A Method for Dissociation of Viable Human Breast Cancer Cells That Produces Flow Cytometric Kinetic Information Similar to That Obtained by Thymidine Labeling

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ABSTRACT

Collagenase dissociation, performed on 40 human breast cancers, yielded between 1 million and 50 million cells from less than 1 g of tissue from each tumor. Approximately 60% of cells (mean) was considered viable as judged by trypan blue exclusion and phase microscopy. On subsequent flow cytometric analysis, 20 cancers (50%) were considered diploid, three were tetraploid, and the remainder, hyperdiploid. Thymidine labeling (TLI) and flow cytometry following mechanical dissociation also were performed on 23 of these 40 tumors. Among this group of 23 cases, the median percentage of S-phase cells obtained by collagenase dissociation was 5.4, by TLI was 5.7, and by mechanical dissociation was 9.7. There was excellent correlation between the percentage of S-phase cells obtained by collagenase and TLI (r = 0.847, p = 0.0001) but only fair correlation between the percentage of S-phase cells obtained by mechanical dissociation and TLI (r = 0.597, p = 0.0027). The percentage of S-phase cells obtained by either collagenase or mechanical dissociation predicted whether a tumor was above or below median TLI in 19 of 23 cases (p = 0.0018). Estrogen receptor positivity or negativity did not predict whether a tumor was above or below median TLI (r = 0.283, p = 0.130) or above or below median S-phase fraction following collagenase dissociation (r = 0.218, p = 0.182), nor did quantitative estrogen receptor correlate significantly with TLI (r = 0.283, p = 0.13) or S-phase fraction (r = 0.218, p = 0.18).

INTRODUCTION

Various investigators have tried to identify characteristics of human breast cancers that will predict subsequent chance of relapse, utilizing morphological techniques, estrogen receptor status, and cell kinetic measurements that use thymidine labeling (8–10, 16, 18, 21). Such information is potentially useful in identifying patients who might profit from adjuvant chemotherapy. Among different morphological parameters that have been studied, the presence or absence and extent of axillary lymph node involvement by metastatic cancer have proven to correlate best with chance of breast cancer relapse (22, 23, 28). Although there appears to be some degree of correlation between estrogen receptor positivity or negativity and chance of relapse, the degree of correlation is insufficient to permit confident identification of a high-risk group (10). In contrast, the percentage of breast cancer cells that are synthesizing DNA (SPF), measured by thymidine labeling, has shown an excellent correlation with chance of relapse. For example, among 227 patients with operable breast cancer treated by total mastectomy and axillary dissection at our institution, those who had tumors with above median TLI had a 52% probability of relapse at 4 years, in contrast to a 20% probability of relapse at 4 years for patients with tumors with below median TLI (p = 0.0001) (18).

Thymidine labeling rarely has been adopted as a routine laboratory procedure, however, in spite of its potential usefulness. Counting of labeled tumor cells in autoradiographs is time consuming and tedious. Furthermore, it is usually done by a pathologist because tumor cells must be distinguished from other cells in the histological sections used for autoradiography. In addition, the results are not immediately forthcoming because as much as a week may be required for exposure of autoradiographs.

As an alternative to thymidine labeling, we have investigated whether similar kinetic information can be obtained by flow cytometry, following mechanical or enzymatic cell disaggregation utilizing collagenase. A relatively large number of cells can be analyzed rapidly utilizing flow cytometry and the results made available within an hour or 2 of receipt of the biopsy specimen. In addition, the collagenase dissociation technique produces a significant yield of intact, viable cells that can be used for other investigative purposes. Flow cytometry also permits us to obtain information concerning tumor ploidy which may be of significance in predicting chance of breast cancer relapse (23).

MATERIALS AND METHODS

Cell Dissociation. Unfixed, 100- to 500-mg samples of tumor were obtained immediately after excision. Specimens were trimmed to remove surrounding breast tissue and fat and cut into multiple 0.5-mm-thick slices, using a Stadie-Riggs microtome (A. H. Thomas Co., Philadelphia, PA). Slices of tumor were pressed onto 3.5- x 7.5-cm Velcro strips (No. 65 hook; Admiral Hospital Supply, St. Louis, MO) which had been placed in sterile 15-ml centrifuge tubes so as to form a lining of the internal surface of the tube, with the hooks facing inward (13). Seven and one-half ml of type IV collagenase (5 mg/ml) (Worthington Biochemicals, Freehold, NJ) in Hanks' balanced salt solution plus 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) at 37° were added to each centrifuge tube. The tubes were sealed with Parafilm and placed in a mechanical shaker (Burrell wrist action) in a 37° water bath. Following 5 min of shaking at approximately 200 oscillations/min, the tubes were removed from the shaker, and the collagenase solution was decanted from the immobilized tissue slices. Cells were pelleted by centrifugation at 200 x g for 30 sec, and cell yield was estimated by examining an aliquot in a hemocytometer counting chamber. The cell-free collagenase solution was then returned to the centrifuge tubes containing the tissue

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slices, and the tubes were resealed with Parafilm and shaken for an additional 5 min. If, after 10-min shaking with the collagenase solution a sufficient number of cells were not dissociated, a fresh collagenase solution (5 mg/ml) was added, and the process was repeated. Breast tumor specimens were almost completely dissociated within 15 min using this procedure. Digestion was monitored by inspecting the tissue slices after each 5-min period of shaking. As digestion progressed, the tissue slices diminished in size until only partially digested collagen fragments remained.

The dissociated cells were pooled and washed once in Hanks’ balanced salt solution plus 10% fetal calf serum (Grand Island Biological Co. Diagnostics, Madison, WI). An aliquot of the cell suspension was incubated at room temperature for 10 min with trypan blue, diluted to a final concentration of 0.2 mg/100 ml in phosphate-buffered saline. Viability was estimated by counting 100 cells, utilizing phase-microscopic microscopic appearance and trypan blue exclusion as indices of viability. A second aliquot was fixed in 70% ethanol and used for CytoSpin (Shandon Southern Products, Ltd., Astmoor, England) preparations that were stained with hematoxylin and eosin and examined by light microscopy. The remaining cells were resuspended at 200 × 10^6 cells/ml for 5 min and resuspended in a modified Krishan’s propidium iodide solution at a concentration of 106 cells/ml reagent (11). The modified Krishan’s propidium iodide solution contained 0.005% propidium iodide (Calbiochem-Behring, San Diego, CA), 0.002% RNase A (Sigma Chemical Co., St. Louis, MO), 3.0% Nonidet P40 (Sigma), and 0.1% sodium citrate. After 30-min incubation at 4°, the resulting nuclei were resuspended at 200 × 10^6 for 5 min and resuspended in fresh modified Krishan’s propidium iodide solution for flow cytometric analysis.

A control for the collagenase method of dissociation was prepared by scraping the freshly cut surface of each tumor with the end of a glass slide held at about a 20° angle from the vertical. Cells were washed into a Petri dish and seeded to Hanks’ balanced salt solution, pelleted at 200 × g for 5 min, resuspended in modified Krishan’s propidium iodide solution, and stained as noted above.

**Ficoll-Hypaque Separation.** To 20.0 ml distilled water were added 42.54 ml Hypaque (sodium Hypaque, 50%; Winthrop Laboratories, New York, NY) and 150.12 ml 9% Ficoll (Sigma) in distilled water (14). Ten ml of the above mixture were placed in a round-bottomed, polycarbonate centrifuge tube (Fisher Scientific Co., Pittsburgh, PA). An aliquot of cells obtained by collagenase dissociation was resuspended in 10 ml Hanks’ balanced salt solution with 10% fetal calf serum and carefully layered on top of the Ficoll-Hypaque mixture. Following centrifugation at 500 × g for 15 min at room temperature, cells at the Ficoll-Hypaque interface were removed with a Pasteur pipet and transferred to a 50-ml centrifuge tube. The cells were washed twice with Hanks’ balanced salt solution plus 10% fetal calf serum and split into 3 fractions for trypan blue exclusion, CytoSpin preparation, and Krishan staining for flow cytometry.

**Flow Cytometry.** Propidium iodide-stained nuclei were filtered through a 44-μm nylon mesh (Small Parts, Inc., Miami, FL) and analyzed on a Coulter EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon laser source. Dye excitation was at 488 nm with fluorescence emission measured through a 515-nm-long pass interference, a 515-nm-long pass absorption, a 590-nm dichroic, and 590-nm-long pass adsorption filters. DNA histograms were derived from an analysis of 100,000 cells.

**Histogram Analysis.** Histogram analysis was performed on a TERA LSI/1123 microcomputer utilizing a software program (PARA 1) written by Dr. C. B. Bagwell (Coulter Electronics). This program was used to determine the channel numbers at which various peaks were located, the coefficient of variation of the G0-G1 peak, and the total number of events in the G0-G1 and the G2-M compartments of the histogram. The percentage of S-phase cells was calculated by means of a rectilinear model that used the mean G0-G1 and G2-M channel numbers as its lower and upper boundaries and an integration of a 7-channel sample taken midway between the upper and lower boundaries of the G0-G1 and G2-M peaks to determine height (2). Initially, chicken RBC were used as an internal reference standard. The 2n channel number was calculated on the assumption that chicken RBC had a DNA content of 35% of the human diploid value (27). More recently, both trout and chicken RBC have been used as internal standards, and the 2n channel number was calculated by establishing a ratio between the trout and chicken RBC channel numbers that would accommodate the assumptions that chicken RBC are 35% human diploid and trout RBC are 80% human diploid (27).

**Estrogen Receptor.** Estrogen receptor was measured by the dextran-charcoal method described previously (20). Cytosols with saturable binding of 10 or more fmol 17β-[3H]estradiol per mg protein were considered positive for estrogen receptor. Cytosols with a saturable binding of 99 or more fmol 17β-[3H]estradiol per mg protein were considered to display high binding.

**TLI.** Thin sections of tumors were incubated with [3H]thymidine, and autoradiographed microsections were prepared by methods described previously (17). The thymidylate synthetase inhibitor, 5-fluoro-2′-deoxyuridine, and hyperbaric oxygen were used to facilitate uptake of [3H]-thymidine by S-phase cells. The TLI for each tumor was derived from a count of 2000 tumor nuclei performed by one of the 2 pathologists (J. S. M., R. W. M.) who participated in the study. Tumor cells that contained 5 grains or more of label overlying their nucleus were considered to have incorporated [3H]thymidine.

**Tumor Morphology.** Hematoxylin and eosin-stained sections from biopsy and mastectomy specimens were reviewed by one of the pathologists (R. W. M.) who participated in this study. Observations recorded for each tumor included maximum diameter; histological type; presence or absence and number of lymph node metastases; histological and cytological grade, number of mitoses per 10 high-power fields, and presence or absence and degree of associated mononuclear infiltrate. Breast cancers were classified according to the system outlined in the Armed Forces Institute of Pathology Fascicle on Breast (15). Histological and cytological grading was done according to standard criteria that utilized 3 different grades (5, 6). Mitotic counts were performed on 10 contiguous high-power fields with the × 40 objective of the microscope. Cellular reaction was evaluated as either absent, slight, moderate, or marked. Information concerning tumor size and axillary nodal status was abstracted from surgical pathology reports.

**RESULTS**

**Pathological Characteristics of Tumors.** Thirty of the 40 breast cancers studied were of infiltration duct type, not otherwise specified. Five cancers were of medullary or atypical medullary type, 2 were invasive lobular carcinomas, 2 colloid, and one tubular. Tumor size varied from a diameter of 1.0 to 10.0 cm with a mean diameter of 3.52 cm. Metastatic carcinoma was found in axillary lymph nodes in 21 cases. Four tumors were histological Grade 1 (most well differentiated), 11 Grade 2, and 25 Grade 3. Thirteen tumors were cytological Grade 3 (least anaplastic), 19 Grade 2, and 8 Grade 1. A varying degree of anaplasia was associated with 33 tumors. In 17, it was slight, in 13 moderate, and in 3 marked. Mitotic rate varied from 0 to 224 counts per 10 high-power fields with a mean of 17.7 counts per 10 high-power fields.

**Estrogen Receptor.** Estrogen receptor was measured in 39 tumors. Twenty-six tumors were estrogen receptor positive, and 13 were estrogen receptor negative. Sixteen tumors displayed high binding.

**Thymidine Labeling.** Thymidine labeling was performed on 30 tumors. The TLI varied from 0.3 to 20.0% with a median of 5.56% and a mean of 7.27%. In 14 tumors, thymidine labeling was less than 5.0%; in 8 tumors, it exceeded 9.9%.

**Flow Cytometry.** Flow cytometry was performed on 40 spec-
imens following dissociation with collagenase. The yield of dissociated cells varied from 1 to 50 million with a mean of 8.5 million cells. Cell viability varied from 13 to 88%, with a mean of 60.6%. Ficoll-Hypaque separation, which was performed on 13 specimens, increased the mean cell viability to 76.1%. Twenty breast cancers (50%) were considered diploid and 3, tetraploid.

Eight hyperdiploid cancers had DNA indices (tumor G0-Gi/2n) between 1.1 and 1.5, and 9 had indices between 1.6 and 1.9. The percentage of cells in S phase varied from 1.6 to 24.6%, with a mean of 6.7%. The percentage of S-phase cells obtained after mechanical dissociation varied from 1.2 to 19.6%, with a mean of 9.5%.

The percentage of S-phase cells after collagenase digestion (r = -0.218, p = 0.184) or with whether a tumor was above or below median TLI (r = -0.212, p = 0.260; r = -0.283, p = 0.130) or above or below median SPF (r = -0.122, p = 0.459; r = -0.218, p = 0.182).

Flow Cytometry and Thymidine Labeling. There was good correlation between collagenase SPF and TLI in 30 cases for which both values were obtained (r = 0.775, p = 0.0001). Among 23 cases for which SPF following mechanical dissociation and TLI were obtained, the correlation was fair (r = 0.597, p = 0.0027) (Chart 1). In this same group of 23 cases, correlation between collagenase SPF and TLI was excellent (r = 0.847, p = 0.0001) (Chart 2). The SPF's obtained by collagenase and mechanical dissociation were equal in their ability to predict whether a tumor was above or below median TLI. Each accurately predicted 19 of 23 cases (83%) (r = 0.652, p = 0.0018). The following formula was used to obtain a predicted value for TLI based on the SPF, TLI = -1.16 + 3.11 (% of S-phase value - median). There was fair correlation between DNA index and SPF (r = 0.407, p = 0.0258) and DNA index and collagenase SPF (r = 0.488, p = 0.0014). Aneuploid tumors tended to have both higher TLIs (r = 0.361, p = 0.0497) and higher collagenase SPF (r = 0.547, p = 0.0003) than did diploid tumors.
DISCUSSION

This study differs from previous flow cytometric studies of human breast cancer in that it correlates the percentage of S-phase cells measured by flow cytometry with percentage of S-phase cells obtained by thymidine labeling of the same tumor specimens. Enzymatic cell dissociation utilizing collagenase produced a better correlation \((r = 0.847)\) with percentage of S-phase cells obtained by TLI than did mechanical dissociation of the same tumor specimens \((r = 0.597)\). In fact, the correlation obtained between collagenase SPF and TLI is not significantly different from the correlation obtained between TLIs counted on the same specimens by 2 different pathologists (R. W. M., J. S. M., \(r = 0.860\)). This suggests that collagenase SPF should not differ significantly from TLI in its ability to predict short-term release of human breast carcinoma. There have been few previous reports comparing SPF and TLI of human solid tumors, although Braylan et al. (7) did find an excellent correlation between the 2 methods in a study of non-Hodgkin’s lymphomas. However, in the latter study, the TLI was derived from labeling of aliquots of the cell suspensions used for flow cytometry. In our study, TLI was derived from microscopic examinations of labeled tumor cells in autoradiographed tissue slices.

In contrast to flow cytometric studies of human breast cancer reported previously, we always dissociated a significant number of normal mononuclear cells along with tumor cells (23, 25). These latter cells produced a distinct peak at the 2n channel obviating the need for us to add lymphocytes or granulocytes to the sample as an external 2n control. The presence of 2 cell populations with different cell and nuclear sizes was apparent on microscopic examination of Cytospin preparations (Fig. 1). The normal cells were further identified by examination of hematoxylin and eosin-stained sections prepared from the Cytospins. Histological sections taken adjacent to the tissue used for flow cytometry provided further confirmation that normal lymphocytes and plasma cells almost always were intimately associated with the infiltrating breast cancer (Fig. 2). When we began this study, we routinely added chicken RBC to our tumor samples and calculated an expected 2n channel based on the assumption that chicken RBC were 35% human diploid. The calculated 2n channel varied somewhat from the 2n channel of our mononuclear cell population and most frequently gave a calculated DNA index for the normal mononuclear cells in the range of 0.9. We assumed that this error was due to the fact that the ploidy of chicken RBC is so dissimilar from that of normal human diploid cells that small adjustments of gain of the flow cytometer slightly change this ratio. Most recently, we have been adding both trout and chicken RBC to our tumor sample and calculating the 2n channel by the method suggested by Vindelev (27). Utilizing trout RBC, which are 80% human diploid, has produced an excellent correlation between calculated 2n channel and the channel number at which the normal mononuclear cells appear. We have not found that an external ploidy marker is needed for analysis of histograms of distinctly hyperploid breast cancers, since the mononuclear 2n channel is obvious. An external marker is useful in identifying the 2n channel in histograms of slightly aneuploid carcinomas, since it is difficult to determine by visual inspection which of the 2 closely spaced G0-G1 peaks is that of the tumor and which belongs to the normal mononuclear component. We have not yet observed a hypodiploid breast cancer G0-G1 peak.

Most previous flow cytometric studies of human breast carcinomas have reported a higher percentage of aneuploid cancers than we report in this study (22, 23, 26). Although one might assume that methods that produced very tight G0-G1, CVs would detect significantly more aneuploid tumors, it is difficult to compare our results with those of previous publications, since G0-G1, CVs are not given in these reports. Raber et al. (25) do state that the DNA index must differ from 2n by at least 10% for them to consider a tumor aneuploid. Using the collagenase method, we are able to visually discriminate 2 peaks that differ from each other by 3.2 mean channel numbers, giving a tumor DNA index of 1.055. Therefore, CV-dependent peak discrimination does not explain this difference. At times we have observed a shoulder on the descending limb of the diploid G0-G1 peak and have suspected that we might be dealing with a slightly aneuploid carcinoma. However, we have not considered such tumors aneuploid, since a similar shoulder could be produced by instrument drift or variation in propidium iodide uptake by tumor cells. Whether previous authors have considered such tumors aneuploid is not known.

Utilizing collagenase dissociation, the CV of our G0-G1, peaks varied by 2.4 to 9.8, with a mean of 4.2. We attribute most of this variation in CV to intrinsic characteristics of the breast cancers rather than to technical artifact, since with rare exception even when the tumor G0-G1, CVs have been relatively high, the CVs of the accompanying mononuclear cell population have been less than 3.0. Visual inspection of histograms with broad G0-G1, CVs often reveals a bimodal configuration, suggesting that we are dealing with 2 cell populations with mean channel number so close that they cannot be resolved.

During the course of our study, we have also been concerned that our dissociation techniques might be selecting out a non-representative population of tumor cells that would produce spurious estimations of percentage of S phase. The similarities in the SPF obtained by mechanical and collagenase methods suggest that these 2 methods are not dissociating strikingly different populations of cells. The correlation between SPF and TLI also, of course, is reassuring. The similarity in histograms obtained before and after Ficoll-Hypaque separation suggests that viable and dead cells do not differ significantly in their ability to intercalate DNA with propidium iodide. We also were concerned that cell clumping might be selecting out a particular type of tumor cell that would bias our estimations of S phase. To test this, in several specimens for which we had a particularly high cell yield, we filtered specimens sequentially through 77-, 44-, and 25-μm filters and obtained histograms after each filtration and on the cell clumps that adhered to each filter. Since we could not detect any significant difference in these histograms, we abandoned our concern.

The ability of the collagenase method to dissociate viable human tumor cells could potentially be exploited for numerous biological purposes that we have not had time to investigate. We recognize that trypan blue exclusion is an imperfect test of viability and tried to refine our estimate of viability somewhat by counting tumor cells under phase-contrast microscopy. We excluded as nonviable any cells that showed cytoplasmic stain with trypan blue, did not fluoresce brightly, or had developed vesicular or otherwise irregular surface membranes. In a few instances in which we had abundant cell yields, we also introduced collagenase-dissociated cells into tissue culture with satisfactory...
short-term viability.

We selected a rectangular method for histogram analysis, since it appeared suitable for analyzing asynchronous breast cancer populations with relatively small SPFs. The attractiveness of a rectangular as opposed to a gaussian method for analysis of this type cell population was confirmed recently in a study of various mathematical methods published by Baisch et al. (1). Although the purpose of our study was not to compare various methods of histogram analysis, we did analyze each tumor with the gaussian model supplied with the Couter software program and found that it gave a significantly higher SPF when the G0-G1 peak was skewed towards the right, since the skew was interpreted as S-phase cells. Use of gaussian methods for histogram analysis may explain why previous flow cytometric studies of human breast cancer report mean percentages of S-phase cells that are somewhat higher than are reported in this study (3, 12, 24).

The correlation we observed between tumor ploidy and TLI has not been reported previously. Some previous authors have observed a correlation between DNA index and SPF; others have not (3, 25). Bichel et al. (4) found a correlation between DNA index and histological grade, but Raber et al. (25) did not. Raber et al. found some correlation between ploidy and cytopathological grade, as did Olszewski et al. (23). Both Olszewski et al. (23) and Bichel et al. (4) found that diploid tumors tended to be estrogen receptor positive, whereas aneuploid breast carcinoma tended to be estrogen receptor negative. Both Raber et al. (25) and Olszewski et al. (24) report that estrogen receptor-positive tumors tended to have low SPF, whereas estrogen receptor-negative tumors had higher SPFs. We found no previous report that investigated the possible correlation between mitotic count and SPF, TLI, or DNA index.

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REFERENCES


Fig. 1. Cytospin nuclear preparation of tumor cells dissociated with collagenase. Tumor cell nuclei (T) are larger and stain less intensely than do the normal lymphocyte nuclei (L) dissociated with the tumor. H & E, × 400.

Fig. 2. Tissue section of the same breast cancer illustrated in Fig. 1. Normal lymphocytes (L) are seen adjacent to the nests of infiltrating tumor (T). Nuclear size and staining quality are similar to that seen in Fig. 1. H & E, × 400.
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