GEF</td>
<td>12.5</td>
<td>6.2</td>
<td>19.2</td>
<td>7.3</td>
<td>0.8</td>
<td>22.5</td>
<td>68.1</td>
<td>31.9</td>
<td></td>
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<tr>
<td>GDF</td>
<td>13.5</td>
<td>2.6</td>
<td>1.3</td>
<td>6.4</td>
<td>0.4</td>
<td>24.9</td>
<td>49.1</td>
<td>50.9</td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>12.0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.2</td>
<td>0.2</td>
<td>23.0</td>
<td>39.4</td>
<td>60.6</td>
<td></td>
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<tr>
<td>Type 2</td>
<td>14.4</td>
<td>1.1</td>
<td>0.2</td>
<td>8.1</td>
<td>0.4</td>
<td>26.4</td>
<td>50.6</td>
<td>49.4</td>
<td></td>
</tr>
<tr>
<td>Type 3</td>
<td>15.7</td>
<td>6.8</td>
<td>3.0</td>
<td>10.2</td>
<td>1.9</td>
<td>28.0</td>
<td>65.6</td>
<td>34.4</td>
<td></td>
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<tr>
<td>Type 4</td>
<td>12.6</td>
<td>6.2</td>
<td>21.8</td>
<td>7.3</td>
<td>1.0</td>
<td>22.2</td>
<td>71.1</td>
<td>28.9</td>
<td></td>
</tr>
</tbody>
</table>
Tumorigenicity

Results indicate the presence of Con A receptors in the plasma-subpopulations (Figs. 4, A and B). Control preparations (incubation without Con A or Con A plus 0.2 M methyl-α-D-mannopyranoside) demonstrated no membrane labeling. These results indicate the presence of Con A receptors in the plasma-lemma of the acinar carcinoma cells regardless of the extent of cytodifferentiation.

Tumorigenicity

All rats (5 for each group) which were given s.c. injections of $5 \times 10^6$ cells of GDF or GEF subpopulations developed tumors within 30 days of injection. The latency period was 4 to 7 days shorter in GDF-injected animals, but the tumor growth appeared similar in both groups once the masses became palpable. Additional studies are needed to examine the GDF and GEF interactions in tumor latency, growth, and differentiation.

DISCUSSION

The isolation of 2 tumor cell subpopulations by a self-generated gradient in Percoll was facilitated by the successful dissociation of the pancreatic acinar carcinoma into single cells by the combined mechanical and enzyme:cation chelator disaggregation method outlined in this paper. The self-generated gradient centrifugation in Percoll was found to be superior to discontinuous or preformed gradient separation of GEF and GDF cells. This procedure provided reproducible separation of 300 million cells in a 28-ml gradient. Although the purity of the GDF and GEF obtained appeared satisfactory, it is possible that the centrifugation of small numbers of cells might result in even highly purified subpopulations. Preparations of homogeneously dispersed single cells are critical to the isolation of relatively pure fractions of cells when the density difference between the GDF and GEF is $\sim 0.02$ g/ml. The pancreatic acinar tumor, which is very susceptible to damage by prolonged treatment with proteolytic enzymes, necessitated utilization of collagenase only, without other enzymes, in a buffer containing high levels of STI. The suspensions of freshly dissociated or gradient-separated GDF and GEF subpopulations of pancreatic acinar carcinoma cells are highly viable (>97%) and maintain the ability to synthesize DNA and protein, as exhibited by isotope incorporation studies. In addition, both GDF and GEF subpopulations obtained by Percoll centrifugation retain the ability to form tumors following s.c. injection.

The densities of 1.0987 and 1.0789 g/ml, respectively, at which GEF and GDF populations banded, were reproducible for each tumor suspension fractionated on self-generated gradients. The relative number of cells banding at each of these densities, however, did vary from tumor to tumor. This is not surprising in view of the heterogeneity observed in this tumor and has been reported for other systems as well (16, 18, 32, 34). Greater variability was noted with s.c. transplanted tumors; therefore, for these studies, only i.p. transplants, which contain less necrotic areas, were used. The density of GEF (1.0987 g/ml) was somewhat similar to that of the zymogen granule-rich acinar cells (1.11 to 1.17 g/ml) of normal pancreas (41), and has been reported for other systems as well (16, 18, 32, 34). Greater variability was noted with s.c. transplanted tumors; therefore, for these studies, only i.p. transplants, which contain less necrotic areas, were used. The density of GEF (1.0987 g/ml) was somewhat similar to that of the zymogen granule-rich acinar cells (1.11 to 1.17 g/ml) of normal pancreas reported by Blackmon et al. (7). In addition to the observed differences in density, the GEF and GDF were found to contain different levels of stored zymogen, as verified by both biochemical and morphometric analyses. Differences in nuclear:cytoplasmic ratio, mean cell volume, size, and distribution of organelles were also evident. No significant differences ($p > 0.05$) were noted between rates and levels of DNA synthesis, protein synthesis, and mitotic indices between control, GEF, and GDF cells.

Reddy et al. (41) discussed the significance of nondiscriminatory mitotic indices for the heterogeneous population of cells in this neoplastic secretory epithelium. It was shown that cells, regardless of the degree of cytodifferentiation, are capable of DNA synthesis and cell division. Accordingly, it was suggested that pancreatic acinar carcinomas are derived from the mature acinar cell and not from an as yet identifiable stem cell. Several studies dealing with the histogenesis of experimentally induced pancreatic carcinomas in the guinea pig (37, 38), rat (8), and hamster (14, 43) appear to substantiate the contention that mature acinar cells can acquire immature phenotypic features as a result of continuous proliferation. The demonstration of Con A receptors on all cells in the GDF and GEF subpopulations of pancreatic acinar carcinoma in the present study further substantiates the dedifferentiation concept, because during normal pancreatic embryogenesis Con A receptors are discerned only in acinar cells containing mature secretory granules (25). Previous studies with $^{125}$I-labeled Con A demonstrated essentially the same total number of plasmalemma Con A receptors for unfractionated acinar carcinoma cells and normal pancreatic acinar cells (49), although only about one-third of pancreatic acinar carcinoma cells contain well-formed zymogen granules (Type 4 cells). Quantitative analyses of cell surface characteristics of the GDF and GEF using a battery of pancreatic cell-specific lectins may provide relevant information about differentiation and malignant phenotype. During early stages of embryogenesis, undifferentiated epithelial cells...
(the protodifferentiated stage) do not possess Con A-binding sites (25). The failure to find neoplastic cells devoid of Con A receptors (i.e., a progenitor cell) among the GDF and GEF subpopulations of pancreatic acinar carcinoma suggests either the absence of a readily discernible stem cell pool in this tumor or that neoplastic transformation results in altered genetic expression whereby the undifferentiated “stem cells” which lack Con A receptors during normal pancreatic embryogenesis acquire such receptors during transformation.

Isolation of morphogenetically and biochemically distinct subpopulations of neoplastic pancreatic epithelial cells should enable delineation of factors controlling differentiation and growth in this neoplasm. Miller et al. (26, 27) recently reported that the growth characteristics of particular subpopulations of a mouse mammary tumor, as well as sensitivity of those subpopulations to certain antineoplastic agents, depends upon interactions between such heterogeneous subpopulations in the same tumor. Whether differentiation potential of individual subpopulations of neoplasms is also influenced by such subpopulation interactions remains to be determined. The wealth of markers that exist for this carcinoma will permit examination of the development of macromolecular processing of exportable proteins (48) and differentiation of plasma membrane receptors involved in its previously documented secretory responsiveness (47, 50). Since this neoplasm is heterogeneous in nature, differential 2-dimensional electrophoretic analysis of the secretory proteins (42) as well as total cell proteins will allow for greater resolution of the differences in genetic expression exhibited by the GEF and GDF. The studies of tumorigenic and metastatic potential of these isolated subpopulations should provide further insight into the role of differentiation in neoplasia and the interaction of host defense mechanisms in their control.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Light microscope appearance of dissociated neoplastic pancreatic acinar cells isolated on Percoll gradient. A, high-power photomicrograph of granule-enriched cells. Note the presence of abundant secretory granules within the polarized cytoplasm and eccentrically placed nucleus. Nomarski differential interference contrast, toluidine blue, × 2300. B, high-power photomicrograph of granule-deficient cells; the cells contain much less secretory product. The nucleus is more centrally located, and cytoplasm is less evident. Nomarski differential interference contrast; toluidine blue, × 2500.
Fig. 2. Electron microscope appearance of granule-enriched cells isolated from Percoll gradient. These neoplastic acinar cells are well differentiated, contain numerous secretory granules, well-formed Golgi complex (arrow), and an eccentrically placed nucleus. × 6500.

Fig. 3. Electron microscope appearance of granule-deficient cells isolated from Percoll gradient. These neoplastic acinar cells are poorly differentiated and contain no secretory granules. Nuclei are more centrally located. × 6500.
Fig. 4. Electron microscope appearance of isolated subpopulation labeled with Con A:peroxidase method. A, GEF cells, × 7400; B, GDF cells, × 6600.
Separation and Characterization of Neoplastic Cell Subpopulations of a Transplantable Rat Pancreatic Acinar Carcinoma

Michael J. Becich and Janardan K. Reddy