DNA in Situ Sensitivity to Denaturation as a Marker of Human Breast Tumors

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ABSTRACT

DNA content and in situ sensitivity to denaturation were analyzed by flow cytometry of individual cell nuclei isolated from 40 breast carcinomas, nine fibroadenomas, and 14 samples of normal breast tissue. The extent of DNA denaturation induced by acid was expressed as $\alpha_2$, which represents the fraction of DNA staining metachromatically red with the fluorochrome acridine orange. In all cases of normal breast tissue DNA was very sensitive to denaturation and the frequency distribution of $\alpha_2$ values was unimodal with over 90% of cells having $\alpha_2$ above 0.6. All fibroadenomas were diploid; four had unimodal $\alpha_2$ as in normal tissue and five had a bimodal distribution with an additional peak below 0.6. Twenty-seven adenocarcinomas (67%) had a DNA index above 1.0; of these 24 had bimodal $\alpha_2$ distributions. Among 13 diploid carcinomas 10 had bimodal $\alpha_2$ distributions. Statistically significant differences were observed in $\alpha_2$ distributions of normal versus tumor breast tissue ($P < 0.005$). In normal tissue and in all tumors a predominant proportion of cells with S and G2 + M DNA content were characterized by DNA resistant to denaturation ($\alpha_2$ below 0.6). Of interest, the diploid cells from aneuploid tumors which may represent reactive host cells often displayed bimodal distributions of $\alpha_2$.

These results may be interpreted in light of earlier studies demonstrating increased resistance of DNA to denaturation in diffuse chromatin of proliferating and/or transcriptionally active cells, and greater sensitivity to denaturation of DNA in condensed chromatin of quiescent cells. Thus, the presence of the second peaks representing cells with low $\alpha_2$ values in breast tumors may indicate a high proportion of proliferating cells, whereas high $\alpha_2$ populations may represent quiescent and differentiating (condensed chromatin) or dying (pycnotic nuclei) cells. It is likely that the low $\alpha_2$ diploid cells detected in aneuploid tumors may represent the reactive (transcriptionally active and/or proliferating) infiltrating host cells (i.e., lymphocytes, monocytes) whose presence may also be of prognostic value. The data suggest that a DNA denaturability assay may be useful to characterize tumor and infiltrating host cell populations.

INTRODUCTION

The need to develop prognostic markers for human tumors is undisputable. In the case of breast cancer, conventional histopathological examinations have limited prognostic information. Evidence regarding the expression of ER, is conflicting. Whereas some reports indicate a weak correlation between ER levels and prognosis (6, 7), more recent observations do not demonstrate a significant difference in outcome of the disease according to histopathological diagnosis: (a) 40 cases of breast carcinoma, (b) nine cases of fibroadenoma, (c) 14 specimens of normal breast tissue. The three lobular carcinomas were not considered to be useful to characterize tumor and infiltrating host cell populations.

MATERIALS AND METHODS

Patients. All of the specimens studied were from women undergoing breast surgery because of a palpable mass or the presence of mammographic abnormalities, ranged in age from 24 to 82 years, with a median age of 58. The tissue specimens were subdivided into three groups according to histopathological diagnosis: (a) 40 cases of breast carcinoma, (b) nine cases of fibroadenoma, (c) 14 specimens of normal breast tissue. Tumor size when present varied from 1.0 to 8.5 cm in diameter. Among the carcinomas there were 36 cases of infiltrating duct carcinoma, three cases of infiltrating lobular carcinoma and one undefined carcinoma. One tumor was histologically low grade, 18 were moderate, and 17 high grade. The three lobular carcinomas were not graded. Among the nine fibroadenomas, five showed active proliferation of the epithelial component. One nonneoplastic, noncarcinomatous breast specimen had atypial duct hyperplasia.

Specimen Preparation. The studies were carried out using fresh, unfixed specimens of breast tissue which were stored in buffered saline at 0°C for up to 72 h prior to isolation of nuclei. Two types of nuclear isolation buffers were used. For measurements of DNA and RNA content nuclei were isolated in 1.0% citric acid (Sigma Chemical Co., St. Louis, MO) and 0.1% NP-40 detergent (Accurate Scientific and Chemical Co., Hickville, NY). To measure sensitivity of DNA to denaturation nuclei were isolated in a buffer consisting of 10 mM acetic acid, 1 mM MgCl2, and 0.1% NP-40, pH 1.5. In both cases the breast tissue was first trimmed to remove necrotic areas, placed in the appropriate isolation medium, and minced with a scalpel. The efficiency of nuclear isolation (lack of cytoplasmic tags) was checked by phase microscopy. The nuclear suspension was then filtered through four
layers of gauze to remove large debris particles, and immediately mixed with an equal volume of buffer consisting of 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (Sigma), 5 mM MgCl2, and 1 mM CaCl2, pH 7.4.

**DNA and RNA Determination.** The DNA ploidy and RNA content of citric acid isolated nuclei from malignant or normal breast tissue were determined using the technique of AO staining described earlier (36). Peripheral blood mononuclear leukocytes from normal donors, isolated by Ficoll/Hypaque gradient centrifugation, served as a normal diploid control. In this technique, the metachromatic fluorochrome AO intercalates into native DNA and fluoresces orthochromatically green with maximum emission at 530 nm; the intensity of green fluorescence is proportional to cellular DNA content. RNA stains metachromatically red with maximum emission at 640 nm; the intensity of red luminescence (phosphorescence) is proportional to RNA content (37).

Briefly, an aliquot of 0.2 ml of nuclear suspension in the buffer (1-4 x 10⁶ nuclei) was mixed with 0.4 ml of 0.08 N HCl, 0.15 N NaCl, and 0.1% Triton X-100 at 4°C. After 30 s, 1.2 ml of the solution containing 0.2 mM Na2HPO4-0.1 mM citric acid buffer, pH 6.0, 1 mM EDTA-Na, 0.15 N NaCl, and 6 µg/ml of AO (Polysciences Inc., Warrington, PA) was added. Measurements were carried out immediately after staining, using an FC-200 cytofluorometer (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, MA). A total of 5 x 10⁶ nuclei per sample were measured and displayed in DNA and RNA histograms.

The DNA index was calculated as the ratio of the DNA content of the G1 cells (peak value) of the tumor to the G1 peak value of the normal unstimulated human lymphocytes, which were used both as an external and internal standard. The mean c.v. value of the lymphocytes was 2.9. Aneuploidy was recognized when, upon addition of lymphocytes to the studied sample either two peaks or clear bimodality of the G1 peak become apparent.

**Acid-induced DNA Denaturation.** DNA denaturability of chromatin was studied in acetic acid isolated fresh nuclei according to the method described elsewhere (25, 26). One million of cell nuclei were treated with 5 x 10⁶ units of RNase (Calbiochem, Behring Diagnostics, La Jolla, CA) at pH 6.5, at room temperature, for 20-30 min; then 0.2-ml aliquots of nuclear suspension (2 x 10⁶ nuclei) were mixed with 0.5 ml of 0.1 M KCl/HCl buffer, pH 1.4, and 30 s later with 1.5-ml AO solution. Acridine orange was used at a concentration of 5 µg/ml in buffer containing 0.1 mM citric acid, 0.2 mM Na2HPO4, at final pH 2.6. All solutions were at room temperature. The KCl/HCl buffer induces DNA denaturation whereas staining with AO at pH 2.6 precludes DNA renaturation, which otherwise, at higher pH, is very rapid (25).

**Luminescence Measurements.** Luminescence of individual cell nuclei was measured as described (35), using an FC-200 cytofluorometer. Red and green luminescences from each nucleus were separated optically and the integrated values of the pulses quantitated by separate photomultipliers (37).

The degree of DNA denaturation in situ was expressed as an α index (the ratio of red luminescence to total cell luminescence) as described (38, 39). Cells from the L1210 cell line were used as an external standard. Photomultiplier sensitivities for red and green luminescence detection were adjusted so that α values of G1 cell population of L1210 cells were always between 0.41 and 0.44, with a mean value of 0.43.

**Statistical Analysis.** Tests for differences in distributions of αi in various subgroups and correlations of this parameter with histopathological variables were carried out employing the two-sample Smirnov-Kolmogorov test, the Mann-Whitney test, and G test for two-way tables (40). These nonparametric tests were used in the absence of any a priori information about the distributions.

**RESULTS**

All specimens from noncancerous breast tissue, fibroadenomas, and 13 out of 40 carcinomas (33%) were diploid, displaying a single G1 peak with DNA index 1.0. Twenty-seven carcinomas had an additional G1 peak and were classified as aneuploid (67%). Among them there was one hypodiploid tumor with DNA index of 0.75, remaining tumors were hyperdiploid with DNA index ranging from 1.15 to 2.4. The distribution of individual cases with respect to their ploidy values is shown in Fig. 1. Analysis of nuclear RNA content of the present material will be the subject of the separate report. Raw data illustrating the frequency distributions of total nuclear luminescence versus αi values, are shown in Fig. 2. As discussed before (38) the total

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**Manuscript in preparation.**

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Fig. 1. Frequency distributions of DNA Index in 40 primary breast carcinomas.

Fig. 2. Representative bivariate total luminescence versus αi distributions of: a, normal breast tissue; b, fibroadenoma-benign tumor; and c, DNA aneuploid breast carcinoma. Cell nuclei were stained with AO following treatment with RNase and partial acid-induced denaturation of DNA. Total luminescence is proportional to total nuclear DNA; the αi index represents the extent of DNA denaturation in situ, known to correlate with a degree of chromatin condensation. a, an unimodal cell distributions with respect to their αi values, which is characteristic to all cases of normal breast tissue; b, bimodal αi observations observed in 5/9 cases of fibroadenomas; and c, a typical cell distributions with respect to αi value characteristic for DNA aneuploid breast carcinomas.

The major cell populations can be distinguished as follows: A, cells with diploid DNA content and high sensitivity of DNA to denaturation; B, diploid cells having DNA resistant to denaturation; C, aneuploid cells with DNA resistant to denaturation (see "Results").
nuclear luminescence (red + green) is a reflection of a total DNA content, i.e., denatured (red) plus double stranded (green) whereas $\alpha_v$ value represents the denatured DNA fraction. The latter can vary, in theory, from 0 (all DNA is double stranded, staining green) to 1 (all DNA is denatured, staining red). Under the present conditions of DNA denaturation, $\alpha_v$ varied between 0.4 and 0.9, indicating that in the studied cells from 40 to 90% DNA was denatured.

Total measurements of DNA content based on sum of red plus green luminescence intensities of cell nuclei, i.e., in which latter can vary, in theory, from 0 (all DNA is double stranded, DNA content, i.e., denatured (red) plus double stranded (green) nuclear luminescence (red + green) is a reflection of a total DNA content, i.e., in which DNA was partially denatured by acid and stained with AO at pH 2.6 were less accurate than measurements based on DNA/RNA staining. Therefore, ploidy was estimated from the later measurements. However, total nuclear luminescence after DNA denaturation was sufficient to discriminate between diploid versus aneuploid cell populations in nearly all cases in which tumor DNA index was different than 1.0 (Fig. 2C), thus making it possible to separately estimate $\alpha_v$ values of diploid cells and aneuploid tumor cells in these cases.

Based on $\alpha_v$ values and total cell luminescence (DNA content) the following cell populations could be discriminated in the studied material:

1. **Nonneoplastic Breast Tissue.** The distributions were generally unimodal; nearly all cells had high $\alpha_v$ values and a diploid DNA content, as seen in Fig. 2A. There were a few cells with low $\alpha_v$ (0.6) usually not exceeding 10%. Among the latter were cells with DNA content equivalent of that of S and G2 cells. An exception to the pattern was a sample with marked duct hyperplasia in which two distinct major subpopulations could be discriminated, differing in $\alpha_v$. Eighty % of the cells in this sample were in the low-$\alpha_v$, subpopulation and among them 5% of the cells had an S + G2-DNA content.

2. **Fibroadenomas.** Five of nine fibroadenomas had bimodal cell distributions, and four were unimodal with respect to $\alpha_v$ values. As with the nonneoplastic breast tissues above, in all unimodal distributions the main cell population was characterized by a high $\alpha_v$ value (>0.6), and fewer than 10% of cells had $\alpha_v$ values below 0.6. When the distributions were bimodal there usually were two well-defined peaks rather than a peak with a shoulder, and in nearly all cases the border between low and high $\alpha_v$ subpopulations was 0.6. The subpopulation with low $\alpha_v$ typically contained cells in G1, S and G2 phases of the cell cycle as determined by DNA content. The subpopulation with high $\alpha_v$ (>0.6) consisted primarily of G1 phase cells though there might be a few cells with S + G2 DNA content.

3. **Diploid Tumors.** Diploid tumors had either unimodal or bimodal cell distributions with respect to $\alpha_v$ values. In the bimodal tumors, the mean $\alpha_v$ value of the low-$\alpha_v$ subpopulation showed substantial variation from tumor to tumor. The mean values of $\alpha_v$ of the high-$\alpha_v$ subpopulation were less variable. In diploid tumors it was impossible to discriminate between host and malignant cells and thus both cell types may have been present in each of the subpopulations discriminated by $\alpha_v$.

4. **Aneuploid Tumors.** The pattern of cell distributions with respect to $\alpha_v$ versus total luminescence was most complex in aneuploid tumors. Based on DNA content (total luminescence) it was possible to distinguish cells with diploid content in G1, and aneuploid cells. Both cell types had subpopulations differing in $\alpha_v$, i.e., low-$\alpha_v$, and high-$\alpha_v$, subpopulations. Furthermore, it was possible to discriminate cells in $S + G2$ in the aneuploid population. The following parameters therefore were measured: (a) percentage of cells in the low-versus high-$\alpha_v$, subpopulations at the diploid DNA level; (b) percentage of cells in the low-versus high-$\alpha_v$, subpopulations at the aneuploid DNA level; (c) the mean $\alpha_v$ value of the cells in the high-$\alpha_v$, subpopulation; (d) with some approximation, percentage of diploid (G1) versus aneuploid cells. In aneuploid tumors it was also possible based on differences in DNA content (total luminescence), to distinguish between tumor and presumed nontumor (diploid) G1 cells and to subcategorize those cells respectively, based on their $\alpha_v$.

Table 1 lists subpopulations of cells discriminated by differences in $\alpha_v$ in all of the studied cases. It is evident that whereas all normal breast tissues had unimodal cell distributions, with nearly all cells having $\alpha_v$ values above 0.6, most tumors had additional subpopulations of cells with low $\alpha_v$ values. The percentage of cells in the low-$\alpha_v$ subpopulations varied from 13 to 99%. Specifically, in 13 cases of normal breast tissue an unimodal $\alpha_v$ distribution was observed with higher $\alpha_v$ peak only. In one other specimen showing substantial duct hyperplasia there was a bimodal $\alpha_v$ distribution.

Among nine fibroadenomas studied a bimodal $\alpha_v$ distribution was apparent in five cases. The difference in proportion of breast carcinomas (34/40) and fibroadenomas (5/9) with bimodal $\alpha_v$ population is of borderline significance (0.05 < P < 0.1), whereas the difference between nonneoplastic breast tissue (1/14) and breast cancer (34/40) is highly significant (P < 0.005).

A statistical analysis of the difference in distributions of $\alpha_v$ between subgroups is presented in Fig. 3. The first index analyzed was the position (mean value) of $\alpha_v$ of the lower of the two $\alpha_v$ peaks, denoted $\alpha_{v_{\min}}$ (if only one subpopulation existed, $\alpha_{v_{\min}}$ was defined, by default, as the position of its peak). Two-sample comparisons demonstrate statistically significant differences between mean $\alpha_{v_{\min}}$ in benign breast tissue (normal and fibroadenomas) versus carcinomas, with significance at P < 0.005 (Fig. 3A). There was also a statistically significant difference (P < 0.005) between $\alpha_{v_{\min}}$ in normal breast tissue and fibroadenomas pooled together versus diploid carcinomas (Fig. 3B) or between normal breast tissue and fibroadenomas (P < 0.05; Fig. 3C). However, there was no difference in $\alpha_{v_{\min}}$ between diploid and aneuploid carcinomas (Fig. 3D), between diploid and tetraploid carcinomas pooled together versus carcinomas with DNA index between 1.0 to 2.0 (Fig. 4A), or between fibroadenomas and carcinomas (not shown). It should be noticed that the demonstrated highly significant differences between the distributions of $\alpha_{v_{\min}}$ in various subgroups is caused primarily by the fact that the normal specimens had mostly unimodal distributions of cells with high $\alpha_v$, whereas cell distributions of carcinomas and some fibroadenomas were bimodal. Additional sets of comparisons were made between the $\alpha_v$ parameters (mean $\alpha_v$ values of the cell subpopulations) and such variables as proportion of cells in S + G2, tumor size and grade, estrogen and progesteron receptors as well as the presence and number of metastases. No significant correlation was found except that the fraction of cells with low $\alpha_v$, correlated (P < 0.05) with absence of progesterone receptors in carcinoma cases (n = 32), (Fig. 5).

In addition, comparisons were carried out within the carci-
noma subsample, relating DNA content classified as (di-, tetra-, and other ploidy) to estrogen and progesterone receptors, involvement of lymph nodes, tumor size and grade. As expected, tumor size correlated with the presence of nodal metastases (borderline significance), and the absence of estrogen receptor correlated with tumor grade (P < 0.001).

DISCUSSION

It was previously shown that sensitivity of DNA in situ to denaturation (α) correlates with a degree of nuclear chromatin condensation and with kinetic properties of the cells (25–36, 39, 41). Generally, the DNA of quiescent cells and/or cells having more condensed chromatin, is more sensitive to denaturation than is the DNA in cycling cells, with diffuse and transcriptionally active chromatin. This evidence suggests that cell subpopulations characterized by high α values may be kinetically and transcriptionally less active compared to cells with low α.

The assay of DNA denaturation in situ was recently adapted to solid tumors (35). In studies of human colon tumors we observed that DNA in cells of adenomas as well as normal colonic epithelium was more sensitive to denaturation than the DNA in nuclei of carcinoma cells, and that the degree of sensitivity correlated with pathologically determined stage of the disease. Thus, significant differences in DNA resistance to denaturation were seen in the noninvasive (adenomas) and superficially invasive (Dukes' A stage) tumors versus more deeply invasive (Dukes' B, C, and D) tumors (35). The data suggested that measurements of the DNA in situ sensitivity to denaturation may provide a novel parameter for assessing tumor prognosis.

In the present study of human breast tumors, the technique was further refined by using human lymphocytes as external and internal standards. The data indicate that there are significant differences in DNA sensitivity between normal breast tissue and most carcinomas. All normal samples, with the exception of a single case diagnosed as duct hyperplasia, were characterized by a unimodal cell distribution with α values above 0.6 (mean value = 0.69).

Most carcinomas were bimodal, having a cell population with DNA resistant to denaturation and at values below 0.6 (mean 0.53). Taking into account previously noted data obtained from in vitro studies, linking the proliferative state with DNA sensitivity to denaturation, it is possible that the observed high α and low α subpopulations represent quiescent and cycling cells, respectively. High α values may also be characteristic of either differentiating, low transcriptional activity or dying cells with condensed chromatin or pycnotic nuclei, respectively (35, 41–43).

In the case of the aneuploid tumors it was possible to discriminate diploid versus aneuploid (tumor) cells based on differences in total cell luminescence (DNA content). In most of the tumors the high α aneuploid (tumor) cells had a G2 DNA content whereas the low α cells may have G1, S and G2 DNA content. This suggests that in these tumors quiescent cells have a G1 DNA content. Occasionally, however, cells were observed with high α and an S or G2 DNA values. Such cells are typical of quiescent cells arrested in S or G2, as described in variety of cell systems (39, 41).

No correlation between cells with low α and proportion of cells in S + G2 was apparent. This may be due to a combination of several factors: (a) the presence of quiescent cells with an S and G2 DNA content, which, as mentioned, may have high α.
(b) the large excess of cycling G$_1$ cells compared to S + G$_2$ cells; (c) possibility that high $\alpha$$_1$ of the cells correlates not only with their cycle kinetics but also with low transcriptional activity (condensed chromatin), nuclear pycnosis (cell death), or differentiation.

In most of the aneuploid tumors the diploid cells were heterogenous with respect to $\alpha$, and it was possible in such cases to distinguish low- and high-$\alpha$$_1$ subpopulations (see Fig. 2C). This is in contrast to normal breast tissue which with the exception of a single case showing duct hyperplasia, had only high $\alpha$$_1$ subpopulations (Fig. 2A, Table 1). It is possible that the low $\alpha$$_1$ diploid cells may be reactive cells (stimulated lymphocytes, macrophages) infiltrating the tumor. So, the number of such reactive host cells may also be of prognostic value.

In the diploid tumors the low and high $\alpha$$_1$ subpopulations may each represent a mixture of tumor and host cells present in unknown proportions. Since the nuclear samples were prepared from tumor tissue, it is expected that tumor cell nuclei predominate in these samples. There is no second marker however, to positively identify these cell populations.

None of the other measured parameters (hormone receptors, ploidy level, etc.) has sufficient prognostic value to assess, by comparison with, the value of the denaturability parameter. Nodal metastases and tumor size represent advancement of tumor growth (a time function) not necessarily related to intrinsic properties of the tumor. Thus, whether the assay of DNA denaturability will eventually prove to be of prognostic value will require prospective studies of a much larger group of patients over a period of years.

REFERENCES

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