Culture of Squamous Head and Neck Cancer on 3T3 Fibroblasts following Isokinetic Velocity Sedimentation

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ABSTRACT

Growth in culture of squamous head and neck cancer is hampered by microbial contamination, low plating efficiency, and cellular heterogeneity within tumors. Furthermore, clumps of cells must be removed if plating efficiency is to be accurately determined. Isokinetic velocity sedimentation was applied to 44 primary tumor specimens in an effort to minimize these problems. Seven fractions were evaluated for cell number, clump number, cell viability, clonogenic growth, plating efficiency, and microbial overgrowth. Unseparated specimens were simultaneously cultured. Microbial growth was significantly associated with the highest gradient fraction. Clumps were significantly associated with the lowest gradient fraction. Colony formation was significantly associated with middle gradient fractions. Cloning efficiency was not significantly improved by isokinetic velocity sedimentation, although seven specimens grew only when fractionated, suggesting the possibility of inhibitor cells within the tumor specimen.

INTRODUCTION

Successful culture of squamous head and neck cancer in a colony-forming assay is challenging. Five major problems can be identified. (a) Limited tissue is available from approximately one-third of tumors that are detected in early stages. (b) Cloning efficiency in primary cultures is low even when colony formation occurs (1). When a colony-forming assay is used to predict chemotherapy sensitivity, large numbers of colonies are necessary to determine sensitivity reliably (2). The problems of low plating efficiency and small tumor size compound each other in this regard. (c) Bacteria and fungi naturally colonize head and neck cancers, and necrotic areas within tumors are often infected. Thus, many cultures are overgrown by microbes (3–6). This problem can be attenuated by adding antibiotics to the culture medium. However, such drugs may be cytostatic for mammalian cells in culture and may compromise plating efficiency. (d) The unrecognized presence of cell clumps in the initial culture suspension renders results of colony-forming assays unreliable (2). Squamous head and neck cancer cells are tightly adherent to each other. Vigorous techniques of dissociation are required to obtain a single cell suspension. However, this may damage cells and lower plating efficiency. Sieves or nylon mesh may be used to reduce the number of clumps, but many single cells are also lost in this process. (e) The population of cells within the tumor is heterogeneous. Tumors contain blood, lymphoid, mast, and stromal cells, in addition to cancer cells. The presence of nonmalignant cells artificially decreases the plating efficiency of primary cultures. Furthermore, some nontumor cells may inhibit colony formation (7, 8).

Isokinetic velocity sedimentation separates particles by density and size. At constant (isokinetic) velocity cells sediment linearly with respect to density and according to the square of their diameters (9). If clonogenic cells have densities or diameters substantially different from nonclonogenic cells, then isokinetic velocity sedimentation should separate clonogenic cells from nonclonogenic (potentially inhibiting) cells. Microbes, which have low densities and small diameters, should sediment in upper gradient fractions. Clumps of cells, which have large diameters, should sediment in lower gradient fractions. We describe culture of squamous cell carcinoma of the head and neck before and after isokinetic velocity sedimentation and show that the technique enriches microbes and cell clumps in fractions which are generally less clonogenic. The results also suggest that a population of cells within the tumor may inhibit colony formation.

MATERIALS AND METHODS

Obtaining Tumor Specimens. Solid tumor specimens were harvested from the surgical block immediately after tumor resection. These specimens were placed in cold McCoy's medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 1% penicillin/streptomycin (GIBCO), and 1% Fungizone (GIBCO) and transported expeditiously to the laboratory. All head and neck cancers studied arose from mucous membrane. All patients received a loading dose of a cephalosporin antibiotic approximately 6 to 8 h prior to specimen harvest. Forty specimens were obtained from the primary site, and four were from metastatic regional nodes.

Disaggregation of the Tumor Specimen. The tumor specimen was carefully dissected free of gross nontumor tissue. Normal-appearing adjacent mucosa was discarded. One g of tumor was cut with scalpels into 1- to 2-mm pieces and suspended in a 25-ml trypsinizing flask which contained 15 ml of an enzyme solution of DNase (800 units/ml; type I from bovine pancreas; 2470 Kunitz units/mg solid; Sigma) and collagenase (800 units/ml; sterile IA-S; 290 units/mg solid; Sigma). The diluent consisted of McCoy's Medium 5A (GIBCO). The flask was placed in an incubator for 90 min on a magnetic stirrer at the lowest possible speed. The incubator was maintained at 37°C, and the internal atmosphere was humidified and supplemented with 5% carbon dioxide. Subsequently the specimen was washed twice in the diluent. Cell viability was assessed by trypan blue exclusion.

Gradient Construction. Ficoll (Pharmacia) is refrigerated as a dry powder and used within 3 mo of purchase. Two hundred g of Ficoll are dissolved in 260 ml of water during a maximum period of 5 h at 4°C in a 1-liter beaker. The cold, sterile water is added slowly over 15 min while stirring constantly. Lumps of Ficoll will rise and can be moistened with an isotonic solution is diluted in 65 ml of sterile water. After bubbles have disappeared 26 ml of Dulbecco's modified Eagle's medium solution are added to the Ficoll solution, and the volume of this final solution is raised to 400 ml with sterile water. Sodium bicarbonate is added according to the package instructions of Dulbecco's modified Eagle's Medium.
medium. Aliquots of the final solution (23%, w/v, of Ficoll) are stored at −20°C for up to 3 mo. Thawed material is used within 2 days.

Sterile 100-ml test tubes (IEC No. 2806) containing 5 ml of the 23% (w/v) solution of Ficoll are clamped to a stand at a 30-degree angle. Using a gradient marker and peristaltic pump, the gradient is constructed at a rate of 0.5 ml per min (10). The linear gradient (g/ml-cm) varies from 2.7% (w/v) Ficoll at the sample-gradient interface (18 cm from the center of revolution) to 5.5% (w/v) Ficoll at the cushion-gradient interface (31 cm from the center of revolution). Gradients are refrigerated and used within 48 hr of construction. A sample of 15 to 30 million cells in 4 ml of cold Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum is slowly layered atop the gradient.

Gradient Centrifugation. Full speed is reached over 45 s, accelerating to a force of 5 × g over the first 15 s, to 25 × g over the next 15 s, and to the desired speed over the remaining 15 s. The specimen is centrifuged for 10 min with a force of 150 × g at the sample-gradient interface. The brake is turned off, and unused cups are filled with full 250-ml centrifuge bottles to maximize rotor mass, thereby minimalizing swirling of the gradient during changes in angular velocity.

Harvesting Cells. Cells are harvested in an ice bath to minimize convection. A previously described tapping cap allows the layering of 60% sucrose beneath the sample and the collection of fractions from the top of the tube (10). Cells from each 12-ml fraction are centrifuged at 200 × g for 5 min, and the pellet is resuspended in squamous culture medium. To assure the linearity of the gradient, the refractive index of each fraction supernatant is checked and converted to percentage of sucrose (Ficoll) using a previously described table (11).

Viability and Recovery of Cells. Duplicate aliquots are taken from each fraction before centrifugation and resuspension and from the unseparated specimen for viability determination and cell enumeration. Cells are incubated for 4 min in trypan blue prior to determining viability. Absolute recoveries are calculated as follows.

\[
\text{Absolute recovery} = \frac{\text{no. of cells recovered from gradient}}{\text{no. of cells layered on gradient}} \times 100
\]

The number of aggregates observed during a 100-cell count is also recorded. A clump is defined as a collection of 2 or more cells that are adherent to each other.

Maintenance of 3T3 Fibroblasts. Swiss mouse embryo-derived 3T3 fibroblasts were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium (No. 320-1965; GIBCO, Grand Island, NY) supplemented with 10% calf serum. Stock cultures were fed twice weekly and subcultured by trypsinization with 0.1% trypsin for 5 min at 37°C and washing vigorously with Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. Stock cultures were not allowed to become confluent.

Preparation of 3T3 Feeder Layers. Fibroblasts were placed in 10- x 35-mm Petri dishes (Lux; Miles Laboratories, Naperville, IL) at one-third confluent density on the day before harvesting the specimen. Confluent density is 55,000 cells/cm². Thus 150,000 cells were placed in each 10- x 35-mm Petri dish (surface area, 8 cm²). The following morning, after fibroblasts had adhered to the dish, 3T3 cells were treated with mitomycin C (Bristol, Syracuse, NY) at a concentration of 4 μg/ml in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum for 30 min at 37°C. This renders the cells incapable of dividing. Mitomycin C was thoroughly washed from the plates using several rinses of Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. One ml of squamous cell culture medium was added, and the plates were returned to the incubator.

Squamous Head and Neck Cancer Culture Medium. Squamous culture medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 25% Ham’s F-12 nutrient (No. 320-1765; GIBCO), 5% fetal calf serum (GIBCO), penicillin (100 units/ml), streptomycin (100 μg/ml) (GIBCO), amphotericin B (2.5 μg/ml) (GIBCO), hydrocortisone (0.4 μg/ml) (Calbiochem, San Diego, CA), regular insulin (5 μg/ml) (Eli Lilly, Indianapolis, IN), 100 μM cholera enterotoxin (Schwartz-Mann, New York, NY), 20 μM triiodothyronine (Sigma, St. Louis, MO), transferrin (5 μg/ml) (Sigma), and 180 μM adenosine (Sigma).

Cell Plating Technique and Culture Conditions. Specimens were cultured in 2 ml of squamous culture medium on mitomycin C-treated 3T3 feeder layers. In order to determine the effect of cell density on plating efficiency, all cells obtained in a given fraction were cultured in the same dish during the first 30 experiments. In the remaining 14 experiments 500,000 viable cells from each fraction were cultured per dish. If fewer than 500,000 viable cells were present in a fraction, then all cells in that fraction were inoculated. An aliquot of 500,000 viable cells from the unseparated specimen was cultured simultaneously. Cultures were maintained in a 5% carbon dioxide in air, humidified, 37°C incubator. Spent medium was replaced twice weekly for 1 mo.

Colony Counting and Definition. Whole plate counts were performed at ×40 magnification twice weekly. A colony was defined as a collection of 30 or more cells growing in a closely knit cobblestone pattern on the surface of the culture vessel (Fig. 1). Colony counting was discontinued when two colonies became confluent (Fig. 2) or if no colonies were observed by Day 28.

Statistical Methods. Cloning efficiencies were calculated by dividing the number of colonies by the product of the number of cells plated and fraction of trypan blue-excluding cells and multiplying this figure by 100%. Means, standard errors of the mean, and standard deviations were calculated for 8 groups of data and 5 variables in each group. The groups were the unseparated aliquot and 7 gradient fractions. Fraction 1 was closest to the center of revolution, and Fraction 7 was farthest, representing the highest and lowest gradient fractions, respectively. The variables were cell number, cell viability as a percentage (the number of cells capable of excluding trypan blue divided by the total number of cells counted, multiplied by 100%), number of colonies formed, number of clumps present, and plating efficiency. Each group was also evaluated
VELOCITY SEDIMENTATION AND CULTURE OF HEAD AND NECK CANCER

Fig. 2. Colony counting was discontinued when two colonies became confluent, as demonstrated here, or when no colonies were observed by Day 28. × 200.

as to presence or absence of microbial overgrowth and presence or absence of colony formation.

The 5 variables were compared by a repeated-measures analysis of variance, \( \alpha = 0.05 \). Dichotomous data (presence or absence of microbial growth and presence or absence of colony formation) were analyzed by the Mantel-Haenszel \( \chi^2 \) test, which allowed control for repeated measurements. If fewer than 25% of the contingency table’s compartments had expected values less than 5, the 2-tailed Fisher’s exact test was applied.

RESULTS

Microbial Contamination. Two specimens from the unseparated group (5%) were overgrown with microbes. Thirty-six fractions were overgrown. Significant differences in microbial overgrowth among groups were confirmed \( (P = 0.0009) \). The most significant differences were found with comparisons to Fraction 1, where microorganisms were highly concentrated. Thirty-six % of first fractions were overgrown, and 42% of microbial overgrowth observed among all 8 groups occurred in Fraction 1 (Table 1).

Cell Number and Viability. The refractive index of each fraction increased linearly from the sample-gradient interface to the gradient-cushion interface. The mean number of cells layered onto gradients was 26.9 million. Table 1 shows that the distribution of cell numbers among fractions is skewed toward the top of the gradient. Fractionation resulted in significant viability differences among groups \( (P < 0.0001) \). Central gradient fractions were more viable than fractions at gradient extremes. Unseparated cell viability was not significantly different from that observed in the fractions with the greatest number of viable cells (Fractions 3 to 6). Recovery of cells layered on the gradient ranged from 21.2% to 99.8%. The mean recovery was \( 48.1 \pm 18.1\% \) (SD).

Cell Clumps. Clumps of cells sedimented through the gradient. As expected (Table 1), lower gradient fractions contained significantly greater numbers of clumps than upper fractions \( (P = 0.0001) \).

Colony Formation. The plating density did not affect the number of specimens which formed colonies. Among specimens which did form colonies there were no differences in plating efficiency for Fractions 1, 2, 4, 5, 6, and 7 \( (P = 0.29 \) to \( P = 0.97) \). However, in Fraction 3 significantly greater plating efficiencies were observed when only 500,000 viable cells were plated (plating efficiency, 0.0013 versus 0.00015; \( P = 0.0003) \). The mean number of viable cells cultured per plate at the higher density was 1,910,908.

Eighteen specimens from the unseparated group formed colonies (43%). There were 65 instances of colony formation among fractions from specimens. The poorest growth was observed in Fractions 1, 6, and 7 (Table 2) and accounted for significant differences among groups \( (P = 0.01) \). Microbial overgrowth did not account for these differences as cultures which were lost to contamination were eliminated from this analysis.

In three instances growth was observed in the unseparated specimen only. In one of these instances every fraction from the gradient was contaminated, but in the other two, no fractions were contaminated. In seven instances growth was observed in fractions, and no growth occurred in the corresponding unseparated control culture. Such growth was observed in

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of specimens</th>
<th>No. with microbial overgrowth</th>
<th>% of specimens in this fraction which were overgrown</th>
<th>% of all microbial overgrowth observed</th>
<th>( 10^{-4} \times ) cell no.</th>
<th>% of viability</th>
<th>( 10^{-4} \times ) viable cell no.</th>
<th>No. of clumps/100 cells</th>
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<td>1</td>
<td>44</td>
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<tr>
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<td>44</td>
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<td>5</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* U. tumors cultured before isokinetic velocity sedimentation.
* Significantly less than Groups 1, 2, 3, and 4.
* Significantly less than Groups 1, 2, 3, and 4.
* Significantly less than Groups 6 \( (P = 0.02) \) and 7 \( (P = 0.0002) \) (based on the Tukey multiple-comparison procedure).
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Tumors were cultured before and after isokinetic velocity sedimentation (Fractions 1 to 7). The presence of colony formation was recorded. Groups which were overgrown by microbes were eliminated from these calculations, since colony formation cannot be observed when specimens are overgrown by microorganisms.

<table>
<thead>
<tr>
<th>Group</th>
<th>U*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>No. of specimens</td>
<td>42</td>
<td>28</td>
<td>38</td>
<td>41</td>
<td>41</td>
<td>42</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>No. with colony formation</td>
<td>18</td>
<td>3</td>
<td>12</td>
<td>14</td>
<td>13</td>
<td>12</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>% of specimens in this fraction with colony formation</td>
<td>43</td>
<td>10</td>
<td>32</td>
<td>34</td>
<td>32</td>
<td>29</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>% of all colony growth observed in this group</td>
<td>22</td>
<td>4</td>
<td>14</td>
<td>17</td>
<td>16</td>
<td>14</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt; x plating efficiency</td>
<td>19.0</td>
<td>6.0</td>
<td>6.9</td>
<td>5.0</td>
<td>7.9</td>
<td>8.4</td>
<td>27.5</td>
<td>31.9</td>
</tr>
<tr>
<td>P value; plating efficiency of U vs. fraction</td>
<td>0.13</td>
<td>0.01</td>
<td>0.04</td>
<td>0.08</td>
<td>0.04</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
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</table>

* U, tumors cultured before isokinetic velocity sedimentation.

Fractions 2 (twice), 3 (6 times), 4 (3 times), 5 (4 times), 6 (once), and 7 (twice). None of the corresponding unfractonated control cultures became contaminated. In addition, there were no significant differences in the percentage of viability or absolute viable cell number between unseparated control cultures and fractions among these seven specimens.

Only one of the specimens taken from lymph node formed colonies, and growth from that specimen was observed only in Fractions 3 and 4. Twenty-five specimens taken from the primary site formed colonies. Higher plating efficiencies were observed in unseparated control cultures compared with Groups 2 to 5 (P = 0.007) (Table 2).

DISCUSSION

Squamous head and neck cancer is notoriously difficult to propagate in vitro. Investigators participating in a multiinstitutional contract study were least successful in growing this solid tumor (2). The human tumor colony-forming assay is suboptimal for culturing squamous head and neck cancer, because it does not provide anchorage or fibroblast support, factors known to enhance growth of squamous cancers (12). Surface culture methods have resulted in successful establishment of squamous head and neck cancer in vitro (3, 4, 6, 12–17). However, even in an assay using fibroblast feeder layers and surface culture (12), growth rates and plating efficiency of primary cultures are low (1). Isokinetic velocity sedimentation has been successfully applied to purification of stromal cells from malignant and parenchymal cells of a variety of tumors and other tissues (18–23). It has also been used to remove contaminating microbes from human colon cancers (18). This is the first time, to our knowledge, that isokinetic velocity sedimentation has been applied to squamous head and neck cancer. Several problems which affect growth rates are addressed by isokinetic velocity sedimentation.

(a) Tumors arising from upper aerodigestive tract mucosa are colonized by bacteria and fungi, and they may be infected as well. We have often observed bacteria within polymorphonuclear leukocytes from these tumors. As in this study, prophylactic antibiotics are frequently recommended for head and neck cancer surgery (24). The number of contaminated cultures is lessened by adding penicillin, streptomycin, and amphotericin to the culture medium, but these antibiotics may decrease plating efficiency. We have effectively enriched microbes in the upper fractions of the gradient for our head and neck cancers using isokinetic velocity sedimentation. Fractions 1 and 2 contained 58% of all microbial overgrowth observed among the eight groups. Isokinetic velocity sedimentation cannot completely eliminate microbial overgrowth, since intracellular microbes and microbes in intercellular locations within clumps of cells will sediment beyond Fractions 1 and 2.

Sterile technique is essential during preparation of Ficoll and Ficoll gradients and during tapping of the gradient. Ficoll is a good culture medium for microorganisms. We observed instances where no microbial growth occurred in unseparated control cultures, but every fraction was overgrown by microorganisms. The gradient was probably contaminated in those cases. Furthermore, a 60% sucrose solution is used to tap off the gradient. Stored at room temperature, this solution can become contaminated and should be prepared in small aliquots to be used only once. While isokinetic velocity sedimentation does not eliminate, it does attenuate the problem of microbial overgrowth in squamous head and neck cancer. It would be interesting to determine the effect of antibiotic concentration on plating efficiency in Fractions 3 to 7, the fractions most depleted of microbes.

(b) Cell clumps artificially inflate plating efficiency, and as a result, the effectiveness of chemotherapeutic agents may be judged incorrectly (2, 25). Isokinetic velocity sedimentation enriches fractions at the bottom of the gradient with cell clumps. The effect of clumps on plating efficiency can be appreciated by examining Table 2. Cell clumps increase progressively from the top to the bottom of the gradient as does plating efficiency. However, the major increase in plating efficiency occurs in Fractions 6 and 7 where multicellular aggregates are found. Our definition of a clump was very rigorous (2 or more cells). It may be that dimers and trimers found above Fraction 6 do not substantially alter plating efficiency. In addition, the plating efficiency in the unseparated fraction was intermediate between plating efficiencies at gradient extremes, suggesting that colonies in the unseparated specimen controls largely arose from clumps.

Clumps are usually removed by passing the dissociated specimen through a metal sieve or nytex mesh. We have used a sieve with 25-μm pores and obtained a relatively pure single cell suspension, but 50% of single cells were lost during the separation (26). Unfortunately isokinetic velocity sedimentation also removes single cells. On the average, 51.9% of single cells were lost. The cells may have adhered to the test tube wall or been destroyed during centrifugation. Perhaps, use of other gradient media, such as Percoll, would improve recovery of cells (27). However, the viscosity of Percoll is not reproducible from batch to batch. The consistent viscosity of Ficoll, when used at concentrations varying linearly between 2.7 and 5.5%, allows cells to sediment at constant velocity through the gradient despite the linear increase in centrifugal force resulting from increments in distance from the center of revolution. Therefore, with isokinetic velocity sedimentation cells are separated purely on the basis of density and diameter (9). This basis for separation is important, since clonogenic cells have been shown to have distinct densities and diameters in at least one human carcinoma (28).

(c) The process of isokinetic velocity sedimentation may affect squamous cell growth. Previously, we cultured 22 squamous cell carcinomas of the head and neck on 3T3 cells. Using
43% of the unseparated specimens formed colonies in the experiment, which may have adversely affected growth rate as the cells are in suspension. Suspension of human keratinocytes may be due to prolonged suspension time. During most of the 5 h present series. The low growth rate observed in this series may well. In our previous series, specimens were processed on the day of surgery. We do not believe that Ficoll is toxic to cells, since many more specimens grew only when fractionated than grew only in the unfractonated control cultures. Plating density in the narrow range studied here had little effect on plating efficiency until almost 2 million cells were cultured per dish (Fraction 3 in the first 30 specimens).

(d) Nontumor cells may inhibit colony formation. There were seven instances where colonies formed only after fractionation. In those cases, the lack of colony formation in the unseparated control cultures cannot be ascribed to microbial contamination or to differences in cell viability, absolute viable cell number, or total cell number plated ($P > 0.05$). Our data suggest that cells which inhibit colony formation may have been removed by isokinetic velocity sedimentation. The same phenomenon has been observed after isokinetic velocity sedimentation of colon cancer and after Ficoll-Hypaque separation of sarcomas, melanoma, and colon cancer (18, 31). Such cells have been identified in other tumor types (7, 8). Purification of those cells and subsequent add-back experiments which demonstrate inhibition of colony formation are necessary to prove this hypothesis in our culture system.

In summary, we have described isokinetic velocity sedimentation of squamous cell carcinoma of the head and neck for the first time. The technique enriches microbes and cell clumps in opposite gradient extremes and enriches viable cells in the center of the gradient. A population of clonogenic cells with distinct physical properties was not identified. Growth occurred only after fractionation in seven of our tumors and suggested that cells may be present in squamous head and neck cancers which are able to inhibit clonogenic growth of cancer cells in vitro.

REFERENCES

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