COMBINED DNA AND PROTEIN CYTOFLUOROMETRY OF GYNECOLOGICAL SMEARS: —QUANTITATIVE IDENTIFICATION OF TUMOR CELLS AND DISCRIMINATION OF INTRAEPITHELIAL NEOPLASIA FROM INVASIVE CANCER—

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Both DNA and protein levels of cells on the cytological specimens obtained from 12 normal individuals, 3 cases of cervical dysplasia, 2 cases of carcinoma in situ, 11 cases of cervical cancer and 4 cases of endometrial carcinoma were measured by combined DNA and protein cytofluorometry.

In cervical dysplasia, the percentage of cells in which DNA values exceeded normal diploid-tetraploid region was high in accordance with the intensity of epithelial atypia. The DNA and protein distribution patterns of severe dysplasia were mostly similar to those of carcinoma in situ. In all the cases of uterine cancer measured, both DNA and protein levels of representative cells were abnormally elevated, exhibiting hyperploidy and aneuploidy in their DNA and protein content. Moreover, a significant positive correlation was observed between DNA and protein values in a linear regression analysis. The parallelism between DNA and protein values was more evident in invasive cervical cancer than in carcinoma in situ. Therefore, it is suggested that an abnormal elevation of both DNA and protein levels in neoplastic cells may be related to tumor invasiveness.

The present study suggests that combined DNA and protein cytofluorometry enables us to identify uterine neoplastic cells quantitatively and facilitates discrimination of intraepithelial neoplasia from invasive cancer with respect to their DNA and protein levels.

Several authors have reported microspectrophotometric measurements of the DNA contents of Feulgen-stained cells on various lesions of histological and cytological specimens in the uterine cervix (8, 9, 10, 11, 12, 13, 14, 17, 18) and endometrium (15, 16). From these studies, it has been shown that uterine cancer cells often have increased DNA amounts compared with those of normal cervical and endometrial cells. However, clear discrimination of normal epithelia, premalignant and malignant lesions has not yet been established with respect to their nuclear DNA levels.

Various techniques applied to DNA microspectrophotometry have some
inherent problems which interfere with the correct measurement of minute amounts of nuclear DNA. For example, factors of distributional error, background and stray light loss due to light refraction and scattering in the object should be corrected when absorbance microspectrophotometry is used. Although these factors are negligible in fluorescence cytophotometry, stabilization of specific fluorescence to DNA as well as elimination of background noise should be mandatory for the validity of DNA measurement. To practically solve these problems, the irradiation method (2, 3), i.e. irradiating the specimens with a light of selected excitation wavelength for a certain fluorochrome previous to the measurement, may be one of the most promising techniques.

Recently, multi-color fluorescence methods (1, 4) have been developed in the field of cytofluorometry for quantification of different intracellular substances in the same cells. Fukuda et al. (4) pointed out that these multi-color fluorescence methods, if they are properly coupled with cytofluorometry, might be useful tools to measure intracellular substances with a very high accuracy and to identify neoplastic cells.

The present study was designed to measure both DNA and protein contents on uterine neoplastic cells of cervical dysplasia, carcinoma in situ, and uterine cancer by the combined DNA and protein cytofluorometry. Specificity and accuracy of the combined DNA and protein cytofluorometry used in this study were previously reported (5, 6).

MATERIALS AND METHODS

Cytological specimens were taken from 11 patients with cervical cancer, 1 patient with mild dysplasia, 2 patients with severe dysplasia, 2 patients with carcinoma in situ, 4 patients with endometrial cancer, and 12 normal menstrual women as controls. Eight of 11 cases of cervical cancer were diagnosed as differentiated squamous cell carcinoma and the remaining were diagnosed as undifferentiated squamous cell carcinoma by postoperative histological examination. Mild dysplasia, severe dysplasia, and carcinoma in situ were histologically confirmed after diagnostic conization. In endometrial carcinoma, 3 cases were grade 1 adenocarcinoma and one was mixed adenosquamous cell carcinoma having the glandular elements of grade 2 adenocarcinoma. Endometrial biopsy specimens taken immediately after endometrial aspiration showed proliferative phase in 3 normal menstrual women and secretory phase of postovulatory day 4 to day 7 in 4 women.

The cells obtained from cervical scraping with a sponge block (1.5×1.5×1.5 cm) or endometrial aspiration with Gravlee jet washer (Bard-Parker Co., U.S.A.) were suspended in lactated Ringer's solution. After centrifugation at 1,500 r.p.m. for 5 min, 50 μl of the sediment phase was resuspended in 2 ml of Saccamanno's solution consisted of 2% polyethylene glycol 1540 in 50% ethanol and gently syringed by 10 strokes to break up clumps of cells. The smear specimens were prepared by centrifugation at 2,000 r.p.m. for 5 min with Sakura Autosmear (Sakura Seiki Co., Japan). The specimens were fixed in Böhmm's fixation mixture (methanol: formalin : acetic acid=85 : 10 : 5) for 45 min at room temperature.

The combined DNA and protein staining was performed using the ninhydrin-Schiff and Feulgen techniques as described previously (5, 6, 7). Briefly, the speci-
mens were washed in running tap water for 2 hr and pre-warmed in distilled water at 60°C prior to hydrolysis. After acid hydrolysis in 1 N HCl for 5 min at 60°C±1°C, the specimens were stained in pararosanilin-Schiff solution (6) for 10 min at 7°C and passed through three changes of 15 min each of sulphurous rinse solution (6) at 7°C and washed in running tap water for 30 min. The Feulgen-stained specimens were subsequently incubated with 0.5% ninhydrin in absolute ethanol for 15 hr at 37°C. After washing the smear specimens in running tap water for 1 hr, protein staining with acriflavine-Schiff reaction was performed for 10 min at 7°C including a thorough rinse in the same way as described above. The doubly stained smear specimens were dehydrated with ethanol and embedded with Entellan (nD20=1.496, Merck Co., West Germany) after replacement of ethanol with xylene.

The specimens were irradiated with an excitation wavelength at λ 543 nm for 6–8 hr and at λ 439 nm for 8–10 hr with a 1 KW high pressure mercury lamp which can yield 10^7 J/M^2 to eliminate background fluorescence and stabilize specific fluorescence of DNA and protein according to the irradiation method (2, 3).

The DNA and protein content of 300 to 500 cells on each specimen were randomly measured by a fluorescence cytophotometer MMSP-RF-II (Olympus Co., Japan) at the wavelength λ 543 nm for DNA and λ 439 nm for protein measurement. The mean DNA-value and the standard deviation as well as the mean protein-value and the standard deviation were calculated from the data obtained from

Figs. 1a, 1b. The DNA and protein histograms of superficial and intermediate cells of normal squamous epithelium of the uterine cervix are shown in Fig. 1a, and those of parabasal and basal cells are shown in Fig. 1b. The dotted columns show the DNA and protein histograms of polymorphonuclear leucocytes in the actual specimens. abscissa; DNA, ordinate; protein. F.I.; fluorescence intensity.
RESULTS

Polymorphonuclear Leucocytes, Normal Cervical Cells and Endometrial Cells

The DNA and protein distribution patterns of about 100 polymorphonuclear leucocytes in the actual specimen showed a single mode occupying 2C and 2P regions in the DNA and protein histograms, respectively. Smear specimens taken from 5 patients with normal squamous epithelium were measured as controls. The DNA contents of superficial and intermediate cells of normal squamous epithelium of the uterine cervix were distributed in 2C region, whereas their protein content ranged from 2P to 4P regions in all cases (Fig. 1a). The parabasal and basal cells ranged from 2C to 4C regions for DNA and from 2P to 4P regions for protein as shown in Fig. 1b. The DNA content of normal endometrial cells in proliferative and mid-secretory phases were distributed from 2C to 4C and 2C regions respectively, while their protein content ranged from 2P to 4P regions in both phases (Fig. 2a, 2b).

Mild Dysplasia

The DNA and protein content of representative cells in mild dysplasia occupied 2C to high-4C regions for DNA and 2P to 6P regions for protein (Fig. 3). The

FIGS. 2a, 2b. The DNA and protein histograms of normal endometrial cells in proliferative phase are shown in Fig. 2a, and those in mid-secretory phase are shown in Fig. 2b. The dotted columns show the DNA and protein histograms of polymorphonuclear leucocytes in the actual specimens. abscissa; DNA, ordinate; protein. F.I.; fluorescence intensity.
Fig. 3. The DNA and protein histograms of mild dysplasia. The percentage of cells in which DNA-value exceeded the tetraploid region was 4.8. Abscissa: DNA, ordinate: protein. F.I.; fluorescence intensity.

Fig. 4. The DNA and protein histograms of severe dysplasia. The percentage of cells in which DNA-value exceeded the tetraploid region was 32.7. Abscissa: DNA, ordinate: protein. F.I.; fluorescence intensity.

Fig. 5. The DNA and protein histograms of severe dysplasia. The percentage of cells in which DNA-value exceeded the tetraploid region was 47.6. Abscissa: DNA, ordinate: protein. F.I.; fluorescence intensity.

Fig. 6. The DNA and protein histograms of the representative case of carcinoma in situ. The cells in which DNA-value exceeded the tetraploid and octaploid region were 52.3% and 6.6%, respectively. Coefficient of correlation (r=0.789). Abscissa: DNA, ordinate: protein. F.I.; fluorescence intensity.
percentage of cells in which DNA-value exceeded the tetraploid region was 4.8.

**Severe Dysplasia**

The DNA content of representative cells in 2 cases of severe dysplasia ranged from 2C to 8C regions having the protein content of 2P to 8P levels or more. The percentage of cells in which the DNA-value exceeded the tetraploid region were 32.7 in the case of Fig. 4 and 47.6 in the case of Fig. 5.

**Carcinoma in situ**

The DNA content of representative cells of 2 cases of carcinoma *in situ* ranged from 2C to 12C regions and their protein contents were distributed from 2P to 12P regions. Although the DNA and protein histograms were almost the same as those in severe dysplasia, there were hyperoctaploid cells which had an abnormally elevated protein levels in carcinoma *in situ* (Fig. 6). The cells in which DNA levels exceeded the tetraploid and octaploid regions were 52.3% and 6.6%, respectively. A correlation coefficient between DNA content and protein content of the measured cells was 0.789 in a linear regression analysis (Fig. 6).

**Uterine Cancer**

In all the cases of cervical cancer, both DNA and protein levels of representative cells were abnormally elevated, ranging from 2C to 24C regions for DNA and from
Additionally, the existence of both hyperploid (more than 8C) and aneuploid cells was confirmed from the DNA and protein histograms (Figs. 7, 8). A significant positive correlation appeared between DNA and protein content in a linear regression analysis ($r=0.9024$). Protein and DNA levels are expressed in an arbitrary unit. F.I.; fluorescence intensity.

2P to low-32P regions for protein. Additionally, the existence of both hyperploid (more than 8C) and aneuploid cells was confirmed from the DNA and protein histograms (Figs. 7, 8). A significant positive correlation appeared between the DNA and protein content of the measured cells in a linear regression analysis irrespective of histological grade. The least square analysis of the DNA and protein values of cells plotted against abscissa and ordinate, respectively brought on a linear function. The slope of the linear function for differentiated squamous cell carcinoma was steeper than that of undifferentiated carcinoma, although clear discrimination between differentiated and undifferentiated carcinoma seemed to be difficult with respect to their DNA and protein values.

In endometrial adenocarcinoma, 3 out of 4 cases were diagnosed as grade 1 adenocarcinoma where the DNA content ranged from 2C to high-16C regions and the protein content ranged from 2P to high-16P regions (Fig. 9). In the 4th case diagnosed as mixed adenosquamous cell carcinoma of the endometrium, the DNA
content ranged from 2C to 24C regions and the protein content ranged from 2P to 16P levels or more (Fig. 10). The cancer cells probably originating from squamous elements showed higher DNA levels than those of glandular elements.

**DISCUSSION**

Many reports have been published on nuclear DNA levels in normal epithelia, premalignant and malignant lesions of the uterine cervix and endometrium by Feulgen cyto- and histophotometry mostly using absorbance microspectrophotometric techniques.

Sandritter (10, 11, 12) measured the nuclear DNA levels of cervical dysplasia, carcinoma *in situ* and invasive cervical cancer on Feulgen stained specimens by absorbance histophotometry and reported that DNA distribution patterns in dysplasia showed regular diploid and tetraploid DNA-values, while carcinoma *in situ* and invasive cervical carcinoma showed abnormal deviation from the diploid-tetraploid DNA-values. Laumonier *et al.* (8) reported the DNA distribution patterns of cervical dysplasia, carcinoma *in situ* and invasive cervical cancer on histological specimens. Again, they concluded that the DNA histograms of
carcinoma in situ were similar to those of invasive cancer. Zelenin et al. (18) presented results obtained from Feulgen cytofluorometry which showed that there was an abnormal increase in the nuclear DNA contents ranging from diploid to octaploid levels or more in 10 out of 12 cases of cervical cancer. Wilbanks et al. (17) reported DNA values in mild dysplasia, moderate dysplasia, severe dysplasia and carcinoma in situ measured on sections by the two-wavelength method. They stated that the DNA histograms of dysplasia were not remarkably different from those of carcinoma in situ, although the degree of deviation of the DNA values from the normal distribution was least in mild dysplasia but was more pronounced in the higher grade lesions. Nishiya et al. (9) studied the DNA values of dysplasia, carcinoma in situ and microinvasive carcinoma by absorbance cytophotometry using scanning techniques and stated that benign proliferation and mild dysplasia cases were within a normal diploid-tetraploidy whereas cases with carcinoma in situ and microinvasive carcinoma exhibited a DNA range which extended beyond hypertetraploidy with many cells possessing a DNA value in excess of hexaploidy. From these results, it seems to be difficult to attain a consensus as to whether the nuclear DNA levels were different among dysplasia, carcinoma in situ, and invasive cervical cancer. Furthermore, there were some discrepancies on DNA distribution patterns among dysplasia, carcinoma in situ, and invasive carcinoma.

In the present study, there seemed to be a clear distinction among invasive cervical cancer, intraepithelial neoplasia such as dysplasia and carcinoma in situ, and normal epithelia with respect to their DNA and protein distribution patterns.
In all cases of cervical cancer examined, representative cells had an abnormal DNA and protein content compared with those of normal cells which occupied the diploid-tetraploid DNA levels. Additionally, it was clearly shown that there were hyperploid and aneuploid cells in the DNA and protein histograms of cervical cancer cases, whereas the DNA values of carcinoma in situ cases did not exceed the hexadecaploid level.

Quantitative differences of nucleoprotein contents between normal and tumor cells were firstly pointed out by Sandritter (12). The present study demonstrated that there was a significant positive correlation between DNA and protein values in a linear regression analysis irrespective of histological grade of cervical cancer. Extension of the parallel relationship between DNA and protein contents in very high regions of DNA and protein distributions was more evident in invasive cancer than in carcinoma in situ (Figs. 6, 7, 8). Therefore, it is suggested that an abnormally elevated DNA and protein content of neoplastic cells to a very high level may be related to tumor invasiveness. It is of interest that the slope of the linear function for differentiated squamous cell carcinoma obtained from the least square analysis of DNA and protein values plotted against abscissa and ordinate respectively, was steeper than that of undifferentiated squamous cell carcinoma. According to Sandritter (12), similar distributional patterns to those of the DNA were shown by measurements on the histone protein content in carcinoma in situ. The increase in protein content in cancer cells may be attributed not only to the increment of nucleoprotein but also to cytoplasmic protein levels.

In endometrial cancer, abnormal DNA and protein distribution patterns were also demonstrated, although the DNA values of cells in endometrial carcinoma remained at lower levels than those of cervical cancer. Again, a positive correlation was observed between DNA and protein levels in a linear regression analysis.

In the intraepithelial lesions such as dysplasia and carcinoma in situ, the percentage of cells in which DNA value exceeded the tetraploid region increased in accordance with epithelial atypia, suggesting a continuum of epithelial changes from mild dysplasia to carcinoma in situ. Although the DNA and protein distribution patterns of severe dysplasia were similar to those of carcinoma in situ, in the latter there were hyperoctaploid cells having abnormally elevated protein levels.

Some previous studies (8, 10, 11, 12, 17) pointed out that hyperploid cells indistinguishable from cancer cells existed in dysplastic epithelia. As cervical scraping was used to collect cells in this study, it is possible that cytological specimens do not necessarily reflect exactly histological features of the lesion concerned. To clarify this point, DNA and protein measurements on the cells dispersed directly from lesions on histological specimens are now under way.

The present study suggests that the combined DNA and protein cytofluorometry is of great value to identify uterine neoplastic cells quantitatively in the gynecological cytology specimens.

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REFERENCES


