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A Simple Method for Cell Isolation from Paraffin-Embedded Tissue Specimens for Flow Cytometric DNA Analysis.

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Abstract Since Hedley et al. developed a cell isolation method using pepsin from a paraffin-embedded material, many archival tissues have been analyzed by flow cytometry. The original and modified methods have greatly contributed to DNA ploidy and cell cycle analyses, in particular, to retrospective studies. However, the methods need repetitive centrifugations and/or long incubation time. For saving time and efforts we aimed to improve the Hedley's method. Tissues used were normal lymph nodes and malignant lymphoma of testis. All cell isolation procedures were done in a 1.5-ml microfuge tube without centrifugation. Tissue sections were minced with scissors, deparaffinized and rehydrated. Subsequently, the tissue was treated with 0.1% pepsin in 0.1N HCl for 90 min at 37℃ and neutralized. After filtering out cell debris, the cell suspension was treated with 0.1% RNase and stained with propidium iodide. The average coefficient of variation for G0/G1 peak of DNA diploid cells was 2.6%, and it was small enough to detect a near diploid DNA aneuploid peak (DNA index: 1.13). All procedures can be completed within 4 hours without difficulty. This method is suitable for lymphocytic tissues.

Key Words: paraffin-embedded tissue ; flow cytometry (FCM) ; DNA aneuploidy ; malignant lymphoma ; DNA content.

Introduction

Since Hedley et al[1)]. developed a cell isolation technique using enzymatic digestion from paraffin-embedded material in 1983, many archival tissues have been analyzed by flow cytometry. The original method used pepsin for digestion and 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining. Afterward, propidium iodide (PI) has been sometimes used for nuclear staining in the method (Hedley's method). The same year, Vindeløv et al[2)]. reported a method using detergent-trypsin treatment and PI staining for DNA analysis of suspended cells. Schutte et al[3)]. modified the Vindeløv's method so that it was applicable to paraffin-embedded material in 1985 (Scutte's methods). These methods have contributed greatly to DNA analysis, in particular, to retrospective studies using clinical materials [4-8)]. Other modified protocols have been reported[9-11)]. Tagawa et al[12)]. compared Hedley's method with Schutte's method using various carcinoma specimens and reported that the latter gave slightly better result.

Scutte's method takes 18 hours for digestion step with trypsin, whereas Hedley's method does 30 min for the step with pepsin. Furthermore, Scutte's method needs complex solution. Thus, the two methods have been used for cell isolation from paraffin-embedded tissues.

Recently, Kamada et al[13]. developed a sophisticated sample preparation method of paraffin-embedded tissues using a grater, which was based on the Hedley's method, for laser scanning cytometric DNA measurement. The enzymatic procedure of Kamada's method uses 0.1 % pepsin for 1 hour. They reported on average coefficient of variation (CV) of 3.9% in carcinoma samples. However, this is not applicable to flow cytometry. In this brief paper, we report a modified the Hedley's method to save time and efforts for flow cytometric DNA analysis.

Materials and methods

Tissues. Formalin-fixed, paraffin-embedded tissue specimens from five separate cases (No.1-5) of lymph node that showed no remarkable histologic changes were selected from the files of Labor Welfare Corporation Yamaguchi-Rosai Hospital. To compare a fresh sample with a paraffin-embedded sample, a case of malignant lymphoma of the testis that showed a near diploid DNA aneuploidy peak was also selected.

Preparation for Single Cell Suspensions from Paraffin-embedded Tissue. Two or three 30-μm paraffin-embedded tissue sections were minced with small scissors in a 1.5-ml microfuge tube. The minced tissue fragments were deparaffinized 3 times with xylene of 1.0 ml for about 10 min at room temperature. The deparaffinized tissue was rehydrated using two changes each of 100%, 95%, and 80% ethanol and three changes of phosphate buffered saline (PBS), for about 5 min at room temperature (Fig.1). The procedure for changing solutions was as follows. After most of the small tissue fragments settled to the bottom, the clear supernatant was removed, and new solution was added with a 3.0-ml transferpipet. After the final PBS wash, the tissue was treated with 1.0 ml of 0.1% pepsin (proteolytic activity 1 : 10,000, Katayama Kagaku, Osaka, Japan), in 0.1N HCl. The tubes were placed in a water bath at 37℃ for 30, 60, 90, or 120 min for pepsin treatment. After the pepsin solution was removed, the tissue was neutralized with three changes of cooled PBS (4℃). The cell suspension was filtered through a 48-μm nylon membrane (Yamagataya, Tokyo, Japan).

Preparation for Single Cell Suspensions from Fresh Tissue. About 30 mm³ of malignant lymphoma tissue was placed in 10-ml

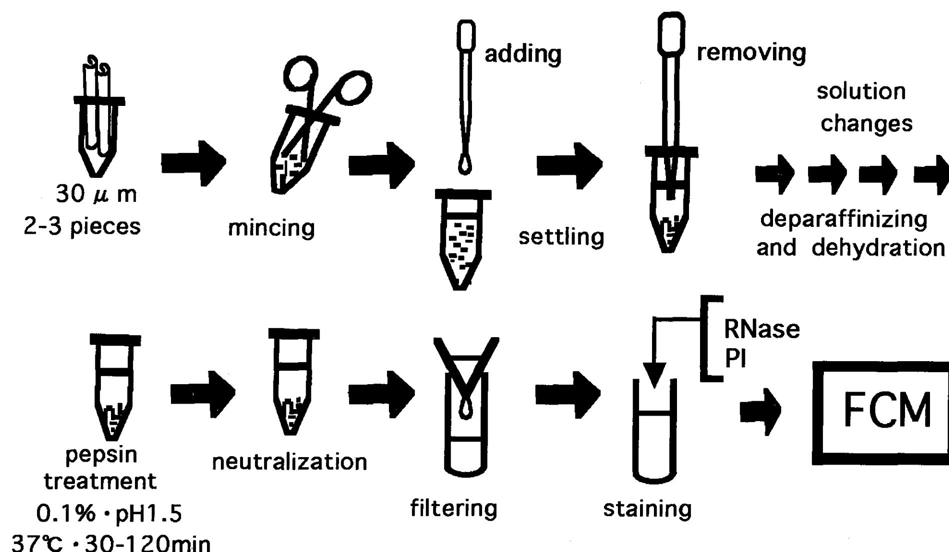

Fig.1 Stages in cell isolation from paraffin embedded tissue.

centrifugation tube, minced with small scissors, and treated with a nonionic detergent (0.1% Triton X-100) for cell isolation. The cell suspension was filtered through a 48 μm nylon membrane.

Preparation for Flow Cytometry. The cell suspension was treated with 0.1% RNase (Sigma, St. Louis, MO), and the cells were stained with PI (final concentration : 50 μg/ml).

Flow Cytometry. Cellular DNA content was measured by FACScan (Becton and Dickinson, San Jose, CA). Ten thousand events were acquired, and analysis was carried out using CellFIT software (Becton and Dickinson) without eliminating cell debris or doublet and triplet cells.

The Method to Estimate DNA Histogram. FACScan-data presented as histogram divided into three regions: region 1 (0 ≦DNA content < 1.8 C), region 2 (1.8 C≦DNA content < 4.4 C) and region 3 (4.4 C ≦DNA content). The best flow cytometric results were considered to be a small population of cells in region 1 or 3 and a small d-CV. Relative amount of DNA in a G0/G1 phase of DNA diploid cell is defined as 2C (a unit).

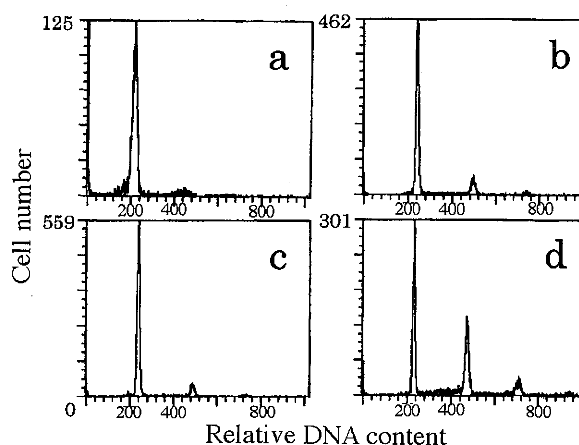

Fig. 2 The effects of pepsin treatment on the DNA histograms of the cells from paraffin-embedded tissue: a) Pepsin treatment for 30 min. (CV of DNA diploid G0/G1 peak, percent of the region 1, percent of the region 3 : 4.6%, 68.0%, 0.6%). b) Pepsin treatment for 60 min. (2.7%, 3.3%, 6.5%). c) Pepsin treatment for 90 min. (2.6%, 4.9%, 2.9%). d) pepsin treatment for 120 min. (2.6%, 2.7%, 20.6%). The tissue used was formalin-fixed, paraffin-embedded lymph node specimens that showed pathologically no remarkable changes.

Results and discussion

There have been major two digestion ways for cell isolation from paraffin-embedded tissue; one is pepsin digestion method and the other is trypsin digestion method. Although the latter generally gives slightly better results for the d-CV[12] than the former does, pepsin treatment is finished generally in a shorter time. To be concrete, digestion step of Hedley's method is done with pepsin for 30 min and the step of Schutte's is done with trypsin for 18 hours or overnight. To finish all procedures within a day we aimed to improve Hedley's method. Tagawa et al. measured DNA histograms of various carcinoma samples using the Hedley's methods and reported that the average d-CVs were 4-7%[12]. Recently, Kamada et al[13]. reported a cell isolation method for laser scanning cytometer, which was a modified Hedley 's method, using scrapped materials of paraffin- embedded tissue by grater. Because Kamada et al[13]. reported that the average d-CV was smaller (3.9%) we used 0.1 % pepsin for enzymatic procedure as they did.

Two-hour pepsin treatment occasionally resulted in increasing of the number of, doublet or triplet, clumped cells (Fig. 2). To determine the optimal protocol, the d-CV and the percentage of the region 1 or 3 were compared (Tables 1,2). The mean d-CV was 2.6% except for the samples treated with pepsin for 30 min. The d-CV for the 30-min pepsin-treatment samples was always larger than that for the over-30-min pepsin-treatment samples. Both the mean population of cells in the region 1 and it in the region 3 was the smallest (region 1 : 7.1%, region 3 : 1.4%) when cells were treated with pepsin for 90 min. The paraffin-embedded malignant lymphoma tissue treated with pepsin for 90 min yielded the smallest d-CV (Table 3) with a near diploid DNA aneuploidy (DNA index 1.13). These results showed that the

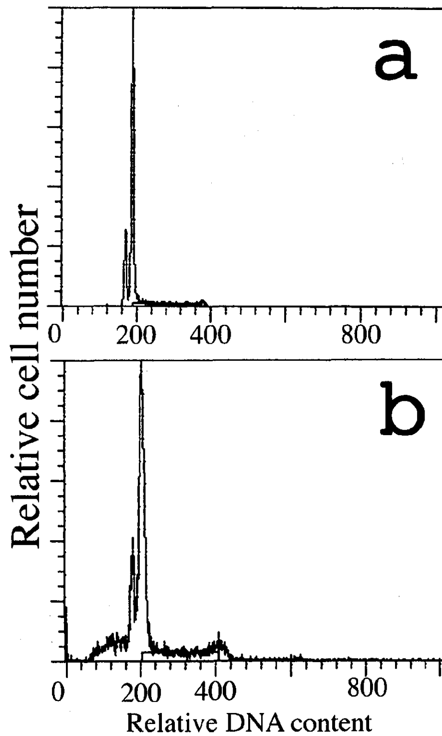

Fig.3 DNA histogram of a case of malignant lymphoma of testis. a) A fresh sample showed DNA aneuploidy (DI: 1.11) and small CV (1.7%) of reference G0/G1 peak. b) Paraffin-embedded material showed DNA aneuploidy (DI:1.13) and small CV (2.2%) of reference G0/G1 peak.

Table 1. Effects of pepsin treatment on d-CV[a] value (%) for normal lymph node[b].

No.	pepsin-treatment time 30	60	90	120 min
1	3.2	2.5	2.1	2.3
2	3.1	2.8	2.8	2.8
3	3.7	2.5	2.3	2.7
4	4.3	2.5	3.3	2.5
5	4.6	2.7	2.5	2.6
mean	3.8	2.6	2.6	2.6

a : The CV for the G0/G1 peak of DNA diploid cells. b:Each tissue (no. 1-5) used was formalin-fixed, paraffin-embedded lymph node specimens that showed pathologically no remarkable changes.

recovery of cells was not insufficient and cell losses were light without a centrifugation step.

We applied this method to malignant epithelial tumor samples. The carcinoma tissues sometimes showed distinct peak of DNA aneuploidy, however, the number of DNA aneuploid cells was usually small and sometimes too small to form DNA aneuploidy peak (data not shown). We think that minor modifications are needed to make this method applicable to DNA analyses of malignant epithelial tumors.

Hedley et al[1]. reported that the d-CV of the

Table 2. Effects of pepsin treatment on the population (%) of the region 1 or the region 3 in DNA histogram[a]

	The region 1				The region 3			
	pepsin-treatment time				pepsin-treatment time			
No.	30	60	90	120min	30	60	90	120min
1	11.7	13.3	7.3	22.9	0.9	0.6	1.4	3.9
2	12.6	15.6	4.8	17.7	1.6	0.5	0.5	1.8
3	18.4	9.5	6.0	6.5	5.2	0.9	1.4	1.0
4	30.8	20.6	12.4	10.0	1.3	1.1	1.0	0.9
5	68.0	3.3	4.9	2.7	0.6	6.5	2.9	20.6
mean	28.3	12.5	7.1	12.0	1.9	1.9	1.4	5.6

a : Each tissue (no. 1-5) used was formalin-fixed, paraffin-embedded lymph node specimens that showed pathologically no remarkable changes. FACScan-data was presented as histogram divided into 3 regions: region 1 (0≦DNA content <1.8C), region 2 (1.8C≦DNA content <4.4C) and region 3 (4.4C ≦DNA content). The relative DNA amount of a normal cell of G0/G1 phase was defined as 2C.

Table 3. Effects of pepsin treatment on DNA histograms of cells from a paraffin-embedded malignant lymphoma tissue[a].

Parameter	30	60	90	120 min
d-CV (%)[b]	3.5	3.0	2.2	3.0
region 1[c]	16.4	14.3	14.3	9.7
region 3[d]	6.1	5.6	4.0	3.8

a : Tissue used was malignant lymphoma of testis. b : The CV for G0/G1 peak of DNA diploid cells. c : Percent total of the region 1 ($0 \leqq$ DNA content $< 1.8C$). d : Percent total of the region 3 ($4.4C \leqq$ DNA content). The relative DNA amount of a normal cell of G0/G1 phase was defined as 2C.

paraffin-embedded tissues stained with DAPI was usually greater than that obtained from fresh tissue (mean±SD : 2.73±0.88 fresh, 3.59±1.05 embedded). Schutte et al[3]. reported that the d-CV (%) of tumor samples stained with PI was 5.1±1.17. Tagawa et al[12]. compared Hedley's method with Schutte's method using paraffin embedded tissues and reported that the average d-CV was 5.5% (Hedley's method) and 4.0% (Schutte's method) in lung cancer. In comparison to the d-CV generated by previous methods for flow cytometry using PI staining, the d-CV generated by our method was smaller (mean 2.6%). The d-CV of paraffin-embedded malignant lymphoma tissue was greater than that of fresh tissue; however, the d-CV was small enough (2.2%) to detect near diploid DNA aneuploidy (Fig.3). Because the lymph node cells used are regarded as DNA diploidy, a smaller number of events in the region 1 or 3 is better. We conclude that the optimal pepsin treatment time is 90 min.

All procedures can be completed within 4 hours, and this method may be adopted for routine hospital examination and may lead to the development of a tool of automatic sample preparation. Because the cells isolated from paraffin-embedded tissues can be used as materials for genetic studies, the method described will be useful in combined cytometric and genetic studies.

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