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Report

Cell dissociation techniques in human breast cancer – Variations in tumor cell viability and DNA ploidy

Britt-Marie Ljung[1], Brian Mayall[2], Chace Lottich[3], Cinda Boyer[4], Steven S. Sylvester[5], George S. Leight[3], Hilliard F. Siegler[3] and Helene S. Smith[5]

[1] Department of Pathology, University of California, San Francisco, CA; [2] Department of Laboratory Medicine, University of California, San Francisco, CA, and Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA; [3] Department of Surgery, and [4] Department of Medicine, Duke University, Durham, NC; [5] Peralta Cancer Research Institute, Oakland CA, USA

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Summary

Approximately 70% of breast cancers contain cell populations with hyperdiploid ($>G_0/G_1$) DNA content; however, cells cultured from breast cancers have only diploid DNA contents and karyotypes. Mechanically dissociated cells rarely, if ever, grow in culture, while enzymatically dissociated cells do grow in most cases. To determine if cell dissociation techniques used to prepare cells for culture and other laboratory procedures select for cells with specific features, and if tumor cells are killed in the process, breast cancer cells obtained by mechanical dissociation and by enzymatic dissociation were examined for DNA content and cell viability (measured by dye exclusion). Mechanical dissociation yielded more dead cells and cells with hyperdiploid ($>G_0/G_1$) DNA than did enzymatic dissociation. Hyperdiploid cells were also found in the dye-excluding population with each dissociation technique, suggesting that the hyperdiploid cells were not always dead.

We conclude that, *in vivo*, tumors contain cellular subpopulations with low viability and hyperdiploid ($>G_0/G_1$) DNA patterns. The extent to which these subpopulations are present in a sample depends on the dissociation technique employed. That only diploid cells are found in cultures of primary breast cancers may be because enzymatic dissociation, used to prepare cells for culture, yields predominantly diploid cells. These observations also have important implications for interpreting measurements made on dispersed cells, *e.g.*, viability, DNA content, and other cytochemical markers.

Introduction

Cellular heterogeneity characterizes most solid tumors [1]. For example, rodent mammary cancers exhibit many properties heterogeneously, including antigenicity, growth rate, immunogenicity, sensitivity to chemotherapeutic drug, and ability to metastasize [2]. Human breast cancers, although more difficult to measure, show similar heterogeneity [3], such as variations in DNA ploidy. DNA ploidy is an important prognostic marker for breast cancers. Numerous cytometric studies have shown that approximately 30% of primary breast cancers contain only cells with 2C stemlines, 40% contain cell populations with both 2C and >2C stemlines, while 30% have stemlines >2C only. The patients who have tumor populations with >2C stemlines tend to have a poor prognosis [3, 4].

Address for offprints: B-M Ljung, Department of Pathology, M-559, Box 0102, University of California, San Francisco, CA 94143, USA

It is generally believed that cells with >2C DNA content represent cells with hyperdiploid karyotypes rather than normal cells in S-phase or G_2 phase because 1) the cells with >2C stemlines often appear as a peak characteristic of G_0/G_1 cells, rather than with the diffuse distribution characteristic of cells in S-phase; and 2) sometimes there are, in addition, cells with obviously abnormal stemlines (i.e., >4C).

In contrast to the cytometric data, when primary human breast cancers are cultured, all the cells are karyotypically diploid or pseudodiploid [5, 6]. In an attempt to reconcile the differences in DNA content before and after culture, we examined the starting population after various dissociation techniques. As previously described by Chassavent *et al.* [7], we found that the population of cells isolated by mechanical dissociation of breast tumor tissue contained relatively more aneuploid and dead cells (measured by dye exclusion) than did cells isolated by enzymatic dissociation. We have extended these studies to show that the dead cells are not an artifact of shear stress or of time delay in specimen preparations, but are apparently present *in vivo*.

Materials and methods

Cell isolation from surgically excised tumors

Mechanical dissociation. Surgically excised, primary human breast tumor tissue was dissected within two hours of surgery. Samples were minced into 1-mm fragments and gently agitated in F12 medium plus 10% fetal calf serum (Gibco, Grand Island, NY) to dislodge cells loosely embedded in the stromal matrix. This process yielded a mixture of single cells, small clusters of cells in suspension, and larger fragments. To minimize the risk of killing cells, we did not attempt to dissociate all the cells, but left some cell clumps intact. The larger fragments were pelleted by unit gravity and separated. The viability of the mechanically dissociated single cells and small clusters was determined by incubation with a 1:1 dilution of fast green stain (20 mg/ml PBS) as described by Weisenthal *et al.* [8]. The cells that absorbed the dye were considered dead. The cells were prepared for microscopy by cytocentrifugation onto glass slides; they were then fixed in methanol, and counterstained with hematoxylin and eosin. As a control for the fast green stain, aliquots of the specimens from five tumors were stained with trypan blue. A minimum of 100 cells were counted in all specimens. We found that both stains revealed a similar percentage of dye-excluding cells.

Enzymatic dissociation. A second fraction was obtained from the pelleted tissue fragments, which were digested overnight with collagenase and hyaluronidase as previously described [9]. These cells, obtained by enzymatic dissociation, were filtered through a 15 μm nylon filter and trypsinized to obtain a single-cell suspension. Staining with fast green for viability and preparation for microscopy was done as described above.

In five additional tumors, mechanical and enzymatic dissociations were done after initially dividing the tumor into two equally sized pieces. This resulted in similar ratios of dye absorbing cells as when the enzymatic digestion was performed on the larger fragments left over after mechanical dissociation.

Cell isolation from needle aspirates of tumors in vivo

To rule out the possibility that the dead cells obtained by gentle mechanical dissociation were artifacts resulting from delay in processing the biopsy material, tumors were aspirated with a 22-gauge needle before surgery. The aspirates were scanty and yielded mainly single cells and only a few small cell clusters. The aspirated material was suspended in culture medium, centrifuged, incubated with fast green stain, and processed in the same manner as the mechanically dissociated cells.

Needle aspiration of cultured cells

To assess whether needle aspiration causes tumor-

cell death, fast green staining was performed after aspiration of a pelleted breast cancer cell line. Cultured human breast cancer cells (cell line 578T) were trypsinized, suspended in medium, and centrifuged into a pellet, and the supernatant was removed. The cell pellet was forced through a 23-gauge needle three times. In addition, a 0.6-cm subcutaneous tumor in a nude mouse was aspirated with a 23 gauge needle. Dye exclusion with fast-green stain and preparation for microscopy was done as described above.

Cytometric evaluation

To evaluate cell viability, a part of each sample was stained with 1% fast green for 10 min followed by hematoxylin and eosin. The remainder was treated with 2.5% DMSO to prevent air-drying and then stained with hematoxylin and eosin. After light microscopic evaluation, both slides were destained and restained for the Feulgen reaction. The fast green dye remained cell-associated throughout this procedure and interfered with Feulgen staining of the cells that took up the fast green. Therefore, identification and measurement of ploidy was only possible in dye-excluding cells. DNA of the entire tumor population was measured using the samples not treated with fast green dye. Benign breast specimens were used to obtain normal diploid cells for controls. After enzymatic dissociation [9], benign breast epithelium was prepared as described above for tumor tissue. One hundred cells on each glass slide were measured for DNA stain content on the TASplus image cytometer as described [10]. All the cells measured had light microscopic features consistent with malignant cells. The nomenclature for DNA content suggested by Hiddeman *et al.* [11] was used; tumor cells were considered to have diploid (G_0/G_1) DNA content when there was a single well-defined mode that corresponded to the mode of the control cells. Any cell with a DNA content significantly higher than the diploid mode was designated as hyperdiploid ($>G_0/G_1$). Aneuploid cells as well as proliferating cells in S-phase were included in this category.

Light microscopic analysis

After staining with fast green and/or hematoxylin and eosin, all glass slides were evaluated for cellularity and preservation. The population of cells taking up the dye was determined in the samples stained with fast green. A minimum of 150 cells were examined per slide. Only cells with morphologic features of breast cancer were included.

Results

Dye exclusion

Markedly fewer dead cells absorbing fast green dye were found after enzymatic cell dissociation than after mechanical dissociation. After enzymatic dissociation, 0–27% (mean 8%) of the tumor cells absorbed the fast green dye. In contrast, after mechanical dissociation, in all but one case, the majority of the tumor cells absorbed the dye. In 17 tumors the proportion of tumor cells absorbing dye ranged from 58–98%. When the remaining tumor, a colloid cancer which had only 1% of cells absorbing dye, was included, the mean was 81% (Table 1).

In the samples obtained from tumors by needle aspirates prior to surgery, the percentage of tumor cells excluding dye was low and very similar to that of the mechanically dissociated cells from the same tumor (Table 2). The cells excluding dye tended to be in the few clusters present, rather than in single cells.

Examination of the aspirated cells from the pelleted breast cancer cell line and from the subcutaneous tumor grown in a nude mouse showed that over 95% of the cells excluded the fast green dye, indicating that there was minimal damage.

Ploidy values

The DNA contents of both the mechanically dissociated and the enzymatically dissociated cells were measured cytometrically. Typical profiles for one specimen are illustrated in Fig. 1, and the re-

sults for 11 tumors are summarized in Table 3. Compared with cells obtained by enzymatic dissociation, the mechanically dissociated cells had not only a larger proportion of dead cells but also more cells with hyperdiploid ($>G_0/G_1$) DNA (Table 3, columns A and D). In all cases, mechanical dissociation yielded a markedly small amount of dye-excluding cells with hyperdiploid ($>G_0/G_1$) DNA (Table 3, column C). This was due mainly to the small percentage of dye-excluding cells overall after mechanical dissociation. There was little difference in the percentage of hyperdiploid ($>G_0/G_1$) cells in the total population of mechanically dissociated cells and in the dye-excluding portion (Table 3, columns A and B).

Similarly, in most cases, enzymatic dissociation yielded a small percentage of dye-excluding cells with hyperdiploid ($>G_0/G_1$) DNA (Table 3, column F). This was due mainly to the small percentage of hyperdiploid ($>G_0/G_1$) cells overall after enzymatic dissociation (Table 3, column D). As in the mechanically dissociated population, there was little difference in the percentage of hyperdiploid ($>G_0/G_1$) cells found in the total population of enzymatically dissociated cells and in the dye-excluding portion (Table 3, columns D and E).

Table 1. Percent dye-absorbing (dead) cells after mechanical and enzymatic dissociation*

Specimen	Mechanical dissociation	Enzymatic digestion
1	79%	1%
2	96%	8%
3	76%	2%
4	88%	0%
5	1%	0%
6	84%	15%
7	82%	1%
8	98%	27%
9	95%	3%
10	88%	1%
11	92%	1%
12	58%	5%
13	84%	4%
14	78%	5%
15	94%	1%
16	93%	5%
17	86%	5%
18	78%	2%

* In all but one case, mechanical dissociation yielded a much greater proportion of dead cells than did enzymatic dissociation. Mechanical dissociation was performed by mincing the tissue and collecting single cells and small clusters in in suspension after larger fragments were pelleted by unit gravity and separated. Enzymatic dissociation was done by digesting the larger fragments separated after mechanical dissociation overnight with collagenase and hyaluronidase, followed by trypsinization. The resulting single cells and small clusters obtained by either technique were stained with fast green stain (absorbed by dead cells) and cytocentrifuged. At least 150 cells/microscopic slide were examined.

Discussion

Compared with enzymatic dissociation, mechanical dissociation of the same tumor yielded more dead cells and more cells with hyperdiploid ($>G_0/G_1$) DNA; this was in spite of using a gentle mechanical dissociation technique to avoid excessive trauma to the tumor cells. These results agree with those of Chassavent [7]. One possible reason for the presence of numerous dead cells after mechanical dissociation is that many cells might have died during the 4–6 hr between disruption of blood supply at the onset of surgery and the processing of the

Table 2. Percent dye-absorbing (dead) cells in preoperative needle aspiration samples and after mechanical dissociation of the same tumors after surgery*

Specimen	Needle aspirate	Mechanical dissociation
CC4	67	81
CC6	>90	>90
CC7	>90	>90
CC9	77	85
CC10	87	85

* Needle aspiration (22 gauge) was done before surgery. The yield was smeared on glass slides and stained with fast green dye. Mechanical dissociation was done by mincing fresh tissue from the excised tumor and collecting single cells and small clusters in suspension after the larger fragments were pelleted by unit gravity and separated. The harvest was cytocentrifuged and stained with fast green dye. Needle aspiration of the tumor *in situ* yielded similar numbers of dead cells as did mechanical dissociation of the surgical specimen.

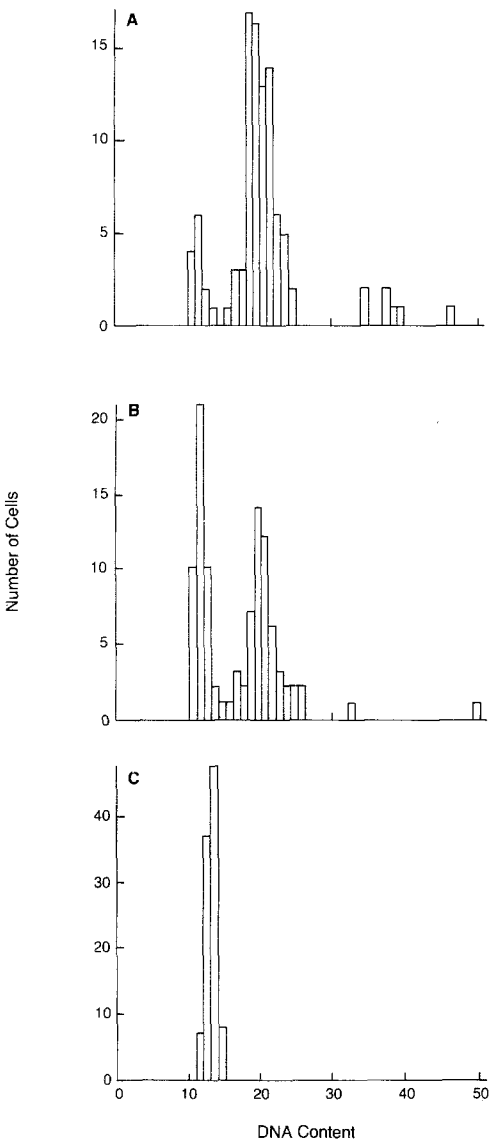

Fig. 1. DNA histograms of cells dissociated from the same human breast tumor (sample 3). A: Sample of mechanically dissociated cells. B: Sample of enzymatically dissociated cells. C: Normal control, enzymatic dissociation of non-malignant tissue adjacent to a carcinoma. Note the increased number of DNA diploid tumor cells in the enzymatically dissociated sample (Fig. 1B).

specimen. To exclude this possibility, needle aspirates obtained from the tumor before surgery were examined and were found to contain a similar proportion of dead cells to that found in the mechanically dissociated fraction. To determine whether the aspiration procedure might have killed the cells, pelleted and cultured cells were aspirated through a 23 gauge needle and stained with fast green. In addition, a subcutaneous tumor growing in a nude mouse was aspirated. In both experiments, the vast majority of the cells (>95%) excluded the dye, thus ruling out that possibility.

Cells obtained by mechanical dissociation are, in our experience, not a good source for culture. We have rarely grown any cells, either diploid or hyperdiploid ($>G_0/G_1$), from this source (unpublished observation). A possible explanation for this is that the vast majority of the cells in the sample, being dead (Table 1), release degrading enzymes that destroy or inhibit growth in the remaining cells. Table 3, column B shows that the percent hyperdiploid cells in the dye-excluding fraction after mechanical dissociation in several tumors is high and, in some cases, higher than the corresponding percentage after enzymatic dissociation. However, in most cases, because the fraction of dye-excluding (viable) cells is so small compared to the fraction of dead cells after mechanical dissociation (Table 1), the overall percent of cells that are both hyperdiploid and dye-excluding is small (Table 3, column C).

On the other hand, cells obtained by enzymatic dissociation provide a good source for short-term culture in many primary breast cancers [6]. After enzymatic dissociation, in the majority of cases, there are few dead cells (Table 1), and most of the cells are diploid (Table 3). In this setting, degrading enzymes would probably not be plentiful enough to interfere with cell growth. We speculate further that diploid cells may outnumber and overgrow hyperdiploid ($>G_0/G_1$) cells, yielding a homogeneous population of diploid cells after several generations in culture.

In the mechanically dissociated fraction, the proportion of hyperdiploid ($>G_0/G_1$) cells was similar in both the dye-excluding population and the total population, which was composed of predominantly dead cells (Table 3). This observation suggests that the mechanism responsible for killing the tumor cells was unrelated to the mechanism responsible for generating hyperdiploidy. The presence of a population with many dead cells was real and not

produced by diminished blood supply or mechanical distortion. Because the dye exclusion test provides only a minimal estimate of dead cells, the percentage of cells in the tumor unable to divide probably was considerably larger than the percentage absorbing dye [12].

The large proportion of dye-excluding cells (over 90% in 16/18 cases) present in the enzymatically dissociated fraction raises the question of what happened to the dead cells presumably present in the tumor fragment before processing. Perhaps the dead cells as well as the cells with hyperdiploid ($>G_0/G_1$) DNA were more vulnerable to enzymes and were lysed in the process, leaving mostly dye-excluding cells and cells with diploid (G_0/G_1) DNA as the final product. Thus, the two most common methods for dispersing tumor cells – mechanical dissociation and enzymatic dissociation – select for differing subpopulations not necessarily representative of the tumor as a whole.

The fact that fine needle aspiration extracted mainly dead cells raises serious concerns about using this technique to obtain material for scientific analysis. However, the aspiration technique used in this study was mainly suction, which produced scanty material consisting mostly of single cells. There is a more aggressive technique that, in addition to suction, uses the needle as a shearing tool and thus, extracts tumor clumps as well as single cells [13]. This latter technique probably yields material more representative of the tumor as a whole.

In summary, we found that breast cancers are heterogeneous with respect to cellular viability and cellular DNA content. Mechanical dissociation selects for dead cells and hyperdiploid ($>G_0/G_1$) cells, while enzymatic dissociation selects for dye-excluding and diploid cells. This may explain why enzymatic dissociation often produces cells that grow in culture while mechanical dissociation does not. It also may explain why cells cultured from

Table 3. Characterization of ploidy and viability as a function of breast cancer dissociation technique[a]

Sample	Mechanical dissociation			Enzymatic dissociation		
	A % Hyperdiploid[b] cells	B % Hyperdiploid of dye-excluding fraction	C % Total cells both hyperdiploid and dye-excluding	D % Hyperdiploid[b] cells	E % Hyperdiploid of dye-excluding fraction	F % Total cells both hyperdiploid and dye-excluding
3	87	85	20.0	60	62	61
9	94	NT[c]	NT[c]	15	NT[c]	NT[c]
10	67	21	2.5	70	63	63
11	9	6	0.5	3	3	3
12	0	5	2.1	2	NT[c]	NT[c]
13	36	88	14.0	8	11	11
14	7	6	1.3	4	1	1
15	87	93	5.6	36	45	45
16	8	8	0.5	2	5	4.8
17	55	68	9.5	21	24	23
18	15	19	4.2	2	2	2

[a] A & D ploidy values measured on cytocentrifuged cells stained with Feulgen reaction and fast green dye; C & F values were calculated as follows:

$$\frac{\text{Fast green-negative cells}}{\text{all cells}} \times \frac{\text{Fast green-negative hyperdiploid cells}}{\text{fast green-negative cells}}$$

Mechanical and enzymatic dissociations were done as described in Table 1 and Methods section.

[b] Hyperdiploid cells = ($>G_0/G_1$).

[c] NT = Not tested.

primary breast cancer are exclusively diploid. Both of these commonly used dissociation techniques fail to consistently produce a population that reflects the tumor as a whole. At present, there appears to be no ideal cell dissociation technique. Enzymatic dissociation works much better for short-term culture than does mechanical dissociation. However, one needs to be aware that the overwhelming majority of the cells submitted for culture are diploid and may not represent the tumor as a whole. On the other hand, mechanical dissociation may be a better choice in studies where one is seeking to identify hyperdiploid cells. Thus, the choice of dissociation technique has important implications for interpretation of, among other things, viability, DNA content, and other cytochemical markers on dispersed cells.

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