

FLOW CYTOMETRIC ANALYSIS OF RELATIVE MEAN DNA CONTENT OF UROGENITAL CANCER CELLS IN FRESH AND PARAFFIN-EMBEDDED MATERIALS*

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ABSTRACT—*The relative mean DNA content calculation was performed by flow cytometry on single cell suspensions prepared from fresh and paraffin-embedded specimens of 10 patients with surgically resected urogenital cancer. Samples were processed by a modified method of Hedley et al. including two hours of pepsinizing time, ribonuclease digestion, and propidium iodide staining. The mean DNA content which is a quantitative description of flow cytometric characteristics was significantly correlated between the fresh and paraffin-embedded materials ($n = 10$, $r = 0.869$, $p < 0.01$). This method allows for the objective, retrospective analysis of DNA content in relation to diagnosis and prognosis of urogenital cancer.*

Measurements of deoxyribonucleic acid (DNA) using flow cytometry (FCM) have allowed the exploration of the abnormal nuclear composition of urogenital cancer cells. The application of flow cytometric analysis of transitional cell carcinoma (TCC) of the urinary bladder makes it possible to discriminate between groups of histologically indistinguishable tumors with different potential for recurrence and progression.¹⁻³ However, clinical use of the FCM technique for the analysis of urogenital tumors has been limited by the requirements for fresh unfixed tissue until recently.

Since Hedley *et al.*⁴ have described methods for FCM determination of DNA contents in formalin-fixed and paraffin-embedded tissue blocks, this allows clinical studies correlating DNA measurements using the archival mate-

rials. With a modified procedure to obtain paraffin-embedded cells, we have succeeded in obtaining reliable DNA content determination. This study has described the calculating of the relative mean DNA content⁵ of both fresh and paraffin-embedded tissue which provides advantage suitable for analyzing paraffin-embedded tissues.

Material and Methods

Ten paraffin-embedded tissue blocks were obtained from the pathology department. The origin of cancer was the bladder in 6, kidney 2, and prostate 2. Fresh tumor specimens were obtained in all of these cases for comparison.

Preparation of cell suspensions from paraffin-embedded materials.

Approximately 100 μm sections were cut using surgical knives. A single section was adequate for kidney and prostate cancer tissue, but

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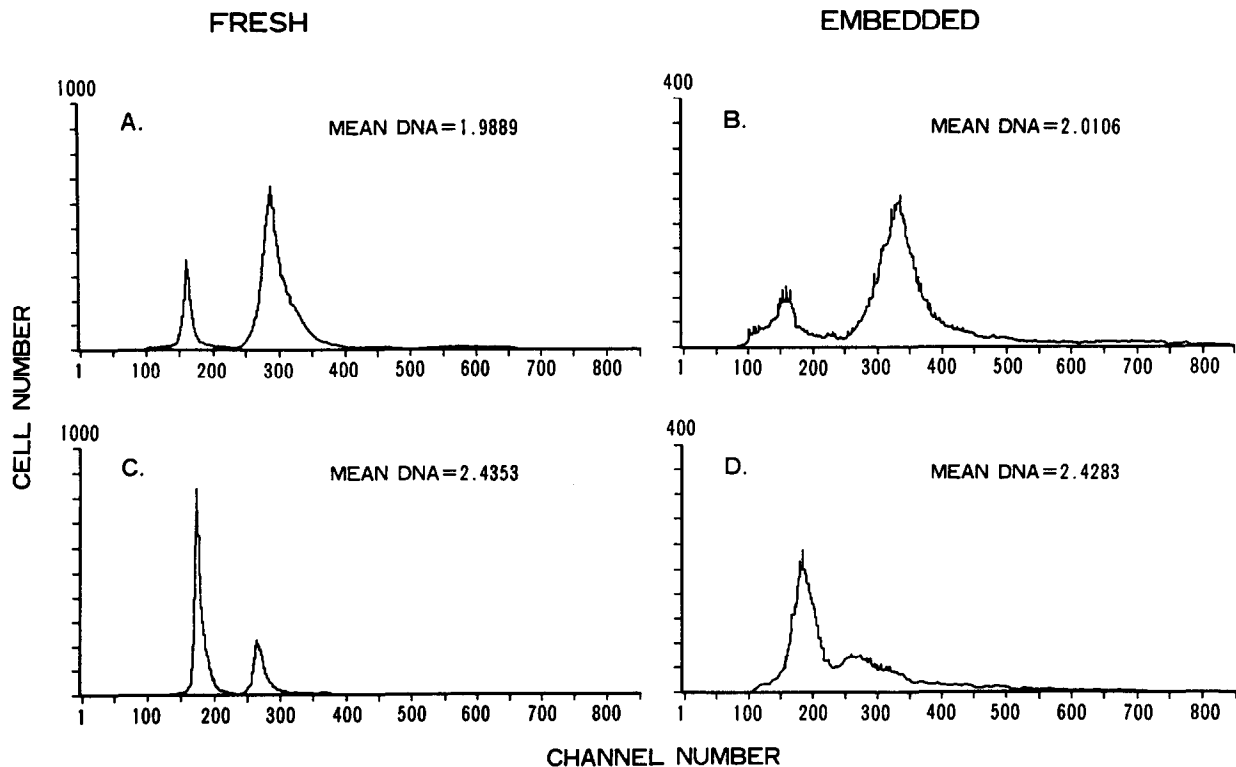


FIGURE 1. Representative DNA histograms and relative mean DNA content of cell suspensions from fresh tissues and paraffin-embedded tissues. FCM histograms (A) prostate carcinoma (Case 1) using fresh tissue and (B) using paraffin-embedded materials; (C) kidney cancer (Case 2) using fresh tissue and (D) using paraffin-embedded tissue.

several sections were required for the small samples of bladder cancer (punched biopsy materials). The sections were placed in 10 mL glass centrifuge tubes, deparaffinized, and rehydrated according to the conventional protocols.⁴ Briefly, the sections were dewaxed using xylene for twenty minutes followed by rehydration for a twenty-minute period in successive immersions of 100, 95, 85, and 50% ethanol. To minimize tissue loss, each step of rehydration was undertaken in the same test tube with distilled water added to dilute the ethanol. The tissue was then resuspended in 2 mL of 0.5% pepsin (Sigma) in 0.9% NaCl adjusted to pH 1.5 with 2N HCl at 37°C for two hours with occasional vortexing. A single cell suspension was made by mincing the tissue and filtering through a 125 μ m mesh filter.

Preparation of fresh tissue

Fresh tissue specimens obtained from ten surgical specimens were immersed in cold RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). The sample was mechanically minced and suspended in RPMI 1640. A cell suspension was nonenzymatically

prepared by filtering through 125 μ m mesh. Specimens were frozen in 10% dimethyl sulfoxide (DMSO) RPMI 1640 at concentration of 10^6 cells/mL and stored at -30°C until the time of FCM analysis.

DNA staining using propidium iodide

Single cell suspensions from fresh tissues were stained according to a modification of the conventional method.^{3,5} Briefly, the fluorochrome stain was prepared by adding 25 μ g/mL of propidium iodide (PI), 500 mg sodium citrate, and 0.5 mL Triton X-100 to 500 mL distilled water. Cells were suspended in 1 mL of the staining solution at 4°C, for thirty minutes. Samples were kept on ice and were filtrated through 30 μ m nylon mesh immediately before analysis.

Single cell suspensions from paraffin-embedded tissue were stained according to a modification of the method of Schutte *et al.*⁶ Briefly, 1 mg/mL solution of ribonuclease (in 0.2 M phosphate buffer, pH 7.0) was added to the cells for thirty minutes at 37°C. Subsequent centrifugation of the sample was followed by a wash in glass-distilled water and staining with 25 μ g/mL of PI solution at 4°C (on ice) overnight.

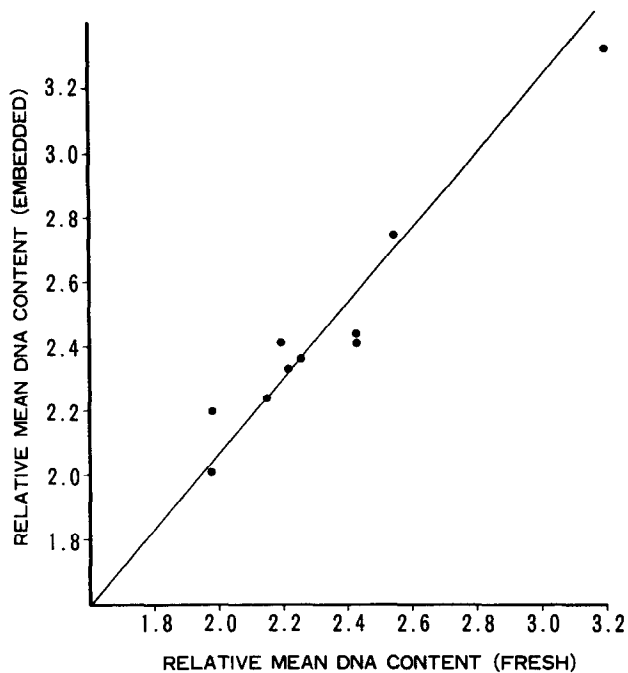


FIGURE 2. Comparison of mean DNA content from fresh or paraffin-embedded tumor tissue. Significant correlation ($r = 0.869$, $p < 0.01$) was observed.

FCM analysis and data analysis

Cellular DNA content was measured in an Ortho System 50-H multiparameter flowcytometer equipped with an Ortho 2150 computer (Ortho Diagnostic Inc., Westwood, MA). Each DNA histogram was obtained, and the calculation of the relative mean DNA content in cell populations was performed by the Pascal program development software package.⁵ Utilizing 1,000 channels to quantify the varying amounts of cellular DNA fluorescence, the total DNA

content in cell population was determined. Then the relative mean DNA content in cell population was obtained from the division of total DNA content by total number of cells analyzed. This calculation allows for the quantitative analysis of DNA histogram.

Results

DNA content from the fresh and paraffin-embedded tissues was analyzed employing a modified technique. Ten different carcinomas were studied, including 6 bladder, 2 kidney, and 2 prostate cancers. In Figure 1 a prostatic carcinoma (frame A and B) and a kidney carcinoma (frame C and D) are shown as examples of the DNA histograms generated from both fresh and paraffin-embedded tissues from the same cases. The comparison of DNA histograms from both sources reveals not only the similar pattern of histogram but also the equivocal value of the relative DNA content, which is calculated by total DNA content in cell populations divided by total number of cells analyzed. The relative mean DNA content was studied in 10 tumors using both fresh and paraffin-embedded materials (Fig. 2). There was a close correlation between the values obtained from fresh and paraffin-embedded tissues ($n = 10$, $r = 0.869$, $p < 0.01$).

In the preliminary study, 17 of 23 cases (74%) of formalin-fixed and paraffin-embedded samples generated by this modified procedure produced interpretable DNA histograms. However, only 6 of 19 cases (32%) of tissues preserved in picric acid-based fixatives were suitable for FCM analysis.

TABLE I. Comparison of methods using paraffin-embedded specimens

Authors	Thickness of Samples	Preparation of Single Cell Suspension	DNA Staining	FCM Machine
Hedley <i>et al.</i> ⁴	30 μm	0.5% pepsin 30 min	DAPI* 30 min	ICP 22 (mercury vapor lamp, 360 nm)
Schutte <i>et al.</i> ⁶	Thin (scraped off with scalpel)	0.25% trypsin overnight	PI* (100-5 μg) 24 hr	FACS IV (argon ion laser, 488 nm)
Stephenson <i>et al.</i> ⁷	5-100 μm	0.5% pepsin 1-2 hr	DAPI	ICP 22
Murphy <i>et al.</i> ⁸	30 μm	0.5% pepsin 30 min	PI (50 μg)	EPICS V (argon ion laser, 488 nm)
Coon <i>et al.</i> ⁹	30 μm	0.5% pepsin 30 min	PI (130 μg) 15 min	EPICS V
Nakamura <i>et al.</i>	Thin (about 100 μm), scraped off with knives)	0.5% pepsin 2 hr	PI (25 μg) overnight	Ortho 50H (argon ion laser, 488 nm)

* DAPI = 4'-6-diamidino-2-phenylindole; PI = propidium iodide.

Comment

In this study, we applied the calculation of the relative mean DNA content to the DNA histogram using paraffin-embedded materials. We have shown a significant correlation of the mean DNA content between the fresh and paraffin-embedded materials (Figs. 1 and 2).

Recently, Hedley *et al.*⁴ developed a new technique to analyze the DNA content by FCM using paraffin-embedded materials. The main advantage of this method is that retrospective clinical studies correlating DNA measurements of tumor cells and overall survival or prognosis become possible. Already in several institutes this technique has been applied with some modifications in thickness of the specimens, preparation of single cell suspensions, and methods of DNA staining (Table I). The modified procedures in our study are two: (1) the longer incubation time (2 hr) with pepsin to make single cell suspensions and (2) ribonuclease digestion and the use of propidium iodide at a concentration of 25 $\mu\text{g}/\text{mL}$ as DNA staining. These modifications allow for the close correlation of the relative mean DNA content in both fresh and paraffin-embedded materials.

The relative mean DNA content was applied to the FCM analysis of both fresh and paraffin-embedded specimens in this study. The reason is because the relative mean DNA content is computer exploration of flow cytometric DNA analysis and has been shown to quantify an objective description of FCM characteristics.⁵

Therefore, using the relative mean DNA content, it becomes possible to represent and compare the quantitative DNA analysis in both fresh and paraffin-embedded specimens. The retrospective study between the mean DNA content and the recurrence or survival of the patients with TCC of urinary bladder using the paraffin-embedded specimens is ongoing in our institute.

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