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COMPARISON BETWEEN FLOW CYTOMETRY AND SINGLE CELL CYTOPHOTOMETRY FOR DNA CONTENT ANALYSIS OF THE UTERINE CERVIX

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Abstract

The DNA content of tumor cells in imprints and biopsies from 33 patients with invasive squamous cell carcinoma and 5 patients with adenocarcinoma of the uterine cervix were analyzed by flow cytometric and single cell cytophotometric methods. A correlation of r=0.83 was found between the modal DNA values obtained by the two methods. Both methods discriminated between diploid and aneuploid tumors and gave comparable but not identical results. Single cell cytophotometric measurements were in this study more reliable than flow cytometric measurements in diploid tumors. Multiple stemlines, small aneuploid peaks and the so called S-phase rate could only be determined by the flow cytometric technique. Flow cytometry seems to be the method of choice in determination of the DNA content in cervical carcinoma cells and cytophotometric single cell measurements are of complementary value.

Both flow cytometric and single cell cytophotometric DNA determinations have been shown to be of prognostic value in certain tumors (1, 4, 6, 8, 13, 16, 18). Moreover, flow cytometry allows an estimation of the relative S-phase rate. In many studies S-phase estimates could be correlated to survival (4, 12, 18, 19), thus enhancing the prognostic usefulness of flow cytometry.

As concerns cervical carcinoma, the results of DNA measurements are contradictory. In 1973 NG & ATKIN (17) published a microspectrophotometric study of Feulgen stained smears, in which carcinomas with near-diploid values had a less favorable prognosis than aneuploid carcinomas with high DNA values. Recently, in flow cytometric studies, TRIBUKAIT (19) has published similar results, whereas the results of JAKOBSEN (14) indicate that grossly aneuploid tumors are more malignant. The diverging results may be due to different follow-up times and different techniques. In the present investigation flow cytometric and single cell cytophotometric techniques were compared from a purely methodologic point of view.

Material and Methods

The material consisted of 33 patients with invasive squamous cell carcinoma and 5 patients with invasive adenocarcinoma of the uterine cervix. All patients were classified according to FIGO (7). From each patient biopsies were taken for histopathology, flow cytometry and single cell cytophotometry. Each specimen was divided into two halves: one for histopathologic examination and the other for flow cytometry and imprints.

Flow cytometry. All the biopsies were preserved in a 12% DMSO (dimethylsulphoxide) solution and instantly frozen at −70°C. Before preparation the biopsies were thawed at room temperature for 60 to 90 min.

Cell suspensions for the flow cytometric analyses were obtained by a technique previously described

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by TRIBUKAIT et coll. (20). The biopsy material was squeezed through a fine-mesh nylon gauze (40–50 mesh counts) with a Tris-EDTA-buffer together with 1 mg/ml RNase to remove all RNA. Suspensions of single cell nuclei were obtained by pepsin treatment (0.5% pepsin 1 000 U/g) to eliminate unspecific cytoplasmic fluorescence. After washing in the buffer the cell nuclei were stained with 2.5×10^{-5} mol/l ethidium bromide in Tris-EDTA buffer with a molality of 395 mOsm, at pH 7.5.

For the analyses of the DNA contents of the cell nuclei a rapid flow cytometer ICP 11 (Phywe, West-Germany) with a flow rate of up to 1 000 cells/s was used. The excitation and emission wavelengths were 455 to 490 nm and 590 to 630 nm, respectively. The obtained signals were sorted by a 256 multichannel analyzer (ND 100, USA). A mean value of 60 000 cell nuclei (with a range of 20 000–80 000 cells) were analyzed from each tumor specimen. From each patient biopsies from tumor-free parts of the cervix were also analyzed.

Human lymphocytes with a diploid DNA content (2c) and with a coefficient of variation (CV) of 2 to 3 per cent were used as an external standard and in peri-diploid tumors also as an internal standard. The modal DNA values of the cell populations were defined by the most prominent aneuploid peak. The DNA content was considered to be aneuploid when a separate peak could be distinguished from the diploid one. If the histopathologic diagnoses were conclusively malignant, samples with diploid DNA contents were regarded as representative for diploid tumors if the deviation was less than 10 per cent from the normal lymphocyte DNA value according to TRIBUKAIT et coll. (21). In each specimen the proportion of cells between the G 1 and the G 2 peaks, corrected for background fluorescence, was calculated according to the method described by BAISCH et coll. (3).

Single cell cytophotometry. Imprints from unfixed, freshly cut biopsies were fixed in acetone-ethanol 1 : 1 for 30 to 60 min and later air-dried. The slides were incubated for one hour at 37°C in 0.5 mg/ml RNase solution (bovine pancreas, Sigma) and then stained in ethidium bromide solution (10 mg/l) buffered with 0.1 mol/l Tris at pH 7.5. The final solution was 0.1 mol/l in NaCl. The preparations were mounted in the staining solution. The DNA measurements were performed in a Zeiss Universal microscope fluorometer equipped with an HBO 100 light source, a BP 516 exciter filter, an FT 580 beam splitter and LP 590+BG 18 barrier filters. The stability of the equipment was checked by an uranyl glass standard. The cells were identified by phase contrast microscopy. Usually about 100 cell nuclei were measured. All measurement values were expressed as DNA ploidies, where 2c corresponded to the mean DNA content of the external reference lymphocytes, 30 of which were usually measured. Ficoll-separated human lymphocytes were used. Internal lymphocyte reference was also used when present. The DNA values were presented in frequency histograms where each class interval comprised 5 per cent (0.1c) of the diploid value. The tumors were considered to be diploid if the deviation was less than 10 per cent from the reference lymphocytes. A few cases with a peak at 2.0c but a median value (P50) of less than 2.4c were also considered diploid. If no distinct peak was obtained, or if the peak was not prominent, the median value (P50) was regarded as the modal DNA value.

Table 1

Distribution of diploid and aneuploid tumors and the proportion of aneuploid tumors with more than one stemline

	Diploid	Aneuploid, total	Aneuploid, >one stemline	Total
Flow cytometry	11	27	6/27	38
Single cell cytophotometry	8	26	1/26	34

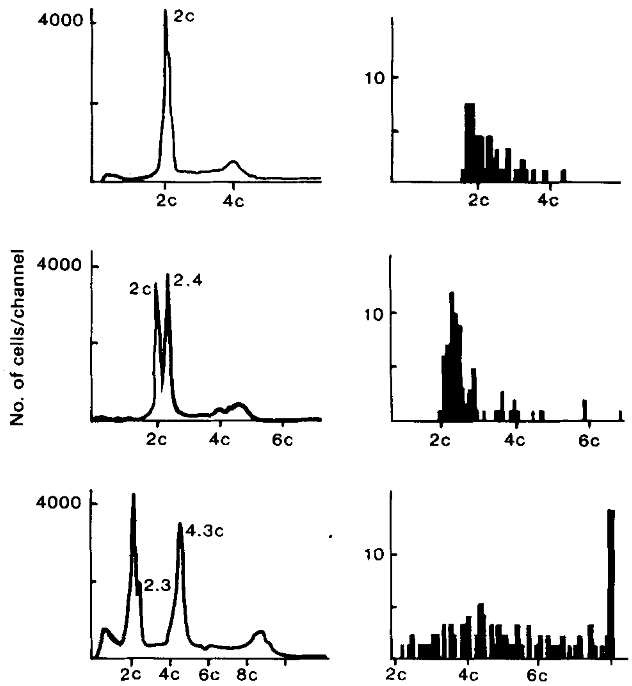
Table 2

Comparison between 2 flow cytometric measurements, performed with an interval of 3 months. Relative DNA values

Case No.	First measurement	Second measurement
1	2.0c (17.5% S-phase)	2.1c (17.5% S-phase)
2	2.0c (16.8% S-phase)	2.0c (16.5% S-phase)
3	1.8c, 2.0c and 3.5c	1.8c, 2.2c and 3.5c
15	2.6c, 5.2c	2.7c, 5.3c
16	4.2c (30.9% S-phase)	4.2c (28.7% S-phase)
17	2.2c (22.9% S-phase)	2.2c (24.7% S-phase)
18	2.2c	2.2c

Results

According to the FIGO classification (7) 21 patients belonged to stage IB, 5 patients to IIA, 8



Three flow cytometric measurements in the left column and the corresponding single cell cytophotometric measurements in the right one. Top: Case 13. Both methods show a diploid tumor with high proliferation. Middle: Case 20. A hyperdiploid (aneuploid tumor), 2.4c, is demonstrated by both methods. Bottom: Case 3. Flow cytometry reveals a marked aneuploidy with two distinct stemlines (2.3c and 4.3c) and high proliferation. Single cell cytophotometry shows clear aneuploidy with increased proliferation.

Table 3

Comparison between 2 single cell cytophotometric measurements performed with an interval of three months. Relative DNA values

Case No.	First measurement	Second measurement
11	4.9c	4.9c
13	2.0c	2.0c and 3.0c
18	2.5c	2.1c and 2.6c
19	3.7c	3.3c
20	2.8c	2.3c
21	2.8c	2.8c

patients to II B and 4 patients to III B. One tumor of the squamous cell carcinomas was well differentiated, 15 tumors were moderately differentiated and 17 tumors poorly differentiated. Four adenocarcinomas were moderately and one poorly differentiated.

A brief summary of the DNA measurements is presented in Table 1.

All biopsies for flow cytometry were evaluable. The mean of the coefficients of variation, CV, was 3.8 per cent (range 2.7–5.1%). Eleven tumors were considered to be diploid and 27 aneuploid. Among the aneuploid tumors 6 specimens contained more than 1 aneuploid stemline. Estimation of the S-phase rate according to BAISCH et coll. (3) was possible in each specimen.

In 7 specimens the cell suspensions were, after fixation, divided into 2 halves, which were measured on two occasions, with an interval of 3 months. This showed good reproducibility of the estimates both as regards the S-phase rate and the relative DNA content (Table 2).

By single cell cytophotometry 8 tumors were regarded as diploid and 26 as aneuploid (Table 1). One of the aneuploid tumors contained 2 separate clones. As a rule, however, multiple stemlines could not be detected by single cell cytophotometry, because of insufficient resolution of the peaks. Proper estimation of the S-phase was not possible and the occurrence of more than one stemline could not be confirmed with accuracy.

Preferably dissociated cells were measured, in order to avoid disturbing fluorescence from surrounding cell nuclei. There was a marked tendency for clustering in well and moderately differentiated carcinomas. Thus 4 of the imprints were not evaluable at all, because of too few dissociated, measurable cells. Further 8 imprints were difficult to measure, mainly for the same reason. Six imprints were measured on two different occasions. In 3 of these there was some degree of variation (Table 3).

The coefficient of correlation between the modal DNA values estimated by the two methods was 0.83. On average single cell cytophotometry gave 0.3c higher modal DNA values than flow cytometry. In 25 of the 34 tumors that could be measured by both methods the agreement was rather good (Figure). Marked differences were observed especially in the 6 cases with more than one stemline. Three histograms were classified as aneuploid by single cell cytophotometry and as diploid by flow cytometry.

The coefficients of variation were lower with the flow cytometric technique than with single cell cytophotometry. In fact, the coefficient of variation was not evaluable in all single cell cytophotometric measurements, since a distinct peak could not be obtained in every case. Thus the modal DNA values were more distinctly estimated by flow cytometry.

Discussion

Previous investigations on invasive carcinoma of the uterine cervix have shown that quantitative DNA measurements are of interest, although the results are conflicting (14, 17, 19). The disparate results may be explained by different follow-up times. As the DNA content can be measured by two principal methods, flow cytometry and single cell cytophotometry, it is of interest to compare advantages and disadvantages of the two techniques.

An advantage of flow cytometry is the rapidity of the method, which allows an estimation of a large number of cells, thus providing a good statistical basis for a proper determination of the modal DNA values and flow cytometric S-phase rates.

In the present investigation flow cytometry was superior in detecting multiple stemlines. Small aneuploid peaks could only be detected with flow cytometric technique. Both the determination of the S-phase rate and the detection of multiple stemlines seem to be of clinical interest (9, 10, 18, 21, 22).

As fresh or frozen biopsies are required for optimum results, flow cytometry is best suited in prospective studies, but a new, promising method for analysis of paraffin-embedded material is developed, thus making retrospective analyses possible (11).

One advantage of the single cell cytophotometry is that each measurement is performed on microscopically identified tumor cells, whereas there always is an admixture of normal cells in flow cytometric measurements. However, discrimination between such cells and tumor cells may be possible by using multiparameter analysis as used by COLLSTE et coll. (5). In aneuploid specimens this admixture is no major problem because the normal cells form a separate, diploid peak. However, in diploid tumors the mentioned admixture is an obvious disadvantage. As almost all cervical cancers show high S-phase fractions, 6 to 30 per cent compared with 4 to 11 per cent in normal samples (mean 6.8%, SD 2%), tumor specimens can, however, usually be discriminated from tumor-free samples.

Single cell cytophotometry is a time-consuming procedure which allows relatively few cells to be measured. In this study preferably dissociated cells were measured. Thus an undesired selection of cells may have occurred.

No satisfactory estimation of the proliferation can be attempted using single cell cytophotometry, because of the limited number of measured cells. If, as in the present investigation, only about 100 cells are measured, the method is not accurate enough to detect multiple stemlines.

Both methods can be used to discriminate between diploid and aneuploid tumors. In accordance with other authors (2, 15) we found a good correlation between the modal DNA values estimated by the two methods. The slightly higher DNA values estimated by single cell cytophotometry may be explained by disturbing, unspecific background fluorescence which in each individual measurement can give a slightly increased modal DNA value. A contributory reason may be that in 10 cases the median value (P50) was used to determinate the modal value.

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