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Flow Cytometric Characterisation of Tumor Associated Changes in Gynecologic Malignancies

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Abstract

Flow cytometry is a rapid and precise technique for the simultaneous measurement of several cell parameters. The flow cytometer used in these studies (1) determines cell volume and simultaneously cell fluorescence. The advantage of a cell volume and fluorescence measurement is that the concentration of intracellular substances and the surface density of membrane structures can be calculated. The present results show, that a simultaneous cell volume / cell DNA measurement is not distinctive enough to clearly identify a few percent tumor cells among many normal cells. The measurement is nevertheless useful since it distinguishes nucleated cells from cell debris, free nuclei and erythrocytes. The determination of the density of Carcinoembryonic Antigen (CEA) on the cell membrane is more informative. The CEA density is significantly increased on a majority of malignant cervical cell samples. A simultaneous three parameter measurement of cell volume, DNA and CEA will improve the CEA density determination on small cells by eliminating the influence of cell debris and erythrocytes in the lower cell volume and cell fluorescence range.

Keywords: Flow cytometry, Gynecologic tumors, DNA, CEA.

1. Introduction

Tumor cells in cervical smears are most often diagnosed from Papanicolaou stained samples. This procedure is time consuming and difficult to quantify. Two new approaches for automation of tumor cell identification have been investigated in recent years. The first and quite advanced technique is image analysis (2-4) and the second is flow cytometry (1,5-8). Flow cytometry does usually not resolve cellular structures, but its advantage is high speed, great precision of the measurement and mainly the capacity to measure several cell parameters simultaneously. The latter capacity opens a new way to use defined biochemical reactions for tumor cell identification. The purpose of this study was to determine to what extent simultaneous two parameter measurements of cell volume / cell DNA and cell volume / CEA were informative parameters with regard to the identification of malignant cells in smears of the cervix uteri.

2. Materials and Methods

Preparation and Fixation of Cells

The smear sample was collected using a cotton wool swab and was immediately transfered with the swab into a 5mM Tris buffered saline solution (TBS) of pH 7.4 containing 20mM EDTA (10). After shaking the cell suspension in plastic tubes in the presence of 2mm diameter spheroid polycarbonate beads for 5 minutes, it was passed through a metal sieve with a hole diameter of 100 microns. The cells were resuspended in TBS after centrifugation (800g, 10 min.) and fixed in 70% ethanol or 2% aqueous glutaraldehyde for 2h at room temperature. Leukocytes were prepared by 15 min. incubation at 0°C of human blood, which was diluted 1+4 (v/v) with a 0.83% NH_4Cl solution containing 10mM Tris/HCl at pH 7.4. The unlysed nucleated cells were washed twice with TBS and fixed in suspension with 2% glutaraldehyde.

Staining of Cells

DNA measurements were performed using ethanol fixed cells, stained with mithramycin (11) at a final concentration of 100 ug/ml in TBS containing 15 mM $MgCl_2$.
Carcinoembryonic antigen on the cell membrane was demonstrated with an FITC-Labelled Anti-CEA serum (Hoffmann La-Roche, Basel, Switzerland) after 15 min. incubation of the cells at 0°C.

The Fluvo-Metricell System and Data Analysis

The Fluvo-Metricell was developed in this laboratory (1) and combines electrical sizing with the measurement of one or two fluorescence parameters. The cells were suspended at a concentration of $1x10^7$ cells/ml in TBS and sized in a cylindrical orifice of 85 µm diameter and 100 µm length, with hydrodynamic focusing at a current of 0.212 mA. The fluorescence was excited by a HBO-100 Hg high pressure lamp using a 500 nm low pass interference filter and a 520 nm high pass barrier filter. The maximal amplitude of the volume and fluorescence signals was stored in a 64 x 64 channel array of a multichannel analyzer (AEG-Telefunken, Ulm, Germany). The experimental histograms were stored on a magnetic tape for later data analysi by a Fortran IV computer program (9). The program plots perspectivic views of the threedimensional histograms (fig.1) and projections of these histograms on the volume/fluorescence plane (fig.2).

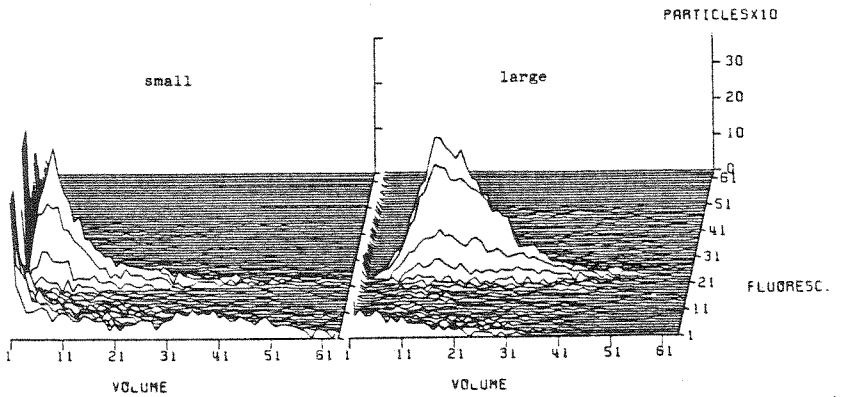

Fig.1 Normal cervical smear stained with mithramycin (cell volume / cell DNA).

The content of each channel was normalized to the maximum channel content (#,100%). The numbers 1 to 9 represent either the amplitude range from 10-90% in steps of 10% or the range of 1-9% in steps of 1%. Channel contents, which are higher than the respective amplitud range are indicated with *. The program calculates in addition the number of particles in limited areas and the fluorescence/cell

surface ratios.

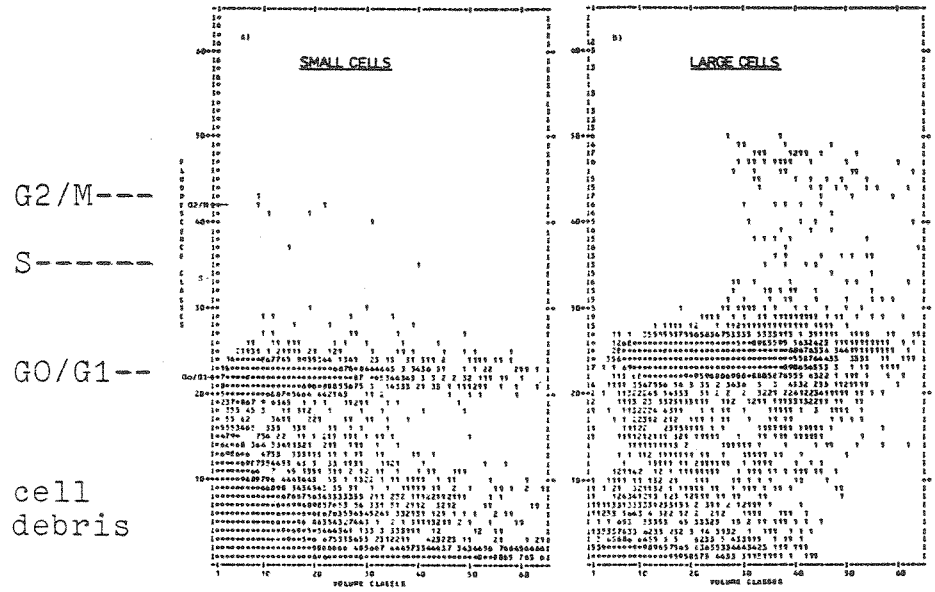

Fig.2 Projection of the histogram of fig.1 into the volume/fluorescence plane (amplitude range 1-9%).

3. Results

DNA Measurements

Two types of nucleated cells were found in a normal cervical smear (fig.1) with regard to the cell volume. The cluster of the large cells represents the superficial cells and the large intermediate cells, whereas the group of small cells comprises leukocytes and cells of the lower cervical epithelium (fig.2). The particles below the GO/G1 phase represent cellular debris. The proliferative activity of the cells is low (fig.3). The number of cells in the S and G2/M phase of the cell cycle in tumor smears is increased (fig.3) and two types of growth patterns have been observed. In some cases (fig.4A) the distinction between S and G2/M phase cells is well marked, while in other cases the G2/M cells can not be clearly distinguished (fig.4B). The GO/G1 phase is often broad in tumor smears suggesting the presence of aneuploidic cells. The proliferative activity of the cells is also increased during pregnancy but to a lesser extent (fig.3).

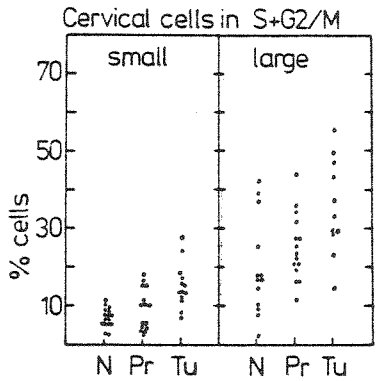

Fig.3: Proliferation patterns of smear samples from normal (N), pregnant (Pr) and tumor bearing women (Tu).

Volume Distributions

Tumor samples show an increase of the number of small cells. Additionally the small cells in the GO/G1 phase exhibit a broader size distribution than normal cells.

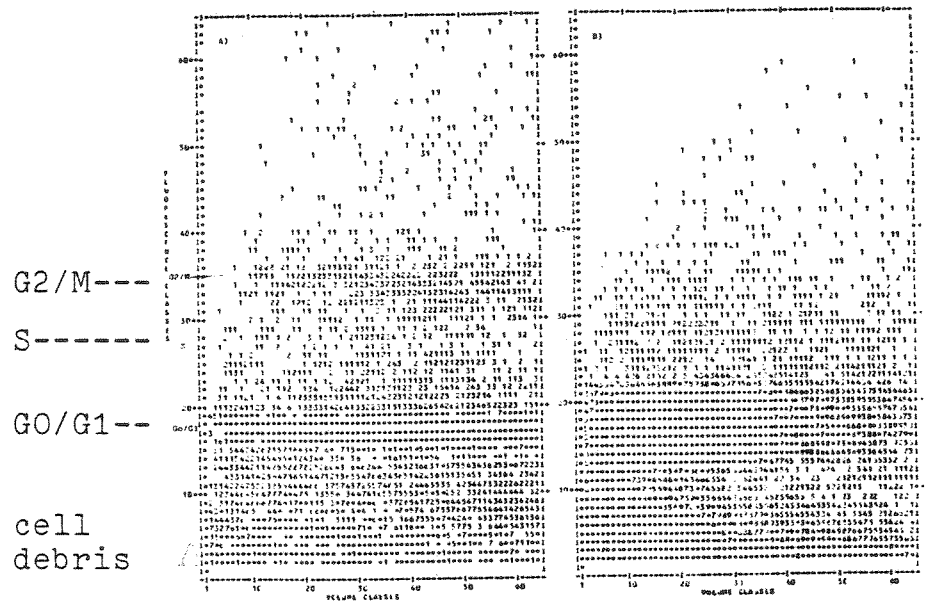

Fig.4: Collum carcinoma cells, mithramycin stained (ampl. range 1-9%) where S and G2/M cells are well distinguishable (A), while in another case G2/M cells are not distinguishable (B).

CEA Density on the Cell Membrane

The total cell membrane bound FITC-Anti-CEA (fig.5) depends of the total cell surface and the density of CEA packing. For reasons of comparison the data were standardized in the following way: The surface (S) of spheroid cells was calculated from the cell volume (V) according to $S=4.84(V)^{0.667}$. The CEA density was obtained as ratio fluorescence / cell surface. The assumption of a spheroid shape for all cells is the most rigorous test for differences between normal and malignant cells. The superficial cells in normal smears are flat and have comparatively more surface than predicted from a spheroid shape i.e. the calculated differences of the CEA density between the normal and the malignant cells are in reality greater than apparent from Tab. 1 and 2.

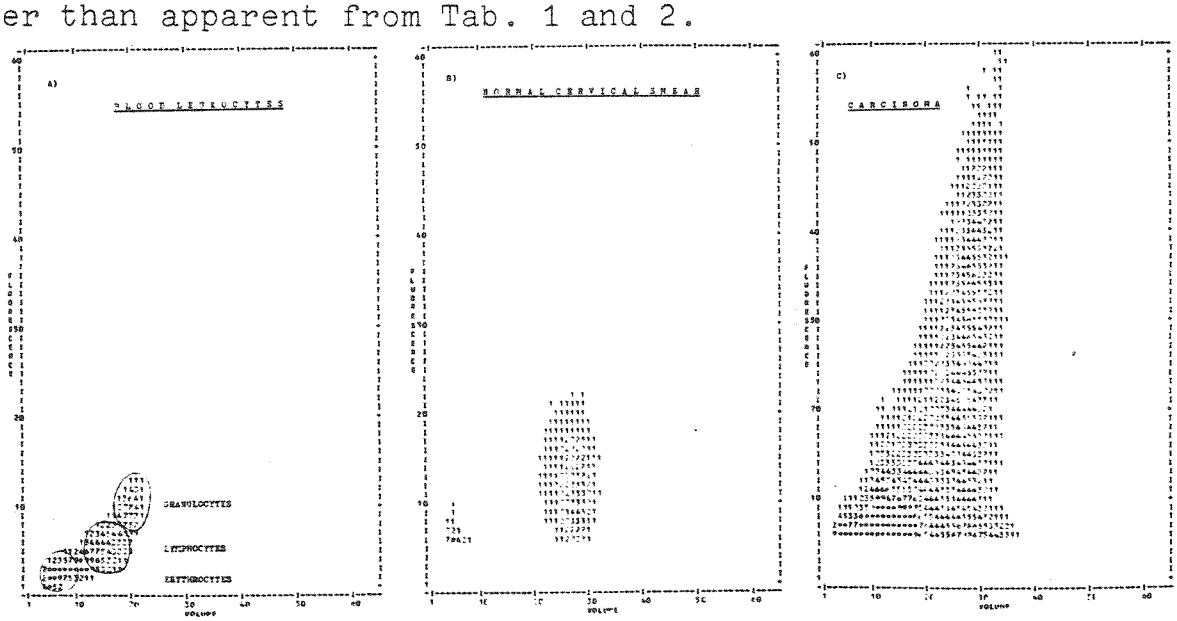

Fig.5: Cell membrane bound FITC-Anti-CEA of blood leukocytes (ampl. range 10-90%) (A), normal cervical cells (ampl. range 10-90%) (B) and collum carcinoma cells (ampl. range 1-9%) (C) (logarithmic amplification of the volume pulses).

There is a significant difference of the CEA density on the cell membrane of tumor cell samples as compared to the normal smear cells (Tab.1 and 2). The superficial and large intermediate cells of a normal smear bind the Anti-CEA in either a comparable or a lower density than blood leukocytes. The fluorescence of the non malignant cells may be due to a non specific absorption of the FITC-Anti-CEA or the presence of crossreacting structures on normal cell membranes (12,13).

Cell membrane bound FITC-Anti-CEA Fluorescence

	Fluorescence / μm^2 cell surface	Cell number x 10^5
Tumor	0.7443 ± 0.001	1.05
Normal smear	0.4356 ± 0.001	0.60
Leukocytes	0.3997 ± 0.001	0.15

Tab.1: Surface densities of membrane bound Anti-CEA for leukocytes, normal and malignant cervical cells of Fig. 5.

Cell membrane bound FITC-Anti-CEA Fluorescence

Exp. No.	Tumor Fl./μm^2 cell sf.	Normal Fl./μm^2 cell sf.
1	0.797 0.598	0.249 0.447
2	0.700 0.648 0.580	0.214 0.448
mean ± s.e.	0.665 ± 0.04	0.340 ± 0.05

Tab.2: Surface densities of membrane bound Anti-CEA for two independent experiments with several different smears.

4. Discussion

Cell volume / cell DNA measurements allow a distinction of normal and tumor cells if a sample contains in the order of 15-30% tumor cells. It is difficult however, to identify a few tumor cells in a majority of normal cells due to the overlap of the position of normal and malignant cells in the two parameter histogram. This is also true for the proliferative activity (fig.3) of the cells, which overlaps between cells of normal, pregnant and tumor bearing women. The broadening of the volume distribution curve of the GO/G1 phase cells is quantitatively more distinctive (not shown), but still does not allow to identify a small percentage of tumor cells. A cell volume/ cell DNA measurement has, however, the important advantage, that morphologically intact cells with nuclei can clearly be distinguished from erythrocytes, broken cells, enucleated cells and free cell nuclei.

The CEA density measurement is more distinctive for tumor cells than the cell volume / cell DNA measurement. Usually CEA is determined from blood serum samples, where it can be detected in amounts above 2.5 ng/ml in the serum of women suffering from squamous cell carcinoma of cervix uteri. The frequency of increased serum

concentrations of CEA is ranging from 9% for the carcinoma in situ up to 88% for the extended metastatic collum carcinoma (14,15). CEA can also be detected on the membranes of collum carcinoma cells by immunohistological methods (16). The flow cytometric results confirm the histological investigation, but the advantage of flow cytometry is that the results can be quantified. A certain problem in the measurement is, that enucleated cells, cell debris and erythrocytes in the lower fluorescence and volume classes may overlap with intact cells. This does not affect the CEA density determination on the malignant cells, but sometimes interferes with the CEA determination on leukocytes. The solution to this problem will be a simultaneous three parameter measurement of cell volume, cell DNA and CEA. The cell volume and cell DNA will serve to identify the nucleated cells and the CEA measurement together with the cell volume to distinguish between normal and malignant cells. CEA density is certainly not the only parameter of interest for the identification of malignant cells. Experiments are in progress where the lectin binding capacity and the charge density of cervical cells are determined, since the glycoproteins of the tumor cell membranes are possibly sufficiently altered to be of value for a flow cytometric diagnosis of malignant cells.

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5. References

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