大韓不妊學會雜誌: 第 23 卷 第 1 號 1996
Kor. J. Fertil. Steril., Vol. 23, No. 1, 1996, 4

Study on the Analysis of Chromosome Abnormality by Flow Cytometric and Cytogenetic Methods

CS Baik, MK Kim, SM Lee, JH Kim, YK Baik*
HT Lee and KS Chung

Animal Resources Research Center, Kon-Kuk University

유식세포분리기와 세포유전학적 방법에 의한 염색체이상 분석에 관한 연구

건국대학교 동물자원연구센터

백청순 · 김묘경 · 이상민 · 김진회 · 백용균* · 이훈택 · 정길생

= 국문초록 =

골수나 유산물질에 대한 세포유전학적 검사에 있어 통상적인 염색체검사는 검사에 적합한 중기핵상을 얻기 어려워 실패하는 경우가 많다. 이러한 경우에 진단이나 치료에 도움을 줄 수 있는 방법으로 유식세포분리기를 사용하여 단일 세포내 DNA량에 따른 aneuploidy를 추적할 수 있는 가를 확인하기 위해 본 실험을 실시하였다. 79 (혈액 30, 골수 37, 유산물 12)예에서 염색체 검사와 유식세포분리검사를 동시에 실시하여 각각의 결과를 비교한 결과 79.7% (63/79)의 일치율을 얻었다. 그러나 염색체의 손실이 없는 전좌와 역위의 경우는 물론 작은 조각의 염색체 부분이 늘어나거나 줄어든 경우에 있어서는 유식세포분리방법에 의해서 추적되지 못하였지만, 염색체 검사의 결과를 얻는데 실패한 경우에는 유식세포분리방법이 DNA량의 변화에 대한 정보를 얻을 수 있다는 것을 확인할 수 있었다. 따라서 본 연구결과는 세포유전학적 검사에서 유식세포분리방법이 염색체 검사보다 신속하며 염색체검사가 불가능한 시료에서도 DNA양에 따른 aneuploidy의 추적이 가능하다는 것을 시사한다.

INTRODUCTION

Cytogenetic studies have been used in diagnosis, prognosis, therapy, and others. The use of bone marrow samples for cytogenetic study is of advantageous immediate harvest due to continuous proliferation. However, the direct chromosome preparation of bone marrow sample has several disadvantages. For example, the cell proliferation of bone marrow is relatively slow in most cases, leading to a poor mitotic index. The condensed chromosome also results in a short and stubby appearance with the diffuse morphology. Furthermore, chromosomes prepared from malignant cells are too sensitive to use in various banding techniques. Cytogenetic studies on spontaneous abortion have provided information of clinical importance for the couple.

Flow cytometry(FCM), which allows the simultaneous measurement of multiple characteristics of a single cell, has recently been used in various studies of cell biology. By this technique, chromosomal DNA could be quantified at any stages of the cell cycle because each cell is evaluated separately and measured total DNA is not changed with whatever the degree of chromosoma contraction. Therefore, FCM has been applied to detect DNA aneuploidy(Barlogie *et al.*, 1980; Murphy *et al.*,

* Hanyang Medical University(Collage of Medicine)

1986 b; reviewed in Lovett *et al.*, 1980; Raffaele *et al.*, 1993), and allowed evaluation of errors of sex determination such as XY sex reversal(Kent *et al.*, 1988). Moreover thousands of cells could be analyzed in a few seconds by FCM. The FCM analysis also provide a simple way of assessing abnormal DNA content. Indeed, the FCM-DNA index has been often adopted to evaluate DNA abnormalities in human tumors(Raffdele *et al.*, 1993). In this study, we determined the possibility of using FCM of cellular DNA to detect the DNA abnormalities of bone marrow and abortive samples.

MATERIALS AND METHODS

Samples

Cytogenetic and FCM analyses were performed on 30 peripheral blood(PB, were used as standard), 37 bone marrow(BM) and 12 abortive samples(AS) of human. These samples were divided into two parts for cytogenetic and flow cytometric analyses.

Cytogenetic analysis

Monocellular suspensions from AS were obtained under the sterile condition by mechanical treatment with scissors and by mild enzymatic treatment with 0.25% trypsin. The cellular suspension was incubated over-night in Ham's F-10 medium at 37℃ and in an atmosphere 5% CO_2 in air. Peripheral blood and BM were cultured for 3 days using micro culture method(Verma and Babu, 1989) in Ham's F-10 medium contained 2%(v/v) phytohemagglutinin in(PB) or 1%(v/v) pokeweed mitogen in(BM). The cells were blocked at metaphase with treatment of colcemid (10µg/ml). After mounting then on the slide, the G-bands by trypsin using Giemsa were observed.

Cytometric analysis

Abortive samples were washed in phosphate buffered saline(PBS) and cell suspensions were obtained by mechanical treatment with scissors. Cell suspension of PB and BM obtained after culture by same methods as those for cytogenetic analysis were used. Cell suspension were fixed in 70% ethanol and stored at 4℃ until preparation for analysis. The fixed samples were centrifuged and resuspended in 0.05% trypsin(Gibco, New York) for 5 min at room temperature for monocellular suspension. The trypsin was inactivated with fetal calf serum(Gibco, New York). Monocellular suspensions were then washed three times with PBS and stained in propidium iodide(PI, Sigma). Final cell concentraion was 1×10^6 cells/ml and final PI concentration was 50 µg/ml according to DNA QC particles (Becton Dickinson, LA). The stained suspension was filtered through a 70µm nylon mesh, and kept at 4℃ at least for 30 min before FCM.

Flow cytometry

The samples were analyzed by a FACStar (Becton Dickinson Immunocytometry, System, LA, USA) with argon ion laser(Coherent Innova 90 argon-ion laser) turned to the 488 nm line at 200 mW output. Approximately 5×10^3 signals were accumulated for each histogram. The level of alteration in DNA content was expressed in terms of the DNA index(DI), calculate the ratio of the G1/G0 model values between the aneuploid and the diploid population as standard. An analysis of DNA histogram was carried out to calculated DI, coefficients of variation(CV), and the relative frequencies of the different cellular compartments.

RESULTS

1. Effect of Fixing Time in FCM analysis

In this study, when we fixed monocells in 70% ethanol, 72-96 hours for fixing time was best for flow cytometric analysis. As shown in Fig. 1, in 72-96 hours group of fixing time, CV was 5.26, but in group of 3 and 24 hours, those were 12.04 and 10.83, respectively.

Table 1. Composition of results from Cytogenetic and FCM Analysis of 79 cases

Samples	Cases	Karyotype by Cytogenetic Analyses	Cytometric Ploidy
PB	30/23	46, Normal	Diploidy
	/1	47, XXY	Hyperdiploidy
	/1	46, X i(Xq)	Diploidy
	/1	46, XX, del(5P)(:p14→qter)	Diploidy
	/1	47, XYY	Diploidy
	/1	46, XX, dir dup(1)(q12 q31)	Diploidy
	/1	46, XX, dir dup(1)(q21 q42)	Hyperdiploidy
	/1	46, XX, inv(9)	Diploidy
BM	37/31	46, Normal	Diploidy
	/2	46, Normal	Hyperdiploidy
	/3	Ph^1 chromosome	Diploidy
	/1	49, XY, +8, +13, +21, 9q+	Hyperdiploidy
AS	12/5	46, Normal	Diploidy
	/1	46, XY, 9q+	Hyperdiploidy
	/3	Failure	Diploidy
	/1	47, XX, +16	Diploidy
	/1	Failure	Tetraploidy
	/1	Failure	Hypodiploidy

PB: peripheral blood
BM: bone marrow
AS: abortive sample

When fixing time was prolonged than 10 days, CV value were decreased dramatically and final cell count was too few to analysis.

2. Flow cytometric and cytogenetic analysis

Flow cytometric analysis was carried out on 79 cases(30 PB, 37 BM, 12 AS). Interpretable DNA histograms were obtained from all samples. Diploidies were found in 28 of in 30 PB cases and remaining 2 cases were hyperdiploidies(Table 1). The latter cases were known as 47, XXY and 46, XX, dir dup(1)(q 12 q31) by cytogenetic analysis. Among 28 cases, there were 46, Xi(Xq), 46, XX, del(5p)(:p14→qter), 47, XYY, 46, XX, dir dup(1)(q21 q42), 46, XX, inv(9), and 23 normal karyotypes according to results of cytogenetic analysis.

In 37 BM cases, 34 cases were found as diploidies and 3 cases were hyperdiploidies(DI>1, Fig. 2). The results of cytogenetic analysis have specified that among the diploidies, 3 cases of karyotypes with philadelphia chromosome were involved and 3 hyperdiploidies were 49, XX, +8, +13, +21, 9q+ -46, XY mosaicism and 2 normal karyotypes.

Cytogenetic analyses of 5/12 AS cases were failed and thus karyotypes of these cases were unknown. However in cytometric analysis these 5 smaples were 3 diploidies, tetraploidy (DI=2, Fig. 3) and 1 hypodipoidy(DI<1). In the other 7 cases, there were identified as 5 normal karyotypes, 46, XX, 9q+, and 47, XX, +16 in the cytogenetic analysis. On the other hand, six diploidies and 1 hyperdiploidy(46, XX, 9q+) were detected by FCM analysis.

DISCUSSION

Cytogenetic technology has been used to detect changes in malignant of bone marrow cell. It is an essential part of the armamentarium of the haematological cytogeneticist, because chromosomal changes of malignant cells and di-

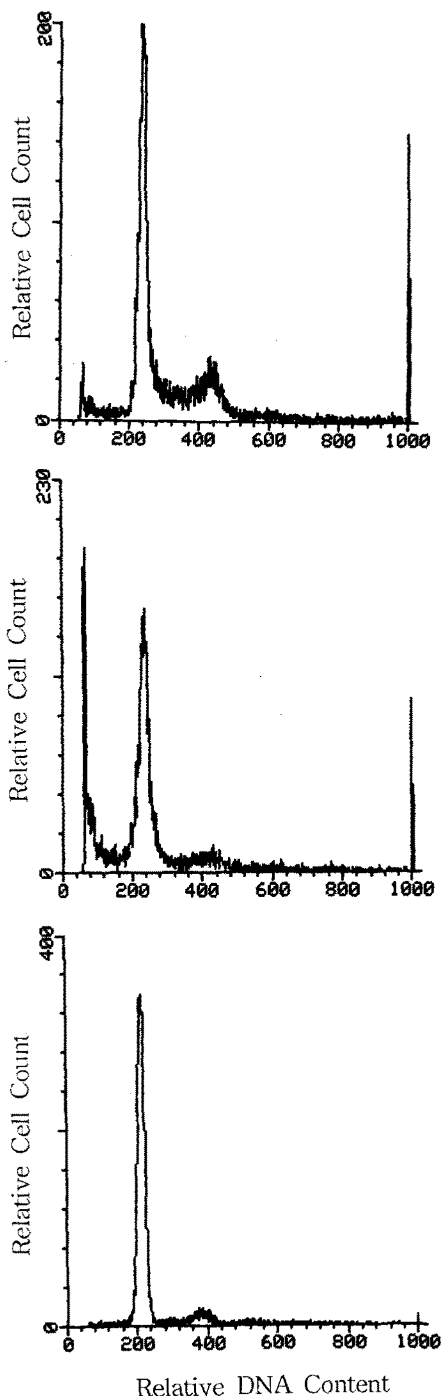
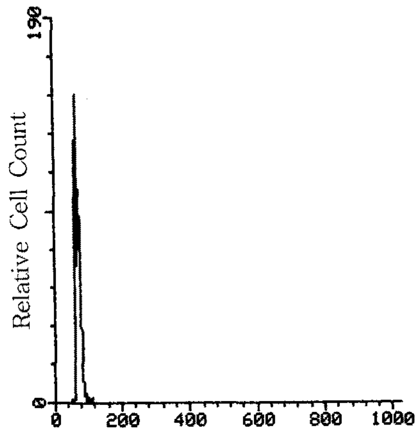


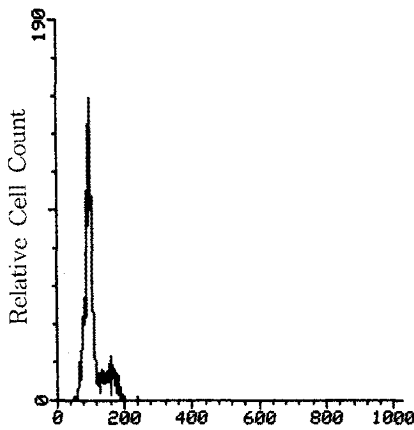
Fig. 1. Flow cytometry analysis with differnt fixing time of peripheral blood samples.
A: 3 hours(CV 12.04), B: 24 hours(CV 10.83),
C: 72-96 hours(CV 5.26).

sorders are related. Accurate information of the abortion karyotype is also important for clinical prognosis and therapy, and for investigating non-chromosomal causes of pregnancy loss.

Numerical changes and autosomal trisomy are the most common abnormalities(Zhou *et al.* 1989). Even with the recent simplified method of study chromosomes taken directly from chorionic villi(Hansmann *et al.* 1986; Eiven *et al.* 1987). Because of several factors, such as the time interval between abortion and the setting up of cultures, the presence of necrotic tissue, and microbial contamination, result from cytogenetic analysis cannot obtained.

In cytogenetic study of bone marrow, metaphase spreads from neoplastic cells are usually not good for analysis regarding quality and number. A simple procedure, especially independent of metaphase spreads, but nevertheless reliable, is necessary to characterize chromosomally normal and abnormal abortions or bone marrows. Recently, complementary techniques in cytogenetics have been applied to assess the presence of numerical or structural chromosome aberrations in specimens of human tumors and amniotic samples. These are radioactive in situ hybridization (RISH, Buckles, 1986), interphase cytogenetics(IC, Anastasi *et al.* 1991), restriction fragment length polymorphism analysis, fluorescent in situ hybridization(FISH, Dekken *et al.* 1990; Roberts 1991), polymerase chain reaction(PCR, Erlich, 1989). These techniques are(especially PCR is 100,000 times) more sensitive than conventional cytogenetic techniques. Therefore, they are powerful tools for the detection of submicroscopic chromosome rearrangements. However, each of them is an enhancement to, not a replacement for, standard cytogenetic analysis, because they only permit the detection of single or a few chromosome numerical or structural aberration. The advantage of these techniques with respect to flow cytometry analysis is the high





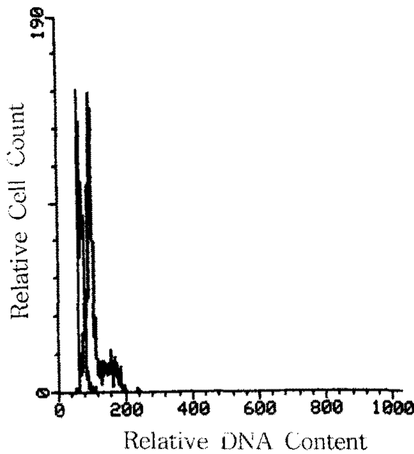
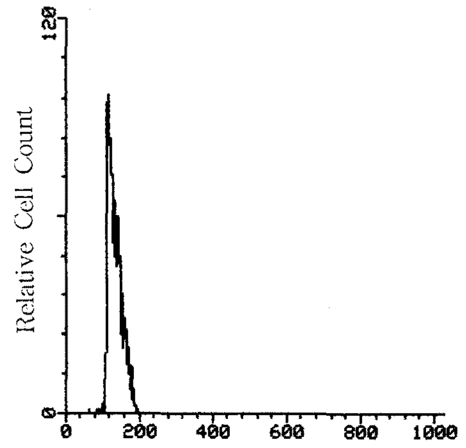
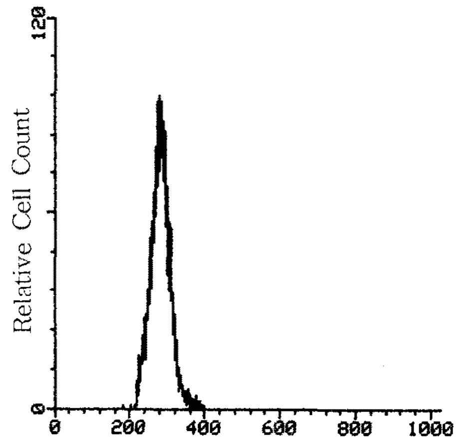


Fig. 2. Flow cytometry analysis of an abortive sample. Panel A, the diploid DNA distribution of standard PB; panel B, the histogram obtained from the abortive sample; panel C, the histogram obtained from the abortive sample together with standard samples(The first peak corresponds to diploid PB and the second hyperdiploid peak correspnds to the G1/G0 cells of the abortive samples).





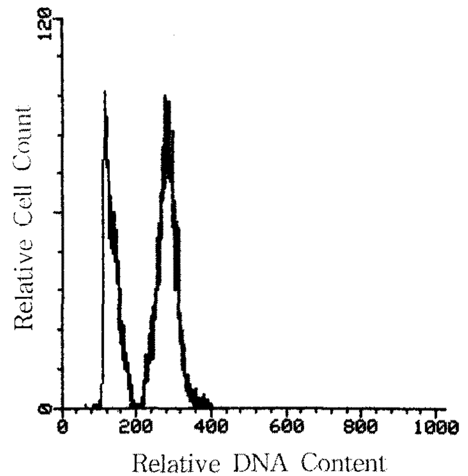


Fig. 3. Flow cytometry analysis of an abortive sample. Panel A, the diploid DNA distribution of standard sample; panel C, the histogram obtained from the abortive sample together with standard PB (In this instance, the first peak corresponds to diploid PB and the second tetraploid peak correspnds to the G1/G0 cells of the abortive samples).

specificity in identifying numerical or structural chromosome aberrations; flow cytometric analysis does not furnish specific chromosomal information but does offers the evaluation of total DNA content alterations independantly of stage of the cell cycle. Therefore, the technique of flow cytometry is characterized by several advantages, such as easy rapid preparation and measurement of samples using biopsies stored in culture medium for up to 48 hours or frozen at -80℃. Moreover, flow cytometric analysis is statistically reliable for the large number of cells measured for each DNA content distribution and is not as costly.

In the present study, the samples were divided and analyzed both by cytogenetic and flow cytometric approaches. The differences in DNA content because of sex chromosomes was from 1.3 to 4.8%, which are comparable with those in former reports(Kent *et al.* 1988; Nakamura *et al.* 1990). In the experiment for decision of fix time in 70% ethanol before PI staining, we obtained well fixed cells after fixation for 72-96 hours. In this group, coefficient of variation of 9.7 on G1/G0 peak of histogram was obtained. The coefficient of covariation in our experiment was slightly wider than those of other report(Raffaele *et al.* 1993). This difference may be resulted from that cells for flow cytometry analysis in this experiment were cultured with mitogen owing to small amount of sample.

Several discordances observed between the results from the two types of analysis. In cases with addition or deletion of a small chromosome(Y or 16) and chromosomal segment (5P- or dir dup 1) was not founded by flow cytometric analysis. However, in spite of a small chromosomal segment, as those,(9q+ or dir dap 1) was detected by flow cytometric analysis. Such discordances are also found in other report(Raffaele *et al.* 1993). In this study, we could not clarify what brought this discordances. This might be low quality of sample, a certain or all of procedure of monocell suspension preparation, or standard. Regarding some disagreements, our result showed similarity with cytogenetic analysis in 79.7%(63/79) of the cases examined. Of course structural anomalies of balanced translocation (Philadelphia chromosome) and inversion could not be detected.

Cytogenetic analysis in this study shows representative chromosomal alterations. The similarity observed between results of cytogenetic and cytometric analysis, led us to conclude that FCM can be employed to obtain information on cellular DNA in cases in which cytogenetic analysis was not possible.

REFERENCE

Anastasi J, Thangavela M, Vardiman, J, Hooberman A, Bian M, Larsoo R, LeBeau M: *Blood* 1991, 77, 1087.

Barlogie B, Drexinko B, Schumann J, Gohoe W, Dosik G, Latreille J, Johnstone DA, Freireichi EJ: Cellular DNA content as a marker of neoplasia in man. *Am J Med* 1980, 69, 195-203.

Buckles V: In Human Genetic Diseases: A practical approach(ed. K. E. Davies). IRL Press, Oxford, Nex York. 1986.

Czepulkowski BH, Bhatt B, Rooney DE: A Practical Approach, Human Cytogenetics, Oxford University Press, New York. 1992, pp 1-25.

Dekken HV, Pizzolo JG, Reuter VE, Melamied MR: Cytogenetic analysis of human solid tumors by in situ hybridizations with a set of 12 chromosome-specific DNA probes. Cytogenet. *Cell Genet* 1990, 54,103-107.

De Vita R, Calugi A, Cavallo D, Eleuteri P, Vizzone A: Flow cytometric and cyto-genetic analysis in human spontaneous abortions. *Hum Genet* 1993, 91, 409-415.

Eiben B, Borgman S, Schubble I, Hansmann I: A cytogenetic study directly from chorionic villi of 140 spontaneous abortions. *Hum*

Genet 1987, 77, 137-141.

Erlich HA, (ed.) PCR Technology: Principles and applications for DNA amplification Stockholm Press, 1989.

Hansmann I, Bartels I, Schubble I, Cytogenetic analysis of early human abotuses after preparation of chromosomes directly from chorionic villi. *Hum Genet* 1986, 72, 189.

Kent M, Chandler R, Wachtel S: DNA analysis by flow cytometry. *Cytogenet Cell Genet* 1988, 47, 88-89.

Lovett Ⅲ EJ, Schnitzer B, Keren DF, Flini A, Hudson JL, McClatchey KD: Application of flow cytometry to diagnostic pathology. *Lab Invest* 1980, 50, 115-140.

Murphy WM, Emerson LD, Chandler RW, Moinuddin SM, Soloway MS: Flow cytometry versus urinary cytology and the evaluation of patients with bladder cancer. *J Urol* 1986, 136, 815-819.

Nakamura D, Tiersch TR, Duglass M, Chandler RW: Rapid identification of sex in birds by flow cytometry. *Cytogenet Cell Genet* 1990, 53, 201-205.

Raffaele DV, Alverto C, Delia C, Patrizia E, Antonio V: Flow cytometric and cyto-genetic analyses in human spontaneous abortions. *Hum Genet* 1993, 91, 409-415.

Roberts L: FISHing cuts the angst in amniocentesis. *Science* 1991, 254, 378-379.

Verma RS, Babu V: Human chromosomes, Manual of basic techniques, Pergamon Press, Nex York 1986, 9-11.

Zhou X, Tong H, Wong S, Shen Q, Fu X, Cui Y: Chromosome abnormalities in early pregnancy analyzed by direct chromosome preparation of chorionic villi. *Human Genetcis* 1989, 83, 277-279.