□원저 : 실험연구□

Single Cell Dissociation Methods for Flow Cytometric Cell Death Analysis of Hypoxia-Ischemia Injured Newborn Rat Pup Brain

Jong Hee Hwang, M.D., Dong Kyung Sung*, Chang Won Choi, M.D., Saem Kang*
Yun Sil Chang, M.D., Won Soon Park, M.D.* and Munhyang Lee, M.D.

Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Samsung Biomedical Research Institute, Seoul, Korea*

Purpose : Newborn brain tissue has to be dissociated into a single cell suspension for flow cytometric analysis of cell death during hypoxia-ischemia. Thus the development of a method to dissociate cells from the brain tissue with least damage and maintenance of membrane and antigen integrity remains the challenge for the *in vivo* application of this technique. We evaluated the efficacy of mechanical or enzymatic (collagenase or tryspin) methods of brain tissue disaggregation.

Methods : The extent of the damage to the plasma membrane and loss of the characteristics of the membrane induced with each dissociation method was determined by comparing the flow cytometric results labeled with both fluorescent annexin V and propidium iodide of the newborn rat pup brain tissue in the control group (n=10) and in the 48-hour after hypoxia-ischemia group (n=10).

Results : In the control group, the cell percentage of damaged, apoptotic and necrotic cells of both hemispheres with the mechanical dissociation method was significantly increased compared to the trypsin or collagenase method. In the 48-hour after hypoxia-ischemia group, the cell percentage of apoptotic and necrotic cells of the right hemisphere with the collagenase method significantly increased, and live cells significantly decreased compared to the left hemisphere, control group. Although the same trend was observed, the extent of alterations made with the trypsin method was significantly less compared to the collagenase method.

Conclusion : The dissociation of neonatal brain tissue for flow cytometric analysis with collagenase was most efficacious with the least cell damage and preservation of the plasma membrane characteristics. **(Korean J Pediatr 2005;48:545-550)**

Key Words : Cell separation, Flow cytometry, Hypoxia-ischemia, Brain, Animals, Newborn

Introduction

Despite continuous improvements in neonatal intensive care medicine and fetal monitoring, hypoxic-ischemic brain injury in neonates still remains a major cause of mortality and neurologic morbidity in survivors such as cerebral palsy, developmental retardation or learning disability[1]. Two alternative modes of cell death, apoptosis and necrosis, can be distinguished during neonatal hypoxic ischemic brain injury[2]. Although the detection of morphological changes using light, fluorescence or electron microscopy is the gold standard method for identifying apoptotic or necrotic cell death, the microscopic method is elaborate, time consuming and cumbersome for quantitative analysis[2, 3]. Furthermore, as this method has difficulty in detecting immediate neuronal cell death, it can detect cell death only at later stages[4].

Recently, flow cytometry with a combination of fluorescinated annexin V and propidium iodide (PI) to measure changes in plasma membrane composition and function, has been established as a method to distinguish live, damaged, apoptotic and necrotic cells[5-9]. As it allows the sensitive detection and rapid quantification of a large population of

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책임저자 : 박원순, 성균관의대 삼성서울병원 소아과
Correspondence : Won Soon Park, M.D.
Tel : 02)3410-3523 Fax : 02)3410-0043
E-mail : wspark@smc.samsung.co.kr

cells, this technique appears to be the methodology of choice to study neuronal hypoxic ischemic cell death[10]. However, besides these advantages, this technique also has some limitations. As it can only work with cells in suspensions, the brain tissue has to be disaggregated into a single cell suspension for flow cytometric analysis. Damage to the plasma membrane or alterations of the biochemical and biological characteristics of the membrane introduced during this procedure might result in decreased numbers of intact and viable cells, thereby posing a technical problem for the subsequent flow cytometric analysis of neuronal cell death[11, 12]. Therefore, the development of the method to dissociate cells from the brain tissue, and retain membrane and antigen integrity and cell viability remains the most significant challenges for the *in vivo* application of this technique.

Although many mechanical and enzymatic methodologies for disaggregation of tissue into a single cell suspension have been applied to solid tumors and normal tissues[13-16], this is the first study, to our knowledge, to assess the various dissociation methods of the newborn brain for the flow cytometry. In the present study, the efficacy of mechanical and enzymatic (collagenase or tryspin) methods of brain tissue disaggregation was evaluated. The extent of the damage to the plasma membrane and loss of the characteristics of the membrane induced with each dissociation method was determined by comparing the flow cytometric results labeled with both fluorescent annexin V and propidium iodide (PI) of the newborn rat pup brain tissue in the control group and in the 48 h after hypoxia ischemia group.

Materials and Methods

1. Hypoxia-ischemia

The experimental protocols described herein were reviewed and approved by the institutional animal care and use committee of the Samsung Biomedical Research Center, Seoul Korea. This study also followed the institutional and National Institute of Health guidelines for laboratory animal care. Seven day-old Sprague Dawley rat pups (Daihan Biolink Co., Seoul, Korea) were anesthetized in a small jar containing cotton soaked with methoxyflurane, and deep anesthesia was maintained during the surgical procedure by placing a small plastic tube containing cotton soaked with methoxyflurane over the nose. The neck was incised in the midline, and the right common carotid artery was permanently ligated with 4-0 silk. Total time of surgery in each animal never exceeded 3 minutes. Approximately 3 to 4 hours of stabilization period after surgery, the animals were exposed to a 90 minute period of hypoxia (8% O_2, 92 % N_2) by placing them in an airtight container partially submerged in a 37℃ water bath. Control animal included littermates that were not subjected to cerebral hypoxia-ischemia but that were quick-frozen at the same intervals as the experimental animals. The rat pups were sacrificed at control (n=10) and 48 h after hypoxia-ischemia (n=10) under deep pentobarbital anesthesia (60 mg/kg, intraperitoneal), and the whole brain tissue was obtained.

2. Brain cell dissociation

The fresh whole brain tissue was placed on a glass Petri dish on ice containing 1-2 mL of phosphate buffered saline (PBS), and three blocks of brain tissue, about 100 mg per block, were obtained from the mid-portion of the cortex of the left and right hemisphere, respectively.

Each block of brain tissue was transferred to another Petri dish, and chopped into small pieces less than 1 mm in diameter using razor blades. Thereafter, one of the following three mechanical or enzymatic (trypsin or collagenase) methods was used to dissociate each block of brain tissue, and the efficacy of each technique was compared. For enzymatic method, 1-2 mL of serum free Dulbeco's Modified Eagle's Media (DMEM) containing 2 mg/mL of type I collagenase or 0.25% trypsin was added to tissue pieces, and incubated at 37℃ for 15 minutes. Thereafter, equal volume of DMEM with 10% fetal serum was added to the dish to neutralize the enzyme activity. For mechanical methods, the tissue pieces were blown in and out of a small bore pipette for 10-15 times to obtain the relatively consistent degree of turbidity of the solution. After each dissociation procedure, the cell suspension obtained was passed through a 40 μm cell strainer (Falcon, Becton Dickinson, Heidelberg, Germany), centrifuged 1,250 rpm for 3 minutes and the pellet was resuspended in 1 mL PBS. All the reagents used in this experiment were purchased from Lifetech Gibco-BRL Co. (Carlsbad, CA, USA) if not specifically mentioned.

3. Flow cytometry

Immediately after brain cell dissociation procedure, 95 μL (containing more than 1×10^6 cells) of cell suspension was labeled fluorescently for detection of apoptotic and necrotic

cells by adding 1 μL of PI (1 mg/mL; Sigma, St. Louis, MO, USA) and 5 μL of annexin V-FITC (fluorescein isothiocyanate) (Pharmingen, San Diego, CA, USA) to each sample. Samples were mixed gently and incubated at room temperature in the dark for 15 minutes. After incubation, each sample was added with 1 mL of annexin V binding buffer (10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-NaOH, pH 7, 140 mM NaCl, 2.5 mM $CaCl_2$) into PAS (Particle Analyzing System) tube (Sarstedt, 1.5 mL, 55×12 mmϕ, Germany). The flow analysis was performed by a PAS (Partec, Munster, Germany) equipped with an argon ion laser tuned at 488 nm wavelength. The green FITC-annexin V fluorescence was measured at 530±15 nm, and the red PI fluorescence was measured at 600 nm. A primary gate based on physical parameters (forward and side light scatter, respectively) was set to exclude dead cells or debris. Electronic compensation was used to eliminate bleeding through fluorescence.

3. Statistical analysis

Data were given as mean±standard deviation. Data were compared using repeated measures analysis of variance with Bonferroni correction. Statistical analysis described above was done using SAS software program ver. 6.04. A *P*-value of <0.05 was considered significant.

Results

Representative analyses of an annexin V versus PI dot plot and regional percentage of each dissociation method of each hemisphere at 48 h after hypoxia-ischemia are presented in Fig. 1. Among the four quadrants, Q1 includes cells that stain negatively for annexin V but are PI positive, and are considered damaged cells. Q2 includes cells that are both annexin V and PI positive, and are considered necrotic cells. Q3 includes cells that stain negatively for both annexin V and PI, and are considered live undamaged cells. Q4 includes cells that stain with annexin V but are PI negative, and are considered apoptotic cells[5-9].

Table 1 presents the regional cell percentage of the left and right hemisphere with each dissociation method in the control group (n=10) and 48 h after hypoxia-ischemia group (n=10). In the control group, the percentage of damaged, necrotic and apoptotic cells in the respective area of Q1 (annexin V^-/PI^+), Q2 (annexin V^+/PI^+) and Q4 (annexin V^+/PI^-) significantly increased, and the live cells in

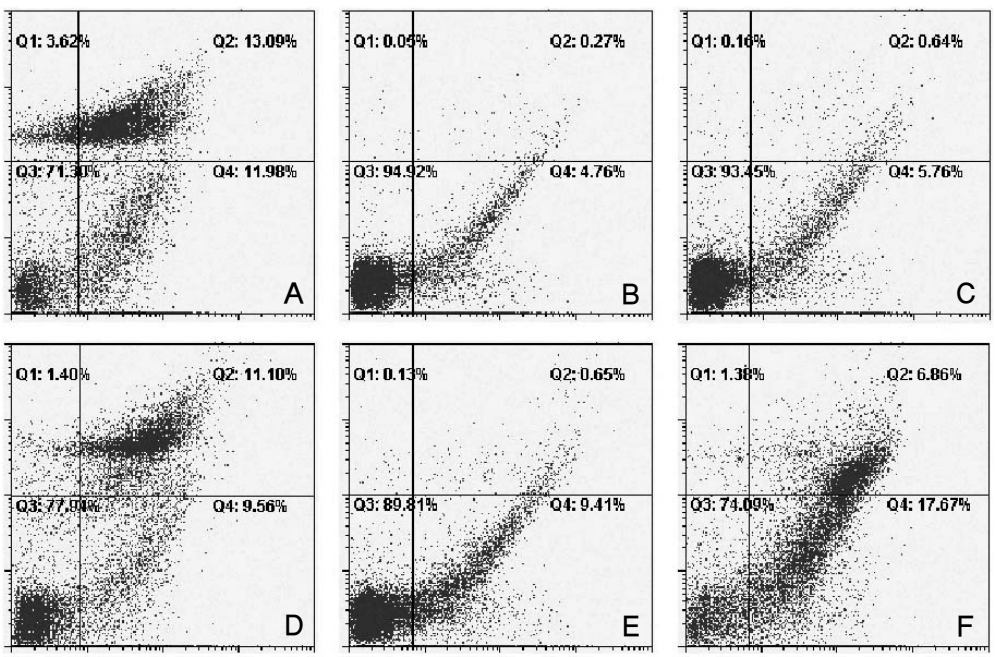

Fig. 1. Representative flow cytogram of an annexin V binding (abscissa) versus propidium iodide uptake (ordinate) in the left (upper row) and right (lower row) hemisphere of the newborn rat brain cells at 48 h after hypoxia-ischemia after dissociation with the mechanical **(A, B)**, trypsin **(C, D)** or collagenase **(E, F)** method. The numbers in the left upper quadrant, right upper quadrant, left lower quadrant and right lower quadrant represent the percentage of damaged (annexin V^-/PI^+), necrotic (annexin V^+/PI^+), live (annexin V^-/PI^-), and apoptotic cells, respectively.

Table 1. Regional Cell Percentage of each Dissociation Method at Control (n=10) and 48 h after Hypoxia-ischemia (n=10)

	Q1		Q2		Q3		Q4	
	LH (%)	RH (%)	LH (%)	RH (%)	LH (%)	RH (%)	LH (%)	RH (%)
Pipette								
Control	5.38±2.72	5.52±2.85	14.24±1.88	12.35±3.67	70.29±2.98	73.27±4.88	10.09±4.49	10.26±4.31
HI 48h	3.17±1.98	3.2±2.02	12.49±2.76	10.42±2.22	71.77±4.20	73.00±3.39	12.56±1.15	13.37±1.96
Trypsin								
Control	0.21±0.19*	0.26±0.15*	0.58±0.05*	0.52±0.14*	94.02±0.84*	93.97±0.63*	5.10±0.66*	5.26±0.50*
HI 48h	0.15±0.09*	0.36±0.19‡	0.13±0.02*	1.03±0.38*	94.15±0.84*	88.76±2.50*, ‡, §	5.39±0.78*	9.86±2.02
Collagenase								
Control	0.43±0.11*	1.35±0.14*	0.56±0.10*	0.87±0.18*	94.68±0.22*	93.94±0.78*	4.51±0.08*	5.10±0.83*
HI 48h	0.27±0.12*	1.05±0.13*	0.61±0.21*	5.87±1.23*	92.40±1.14*	77.71±3.28†, ‡, §	6.70±0.92*	15.07±2.03†, ‡, §

Q1 : Damaged (Annexin V^-/Propidium $iodide^+$) cells, Q2 : Necrotic (Annexin V^+/Propidium $iodide^+$) cells, Q3 : Live (Annexin V^-/Propidium $iodide^-$) cells, Q4 : Apoptotic (Annexin V^+/Propidium $iodide^-$) cells, LH : Left hemisphere, RH : Right hemisphere, HI 48h : 48 h after hypoxia-ischemiaValues given present mean±standard deviation
*$P<0.05$ compared to pipette, †$P<0.05$ compared to trypsin, ‡$P<0.05$ compared to control, §$P<0.05$ compared to LH

Q3 (annexin V^-/PI^-) significantly decreased with the pipette dissociation method compared to the trypsin or collagenase dissociation method. There were no significant inter-hemispheric differences in regional cell percentage with each dissociation method. In the 48 h after hypoxia-ischemia group, the percentage of necrotic and apoptotic cells in the respective area of Q2 and Q4 significantly increased, and the live cells in Q3 significantly decreased with the collagenase dissociation method in the right hemisphere compared to the left hemisphere or to the data of the control group or the pipette dissociation method. Although the same trend has been observed, the extent of alterations made with the trypsin dissociation method was significantly less compared to the collagenase method. There were no significant differences in regional cell percentage between the control and 48 h after hypoxia-ischemia groups with the pipette method.

Discussion

Exposure of phosphatidylserine on the outside of the plasma membrane, which exists almost exclusively on the inner surface of the lipid bilayer in live cells, is the early and most characteristic change of apoptosis[5-9]. This change can be detected by annexin V, which preferentially binds to negatively charged phospholipids such as phosphatidylserine[5-9]. The disruption of the plasma membrane and alterations in permeability, observed in necrotic cells, could be detected by staining with PI. Therefore, by the combined staining of neuronal cells with both annexin V and PI as done in the present study, it was possible to distinguish live cells, apoptotic cells, damaged cells, and necrotic cells by flow cytometry[6-12]. Furthermore, this technique is very sensitive, rapid and objective compared to alternative methods such as morphological analysis. Taken together, these findings implicate that flow cytometry would be the methodology of choice for the quantitative analysis of cell death during neonatal hypoxic-ischemic brain injury.

A critical step in the flow cytometric analysis of neonatal brain injury in the present study is the disaggregation of the tissue into a single cell suspension[13-16]. Brain cells, especially fetal and neonatal, are easy to dissociate because brain tissue hardly has a tight junction. However, brain cells are very sensitive to physical and chemical stresses imposed during the dissociation procedure. Damage to the plasma membrane introduced during this dissociation procedure would allow access of annexin V to the internally located phosphatidylserine and, hence, give false positive results in the subsequent flow cytometric analysis of neuronal cell death[11, 12]. In the present study, the percentage of cells in Q1 (damaged cells, annexin V^-/PI^+), Q2 (necrotic cells, annexin V^+/PI^+) and Q4 (apoptotic cells, annexin V^+/PI^-) was markedly increased, and in Q3 (live cells, annexin V^-/PI^-), there was significant reduction with the mechanical disaggregation method using a small bore pipette compared to the enzymatic trypsin or collagenase method in both the control and 48 h after hypoxia-ischemia group. No significant interhemispheric differences in the cell percentage were also observed. Our data indicate that cell damage induced by the mechanical method is so great that technical sensitivity of the flow cytometry to the analysis of hypoxic-ischemic neuronal cell death is lost.

In the present study, the cell percentage with both the trypsin and collagenase methods was significantly lower in Q1, Q2 and Q4, and higher in Q3 compared to the mechanical method in both hemispheres of the control group. Our data indicate that the stress and cell damage induced with the enzymatic dissociation methods was much less compared to the mechanical method, resulting in greater cell viability. Furthermore, the cell percentage of the right hemisphere with the collagenase method in the 48 h after hypoxia-ischemia group was significantly higher in Q2 and Q4, and lower in Q3 compared to the left hemisphere of the same group or control group. Although the same trend was also observed with the trypsin method, the extent of alterations in cell percentage was significantly less compared to the collagenase method. These findings indicate that although the enzymatic methods are much more efficient than the mechanical approach, trypsin underestimated the apoptotic or necrotic cell death by removing phosphatidylserine or bound annexin V from the cell membrane surface by proteolysis[12] or altering the biochemical and biological characteristics of the plasma membrane[11, 16], thereby reducing the technical sensitivity of this method in the subsequent flow cytometric detection of the cell death. Our data implicate that the dissociation of the neonatal brain tissue with collagenase seems to be the best method with least cell damage and preservation of the features of the plasma membrane.

Collagenase I is a unique protease that specifically hydrolyses the bond between a neutral amino acid (X) and glycine in the sequence of Pro-X-Glyc-Pro. This sequence is found in high frequency in collagen fibrils in the connective tissue. The collagenase I used in the present study is a crude preparation from Clostridium histolyticum, containing clostripiopeptidase A and a number of other proteases, polysaccharidases and lipases. Therefore, although our data indicate that collagenase is the most efficacious method in dissociating neonatal brain tissue, it is not clear which of the active agents are involved in this dissolution[16]. Further studies will be necessary to clarify this.

In summary, the dissociation of neonatal brain tissue for flow cytometric analysis with collagenase was most efficacious with least cell damage and preservation of the plasma membrane characteristics.

국문 요약

저산소성 허혈성 뇌손상이 유발된 신생백서에서 단일세포의 분리

성균관대학교 의과대학 삼성서울병원 소아과,
삼성생명과학연구소*

황종희 · 성동경* · 최창원 · 강 샘*
장윤실 · 박원순 · 이문향

목 적 : 저산소성 허혈성 손상을 받은 신생아의 뇌조직에서 유세포 방법을 통해 세포의 사멸을 분석하기 위해서는 단일세포의 분리가 이루어져야 한다. 본 연구는 세포 분리에 있어서 세포막의 소실을 최소화하고 항원성을 유지하기 위하여 물리적인 방법과 효소 처리를 통한 세포 분리방법의 효율성에 대해 알아보고자 하였다.

방 법 : 생후 7일된 10마리의 SD 신생백서에 우측 경동맥 결찰 후, 8% 산소에 노출시켜 저산소성 허혈증의 손상을 유발하였으며 48시간이 지난 후 뇌조직을 얻어 같은 수의 정상 대조군과 비교하였다. 세포 분리는 물리적인 방법(pipette)과 효소 처리(trypsin 및 collagenase) 방법을 통하여 이루어 졌으며, 세포막의 손상 정도와 범위에 대해서는 annexin V 및 propidium iodide의 형광 염색을 통한 유세포 분석방법을 이용하였다.

결 과 : 정상 대조군에서, 물리적인 방법을 통한 세포 분리가 양반구 모두에서 효소 처리를 한 경우에 비해서 세포의 사멸과 괴사가 통계적으로 유의하게 증가하였다. 저산소성 허혈증을 유발한 군 중, collagenase를 이용하여 세포 분리를 시행한 경우에서 우측 반구의 세포 사멸과 괴사의 비율이 좌측 반구 및 정상 대조군 보다 유의하게 증가하였다. 효소 처리를 통한 세포 분리에서는 서로 유사한 경향을 보였으나, trypsin을 이용한 경우가 collagenase를 이용한 경우에 비해 세포 변화의 정도가 유의하게 감소하였다.

결 론 : 신생아의 뇌조직에서 collagenase를 이용한 단일 세포 분리방법이 세포막의 손상을 최소화하면서 세포막의 성상을 보존할 수 있는 가장 유용한 방법이었다.

References

1) Robertson C, Finer N. Term infants with hypoxic-ischemic encephalopathy : outcome at 3.5 years. Dev Med Child Neurol 1985;27:473-84.
2) Pleura MR, Adams LM, Liu H, Santos DG, Nishimura RN, Yang F, et al. Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. Stroke 1998;29:2622-30.
3) Kerr JFR, Gobe GC, Winterford CM, Harmon BV. Anatomical methods in cell death. In : Wilson L, Matsudaira P, editors, Methods in cell biology. San Diego : Academic Press Inc., 1995;46:1-26.

4) Steinbach JP, Weissenberger J, Aguzzi A. Distinct phases of cryogenic tissue damage in the cerebral cortex of wild-type and c-fos deficient mice. Neuropathol Appl Neurobiol 1999;25:468-80.
5) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis : flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J Immunol Methods 1995;184:39-51.
6) Honda O, Kuroda M, Joja I, Asaumi J, Takeda Y, Akaki S, et al. Assessment of secondary necrosis of Jurkat cells using a new microscopic system and double staining method with annexin V and propidium iodide. Int J Oncol 2000; 16:283-8.
7) Darzynkiewicz Z, Bender E. Analysis of apoptotic cells by flow and laser scanning cytometry. Methods Enzymol 2000; 322:18-39.
8) Walsh GM, Dewson G, Warlaw AJ, Levi-Schaffer F, Moqbel R. A comparative study of different methods for the assessment of apoptosis and necrosis in human eosinophils. J Immunol Methods 1998;217:153-63.
9) Schutte B, Nuydens R, Geerts H, Ramaekers F. Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. J Neurosci Methods 1998;86:63-9.
10) Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell neurobiology : analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997;27:1-20.
11) Zamai L, Canonico B, Luchetti F, Ferri P, Melloni E, Guidotti L, et al. Supravital exposure to propidium iodide identifies apoptosis on adherent cells. Cytometry 2001;44:57-64.
12) Van England M, Ramaekers FCS, Schutter B, Reutelingsperger CPM. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996;24:131-9.
13) Visscher DW, Crissman JD. Dissociation of intact cells from tumors and normal tissues. Methods Cell Biol 1994;41: 1-13.
14) Cunningham RE. Tissue disaggregation. Methods Mol Biol 1999;115:257-60.
15) Serna J, Pimentel B, de la Rosa EJ. Flow cytometric analysis of whole organs and embryos. Curr Topic Dev Biol 1998;36:211-22.
16) Zuccotti M, Urch UA, Yanagimachi R. Collagenase as an agent for dissolving the zona pellucida of hamster and mouse oocytes. J Reprod Fert 1991;93:515-20.