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MIFlowCyt

Optimal Percoll Concentration Facilitates Flow Cytometric Analysis for Annexin V/Propidium Iodine-Stained Ischemic Brain Tissues

Wei-Sheng Juan,[1] Hsiao-Wen Lin,[1] Ying-Hsin Chen,[1] Hung-Yi Chen,[2] Yu-Chang Hung,[1] Shih-Huang Tai,[1] Sheng-Yang Huang,[1] Tsung-Ying Chen,[2] E-Jian Lee[1]*

[1]Neurophysiology Laboratory, Neurosurgical Service, Department of Surgery, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan

[2]Institute of Pharmacy, China Medical University, Taichung, Taiwan

[3]Department of Anesthesiology, Buddhist Tzu-Chi University and Buddhist Tzu Chi General Hospital, Hualien, Taiwan

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*Correspondence to: Dr. E-Jian Lee, Department of Surgery, National Cheng Kung University Medical Center and Medical School, 138 Sheng-Li Road, Tainan 70428, Taiwan

Email: ejian@mail.ncku.edu.tw

Wei-Sheng Juan and Hsiao-Wen Lin contributed equally to the work.

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Abstract

We sought to determine the optimal Percoll concentration for ischemic rat brain prepared for flow cytometric (FC) measurements. Animals were subjected to the right middle cerebral artery (MCA) occlusion, and were euthanized at 3, 12, 24, and 72 h after reperfusion onset. The brains were processed by different concentrations (unisolated, 20, 25, 30, or 40%) of Percoll and stained with annexin V/propidium iodine (PI). Ischemic brain damage was evaluated by FC analysis and image analysis for histologic sections. The relative susceptibility of different phenotypes of cells to necrotic and apoptotic damage were evaluated by the FC analyses for the immunohistochemistry, PI, and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-processed brain tissues. Our results showed that FC analysis effectively detected the extent and maturation of apoptotic/necrotic brain damage, and the results were consistent with those determined from histologic brain sections. Neuron was more vulnerable to apoptosis than glia, whereas both cellular phenotypes were compatible in susceptibility for necrotic cell death. Percoll at a low concentration (20%) could effectively remove tissue debris without affecting membranous integrity of the injured neurons. Conversely, high percentages of Percoll (30–40%) substantially increased membranous damage for the injured cells. These results supported the application of FC to determine the extent and progression in time, as well as relative phenotypes of apoptotic/necrotic cell deaths following ischemic damage. We highlighted the use of Percoll at low percentages to facilitate the removal of tissue debris and to improve membrane integrity preservation for the injured neurons. © 2012 International Society for Advancement of Cytometry

Key terms

stroke; apoptosis; necrosis; phosphatidyl serine; flow cytometry

Brain ischemia induces numerous deleterious cascades such as ionic imbalance, receptor activation, and excessive production of excitatory amino acids and free radicals, each of which may lead to cellular necrotic, apoptotic, or even autophagy injury. Necrosis results when the integrity of cytoplasmic membranes become compromised, and, thus, the event can easily be detected by staining with propidium iodine (PI). In contrast, apoptotic cells are characterized by loss of membranes phospholipid symmetry and exposure of phosphatidyl serine (PS) at the cell surface, followed by condensed chromatin structure, reduced cell volume and DNA fragmentations (1,2). Annexin V and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) techniques, which selectively binds to negatively charged phospholipids and 3′-hydroxyl terminal of DNA strand breaks, respectively, are therefore, widely used to identify apoptotic cell injury (3,4).

Histologic assessment in experimental stroke is largely dominated by the use of traditional brain section staining for the damage to neuronal perikarya and labeling

for DNA breaks. With the development of flow cytometry (FC), this technique has been increasingly used to characterize a variety of subgroups of cells in different kinds of blood, cell, or tissue experiments. Rare however, is the use of FC examinations to define necrotic and apoptotic cell deaths following ischemic brain damage, because a large amount of noncellular (infarct) tissue debris needs to be removed prior to the FC analysis. Alternatively, it is challenging to preserve membranous permeability integrity for the injured neural cells and, therefore, to avoid confounding of cell loss during cell harvesting. Accordingly, even though discontinuous Percoll gradient has been developed to isolate immune cells from diseased rodent brains based on their buoyancy or density (5–7), there is a need to determine the optimal Percoll concentration for ischemic brain tissues through which the FC analysis can effectively detect different phases of necrotic and/or apoptotic cell damage of the brain.

In the study, we therefore examined the optimal percentage of Percoll by which the injured cells in the ischemic brain tissues could be separated from noncellular components or tissue debris, and also determine whether the use of Percoll and FC techniques could effectively detect the progression of necrotic and apoptotic cell death over time for ischemic brain tissues concomitantly stained with annexin V and PI. In addition, we examined the consistency between the FC results and those data obtained from transitional image analysis for time-compatible, cresyl violet-stained and TUNEL-processed brain sections. Finally, we sought to determine the efficacy of FC analysis for relative susceptibility of different phenotypes of neural cells (neurons or glia) to necrotic and apoptotic cell death following ischemic stroke.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise indicated. All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center under the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals).

Transient Middle Cerebral Artery (MCA) Occlusion

Male Sprague-Dawley rats, weighing 240–290 g, were supplied by the University Laboratory Animal Center, and were allowed free access to food and water before and after surgery. Animals were anesthetized with 1–2% halothane in 70% N_2O/30% O_2. During surgery, body temperature was maintained at 37.0 $\pm$ 0.5°C using a thermostatically controlled heating blanket and rectal probe (Harvard Apparatus, South Natick, MA). Focal cerebral ischemia was employed by intra-arterial suture occlusion of the proximal right MCA by the method described previously (8–11). Briefly, bifurcation of the right common carotid artery was exposed under an operating microscope. A 4-0 nylon suture, with its tip rounded by heating over a flame and subsequently coated with silicone (Merck KGaA, Darmstadt, Germany), was advanced 17.5–18.5 mm from the external into the internal carotid artery till the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awake from anesthesia and temporarily transferred to a cage with a heating lamp (ambient temperature $\sim$ 26 $\pm$ 1°C). After 90 min of ischemia, the suture was gently removed during another brief period of anesthesia. After surgical procedures, animals were kept in their home cages. The quality of ischemia and subsequent reperfusion was ensured by a drop of local cortical cerebral perfusion (LCCP) at a defined area of the ischemic core cortex to about 20% of baseline during ischemia, followed by an improvement of LCCP >50% of baseline after reperfusion onset, as determined by Laser-Doppler flowmetry (Laserflo BMP2, Vasamedics, St Paul, MN). The right femoral artery was cannulated for measuring arterial blood gases, glucose, hematocrit, and blood pressure.

Histology

At 3, 12, 24, or 72 h after reperfusion onset, animals were sacrificed under anesthesia and transcardiacly perfused with ice-cold phosphate-buffered saline (PBS; Molecular Probes, Eugene, OR) and 4% paraformaldehyde. The brain was quickly removed, dehydrated in 15 and 30% sucrose sequentially, and then embedded in optimal cutting temperature compound (OCT, Miles, Elkhart, IN) and frozen in liquid nitrogen. Animals' brains were sectioned coronally on a cryostat (HM-500O, Microm International GmbH, Walldorf, Germany). Serial sections of 40 μm at the preselected coronal levels, with 1-mm interval from the stereotaxic coordinates of the Bregma AP +4.22 to −6.78 mm, were mounted on poly-L-lysine coated slides and dried at 37°C overnight (12).

One set of sections was stained with 0.5% cresyl violet. Sections were viewed under light microscopy, and necrotic cells were identified as cells that did not contain the cresyl violet–stained Nissl bodies and nucleoli. Areas of gray matter damage, in which neuronal perikarya displayed typical morphological features of ischemic damage, such as shrinkage in the size, triangulation in the shape, decreased stainability of cellular perikarya, vacuolation (sponginess) of the neuropil and chromatin condensation, were delineated (9,11,13). Video images of coronal sections were simultaneously taken with a 3-CCD color camera (Sony power-HAD, model DXC-390, Tokyo, Japan) equipped with a Micro-Nikon 55 mm f2.8 Lens. Brain areas were traced and measured using a computerized image analyzer (MCID Elite, Imaging Research, Ontario, Canada). The calculated infarction areas on the 12 predetermined coronal sections were then compiled to obtain infarct volume (in mm^3). Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using an "indirect method" (8–11).

Tunel

A commercially available in situ cell death detection kit (Calbiochem, Merk Biosciences, Bad Soden, Germany) was used. In this kit, TdT is used to label the DNA strand breaks and apoptotic bodies (14). Sections were soaked for 20 min in 0.03% H_2O_2 in deionized distilled water containing 0.1 M sodium azide to quench endogenous peroxidase activity. After substrate reaction, sections were coincubated with DAPI (2

μM). Fluorescence was assessed at excitation 450–490 nm and emission >515 nm for labeled incorporated nucleotides and at excitation 365 nm and emission >420 nm for DAPI. The number of cells stained with apoptotic bodies was counted by using a Zeiss Axioskop 2 Mot fluorescent light microscope (HBO 100). Since TUNEL staining may detect cells undergoing necrotic cell death as well, only cells showing typical features of apoptosis (condensation and fragmentation of nuclei, apoptotic bodies around the nuclear membrane and absence of cytoplasmic staining) will be counted as TUNEL-positive cells. Necrotic cells, showing weak diffuse cytoplasmic staining without nuclear condensation or apoptotic bodies, will not be counted. The TdT enzyme was omitted in sections for negative controls, and sections of normal developing intestine were used as positive controls.

Percoll Isolation

At 3, 12, 24, or 72 h after reperfusion onset, the animals were anesthetized, followed by cardiac perfusion with ice-cold PBS. The brains were harvested, separated into right (ischemic and ipsilateral) and left (nonischemic, contralateral) hemispheres, and then incubated with 30 mg/mL papain (P4762, Sigma-Aldrich) and 0.21% deoxyribonuclease I (DNase I; D5319, Sigma-Aldrich) in 37°C water bath for 30 min (13). Brain tissues were mechanically dissociated by trituration and passed through 70 μm mesh screens. Cells were washed with Tris buffered saline (TBS) and then resuspended in 8 mL of different percentage of Stock Isotonic Percoll (SIP), which was prepared by mixing nine parts of Percoll (#17-0891-01, GE Healthcare, Piscataway, NJ) with 1 part of 10× Hanks' Balanced Salt Solution (HBSS; #14185, Gibco, Grand Island, NY), overlaid with 2 mL of 1× HBSS in 15 mL conical tubes. Since no apparent separation of layers can be produced by using Percoll at a concentration <20%, Percoll at concentrations greater than 20% were chosen. Accordingly, brain homogenates were equally distributed in five different tubes after papain digestion. One was kept as original homogenate (unisolated) while the other four tubes were resuspended in 20, 25, 30, or 40% of Percoll overlaid by a layer of HBSS. The tubes were centrifuged at 400 × g at 22°C for 25 min. After centrifugation a whitish puffy layer, which stayed above the Percoll and underneath the HBSS layer, was removed, and the pelleted cells were collected.

Flow Cytometry

After removal of the upper aqueous and Percoll layers containing tissue debris, cell pellets were washed twice and resuspended in TBS and stained with FITC-conjugated annexin V (1:100 diluted, AVF250, Strong Biotech Corporation, Taipei, Taiwan) and PI (50 μg/mL, P4170, Sigma, St. Louis, MO) for 30 min at room temperature in the dark. Flow cytometric (FC) analyses were performed on FACSCalibur™ cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software (Becton Dickinson) for acquisition and analysis. The light scatter channels were set on linear gains and the fluorescence channels were set on a logarithmic scale. At minimum of 10,000 cells in the predefined R1 gating on the forward scatter/side scatter (FSC/SSC) were analyzed in each condition (13).

Immunohistochemistry

Resuspended cell pellets were processed with rabbit anti-MAP2 polyclonal antibody (1:100, Millipore, Boston, MA) or rabbit anti-GFAP polyclonal antibody (1:100, DAKO A/S, Glostrup, Denmark) at 4°C for 1 h, whereas brain sections (10 μm) were incubated with mouse anti-NeuN monoclonal antibody (1:200, Millipore-Chemicon, Billerica, MA) or anti-GFAP monoclonal antibody (1:200, eBioscience, San Diego, CA) at 4°C overnight (9,11). Appropriate secondary antibody (AlexaFluor 546 goat anti-rabbit IgG, 1:100; Invitrogen, Carlsbad, CA or Texas red anti-mouse, 1:100; Vector Laboratories, Burlingame, CA) was then added for 1 h. Cells and brain sections were coincubated with PI or TUNEL. Negative controls with preimmune serum were included, and no immunoreactivity was detected in each protocol.

Statistical Analysis

Numeric results were expressed as the mean±standard deviation (S.D.). Paired Students' t test was used to evaluate the response to a change in conditions, and one-way ANOVA with Tukey's *post hoc* comparison/unpaired Students' t test was used to evaluate differences among groups. $P < 0.05$ was selected for statistical significance.

Results

Physiological Parameters and LCCP

Animals' core temperature and other physiological parameters were kept within normal limits at various time intervals of experiments (data not shown). The LCCP recorded at the ischemic core and the penumbral area in the ipsilateral hemisphere decreased to 15.5 ± 2.5% and 40.7 ± 8.7% of baseline, respectively, after ischemia, which maintained at consistent low levels throughout the course of ischemia and returned to 61.7 ± 16.6% and 89.4 ± 16.7% of baseline, respectively, within 40 min after reperfusion onset.

Flow Cytometry Analysis for Neural Cells Stained with Annexin V and Propidium Iodide (PI)

Neural cells stained with annexin V and PI were gated in R1 on the FSC/SSC plot (Fig. 1). We found that unisolated crude brain homogenates contained a substantial bulk of noncellular debris, which exhibited larger SSC and smaller FSC and were excluded by R1 gating (Figs. 1A and 1B). In contrast, the use of Percoll isolation would remove most of the debris (Figs. 1C–1J). The puffy layer that contained noncellular components was confirmed by its unstainability with either annexin V or PI.

To understand the effect of Percoll on annexin V and PI signals, rat brains obtained at 3, 12, 24, or 72 h after reperfusion onset were analyzed (Figs. 2A–2J). We found that the use of low percentages of Percoll not only facilitated the removal of tissue debris but also improved the preservation of membrane integrity for the injured neural cells. At 24 h after the

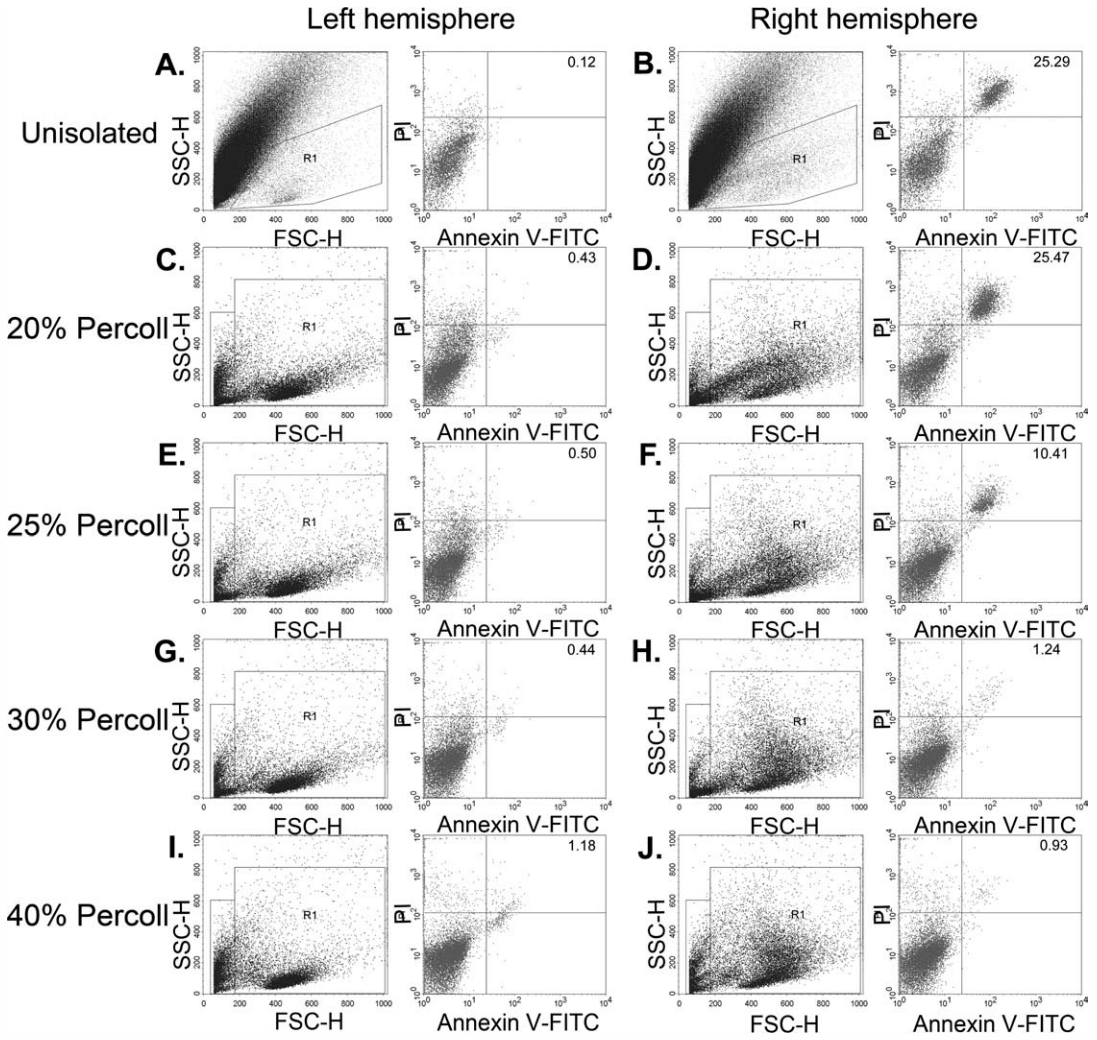

Figure 1. Representative flow cytometry dot plots of rat brain tissues isolated with different percentages of Percoll or unisolated at 24 h following transient focal cerebral ischemia for 90 min. Representative FSC versus SSC and the annexin V-FITC (FL1) versus propidium iodide (FL3) plots are shown. Panels **A**, **C**, **E**, **G**, and **I** show results from the left, nonischemic hemisphere either unisolated or isolated by 20, 25, 30, or 40% Percoll, respectively, while panels **B**, **D**, **F**, **H**, and **J** show those from the right, ischemic hemisphere either unisolated or isolated by 20, 25, 30, or 40% Percoll, respectively. Values in the upper right quadrant are percentage of cells positive for annexin V and PI based on R1 gate.

onset of reperfusion, unisolated brain homogenate obtained from the ischemic right hemisphere contained 25.2 ± 3.7% cells staining positively for both annexin V and PI, while only 3.1 ± 0.9% and 0.5 ± 0.2% cells were annexin V−/PI+ or annexin V+/PI−, respectively (Fig. 2F). On contrast, cells processed by 25, 30, or 40% of Percoll had significantly lower percentage of annexin V+/ PI+ cells at 12, 24, and 72 h after reperfusion onset, compared with the unisolated cells (Figs. 2D, 2F, and 2H). In contrast, Percoll-processed tissues had a larger proportion of annexin V−/PI+ (necrotic) cells rather than annexin V+/PI+ (late necrotic or apoptotic) or annexin V+/PI− (apoptotic) cells identified in the right hemisphere. Less than 1% cells obtained from the intact, left hemisphere showed annexin V+/PI+ at 3, 12, 24, or 72 h after reperfusion onset (Figs. 2A, 2C, 2E, and 2G).

The time-course changes in ischemic brain damage were evaluated by summing the annexin V+/PI−, annexin V−/PI+ and annexin V+/PI+ cells in the right (ischemic) hemisphere. We found that the maturation of brain infarction, as evident by the presence of maturation in ischemic brain damage at 24–72 h after reperfusion, could be identified either by the unisolated or neural cells isolated with 20 or 25% Percoll, but not those by 30 or 40% Percoll. On the contrary, the left, intact hemisphere revealed an acceptable, low proportion of damaged cells at various time intervals postinsult (Fig. 2I). The finding further justified that high percentages of Percoll (30–40%) substantially exaggerated membranous damage and cellular loss for the damaged (ischemic) neural cells, but did not appear to affect the membrane integrity of intact neurons (Fig. 2J).

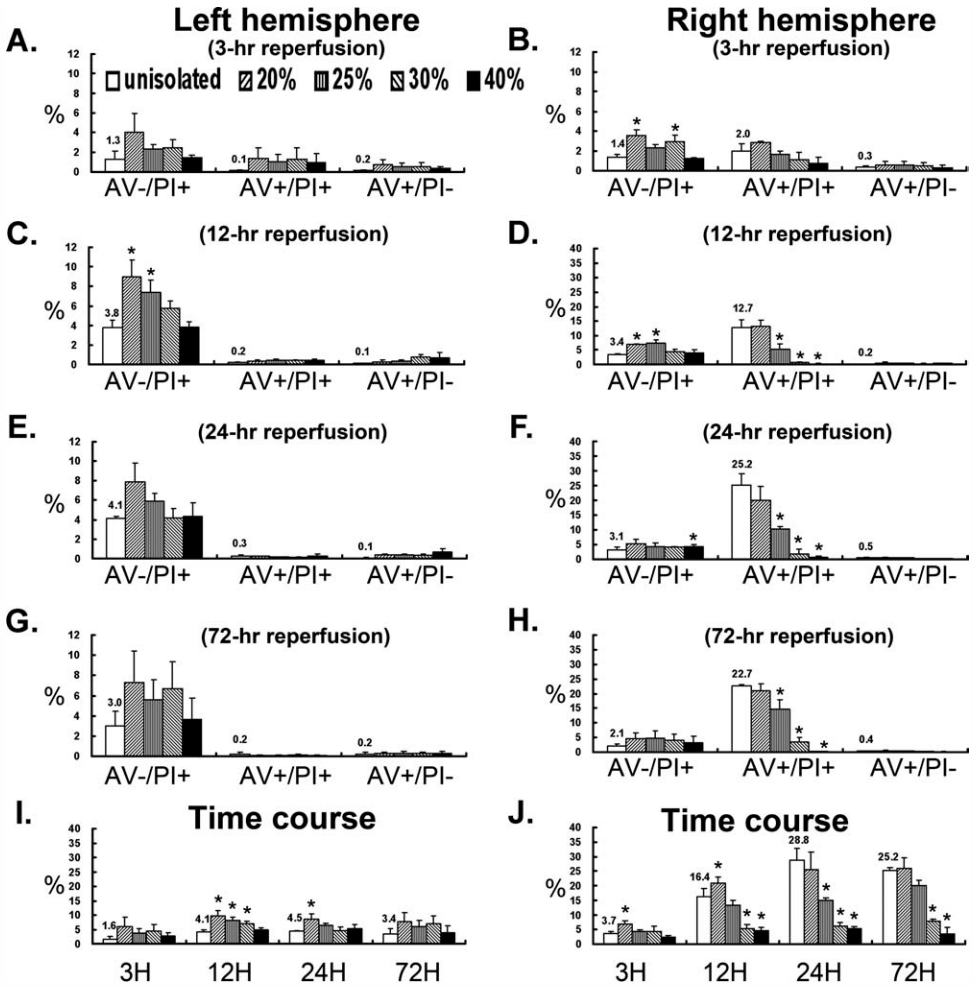

Figure 2. FC analyses for the time-course annexin V (AV) and propidium iodide (PI) signaling of the left (intact) and the right (ischemic) brain hemispheres following transient focal cerebral ischemia. Rat brains were collected at 3, 12, 24, or 72 h after the onset of reperfusion. Cells either unisolated or isolated by different percentages of Percoll were stained with annexin V (AV) and propidium iodide (PI) for subsequent FC analyses. Panels **A**, **C**, **E**, and **G** were results from the left, intact hemispheres at 3, 12, 24, and 72 h, respectively, whereas panels **B**, **D**, **F**, **H**, and **J** were from the right, ischemic hemispheres at 3, 12, 24, and 72 h, respectively. Values are shown in the mean ± standard deviation of the mean (S.D.); $n = 5$. $^*P < 0.05$ compared with unisolated cells as determined by one-way ANOVA analysis followed by Tukey's *post hoc* test.

The Comparison of Ischemic Brain Damage as Determined by Annexin V and PI Staining Using FC Techniques to the Data from the Nissl/TUNEL-Stained Brain Sections

The analysis for histological sections revealed the progress of brain infarction at 24–72 h after reperfusion onset. The infarct volume in the right hemisphere was 7.1 ± 2.6, 15.6 ± 12.3, 37.1 ± 4.2, and 32.9 ± 6.1% at 3, 12, 24, and 72 h after the onset of reperfusion, respectively (Fig. 3). Since the use of Percoll at 20%, but not at 25, 30, or 40%, yielded results most similar to unisolated brain homogenates, we opted to choose to process annexin V and PI-stained brain tissues either by using 20% Percoll or no isolation. The time course changes in ischemic brain damage were compared between those data determined by FC approach and the results collected from the Nissl-stained (Fig. 4A) and TUNEL-labeled brain sections (Fig. 4B). Data obtained by the three methods revealed a similar extent in ischemic brain damage over time. All three methods also showed typical time-course changes in ischemic brain damage that progressed over time and reached the plateau at 24–72 h after reperfusion onset (Fig. 4A). Only at 24 h after reperfusion, ischemic brain damage as determined by annexin V+/PI + cells by using 20% Percoll isolation and flow cytometry analysis was significantly lower than the result obtained from image analysis for the Nissl staining-sections. Thus, a similar trend and extent of ischemic brain damage could be effectively determined by flow cytometry analysis for V+/PI + cells either with no isolation or 20% Percoll isolation, compared to those outcomes evaluated by traditional Nissl-stained techniques. Moreover, there was a similar trend of apoptotic damage by using flow cytometry analysis for annexin V+/PI+ cells either with no isolation or 20% Percoll isolation, when compared with those data obtained from the image analyzed, TUNEL-labeled brain sections, especially at 24 and 72 h after

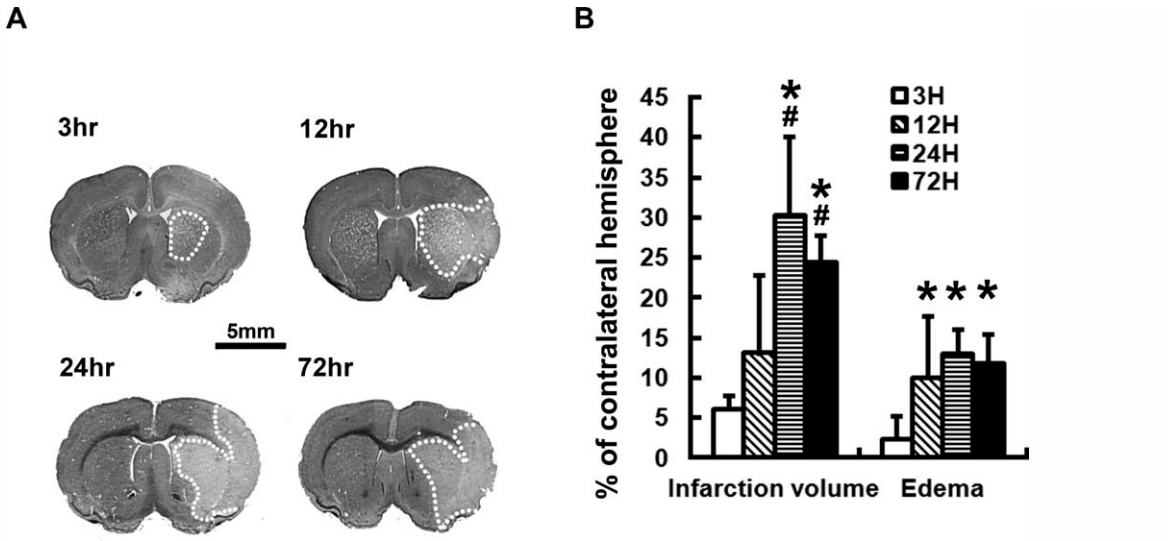

Figure 3. The time-course changes in ischemic brain damage after transient focal cerebral ischemia. **A**: The representative Nissl-stained coronal brain sections were obtained at 3, 12, 24, and 72 h after reperfusion onset, respectively. **B**: The data of brain infarction and edema are expressed by the mean ± standard deviation of the mean (S.D.; $n = 5$). *Means $P < 0.05$ versus 3-h group; #means $P < 0.05$ versus 12-h group.

the onset of reperfusion (Figs. 4B and 4C). However, it appeared that early apoptotic damage could be more sensitively detected by FC analysis for annexin V+/PI+ cells, compared to the TUNEL-labeling techniques (Fig. 4B).

The Susceptibility of Neurons/Glia to Apoptotic Cell Death

At 72 h following ischemic brain damage, flow cytometry analysis showed that 77.6 ± 10.9% of cells positively labeled with TUNEL was proven to be a neuronal phenotype, as determined by MAP2 immunohistochemistry (Fig. 5A; $n = 6$). Only 12.1 ± 3.1% of cells positively stained with TUNEL exhibited immunopositive reactions for GFAP (Fig. 5A; $n = 6$), indicating that glia were more resistant to apoptotic cell death induced by the ischemic insult, compared to neurons. The susceptibility for necrotic cell deaths was, however, compatible for neurons and glia (Fig. 5B, 43.5 ± 4.2 vs. 47.4 ± 3.1%, $n = 6$, respectively). This relative vulnerability of neurons and glia to ischemic apoptotic cell death determined by flow cytometry analysis was consistent with those data obtained from the image analysis for the TUNEL-labeled brain sections (Fig. 5C).

Discussion

This study supported the application of FC for detecting ischemic brain pathology in rats subjected to transient focal cerebral ischemia. In particular, we found that FC analysis effectively detected the extent and maturation of ischemic brain damage, as well as different proportions and phenotypic populations of apoptotic/necrotic cell death following ischemic brain damage, and the results were consistent to those determined by the cresyl violet- and the TUNEL-stained brain sections. We have also observed that the use of FC analysis for ischemic brain tissues tends to underestimate the signal positive cells even with reasonable gating in the FSC/SSC plot, primarily because crude brain homogenate contains a large amount of tissue debris. The use of Percoll at a low concentration (20%), however, could effectively remove tissue debris but well preserve the injured neural cells. Conversely, high percentages of Percoll (30–40%) might have increased membranous permeability damage for the injured (ischemic), but not the intact, neural cells and, consequently, resulted in a large amount of cellular loss. Moreover, our results have shown that the use of Percoll did not significantly affect the annexin V signals in both the ischemic and the non-ischemic brain hemispheres, indicating that the use of Percoll did not increase the proportion and/or severity of the PS extrusion for damaged neural cells following ischemic stroke. Furthermore, we showed that the FC techniques could effectively detect the relative vulnerability of various phenotypic neural cells to either necrotic or apoptotic cell death following ischemic stroke.

A combined staining of annexin V and membrane permeable dyes, such as propidium iodide, ethidium bromide, and trypan blue, has been developed to distinguish apoptotic from necrotic cell damage (15,16). However, it is known that annexin V- and PI-double positive cells could either be cells in late apoptotic (undergoing secondary necrotic) or necrotic stages (15,16). Thus, it should be emphasized that annexin V positive/membrane-permeable dye negative cells are exclusively in the early stage of apoptosis, whereas double positive cells may be either at later stages of apoptosis undergoing secondary necrosis or at various stages of necrosis.

In the study, we showed the FC analysis could effectively detect the maturation of ischemic brain damage over time following MCA occlusion. Consistently, Xu et al. (17) have shown increased annexin V+/PI+ cells over time following

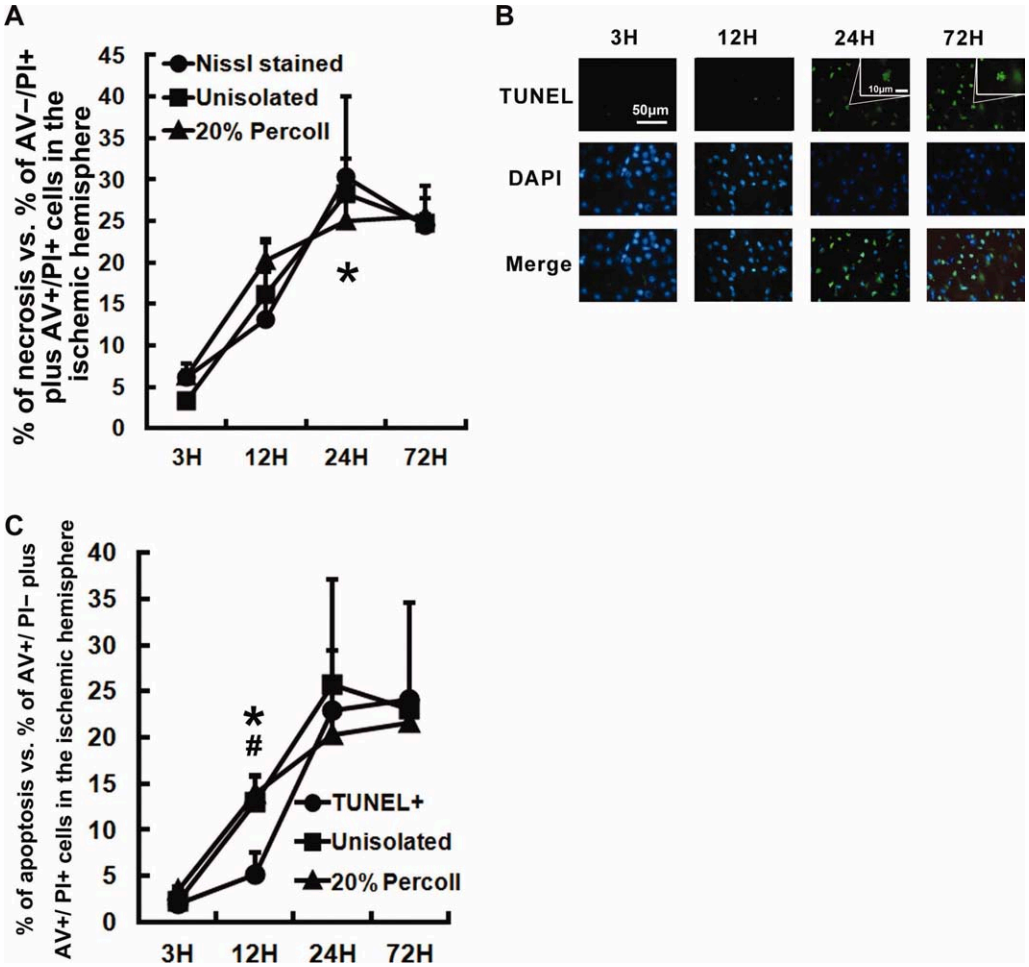

Figure 4. Correlation of necrotic and apoptotic brain damage determined by FC analysis for the annexin V/PI labeling brain tissues with the data obtained from the Nissl- and TUNEL-stained sections. **A**: Data obtained by FC analysis for the annexin V+/PI + cells, either pretreated by 20% Percoll or no isolation, revealed a similar extent in ischemic brain damage over time, when compared with the data from the Nissl-stained section. **B** and **C**: There also was a compatible apoptotic damage over time by using flow cytometry analysis for V+/PI + cells, either pretreated with no isolation or 20% Percoll isolation, when compared with those data obtained from the image analyzed, TUNEL-labeled brain sections. Values are shown in the mean ± standard deviation of the mean (S.D.; $n = 5$). In figure A, * means $P < 0.05$, when compared with Nissl staining data using one-way ANOVA analysis followed by Tukey's *post hoc* test. In figure C, * means $P < 0.05$, when compared between unisolated group and TUNEL group; # means $P < 0.05$, when compared between 20% Percoll group and the TUNEL group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cerebral ischemia-reperfusion. In addition, they showed increased annexin V+/PI− (early apoptotic) signaling in the ischemic striatum over time (17,18). We, however, failed to detect a high level (>10%) of annexin V+/PI− cells in both the ischemic and the contralateral, intact hemispheres. Curiously, it has been demonstrated that mechanical dissociation and the use of trypsin significantly increased the proceeding of the PS extrusion ex vivo (19,20). In contrast, the use of papain and DNase I could effectively dissociate brain tissues whereas well preserved cellular membranous structures of damaged cells, as compared with other methods such as trypsin, collagenase, or mechanical dissociation (20). Accordingly, it was likely that the discrepancy between low percentages of early apoptotic cells observed in this study and relatively high prevalent rates reported in previous reports (17,18) might have been derived from the use of papain (this study) rather than trypsin (previous studies) as digesting enzyme prior to the FC analysis.

It was noteworthy that annexin V−/PI+ cells might represent both necrotic cells and those broken cells with isolated nuclei because of mechanical damage during cell isolation. Incubation of cell suspension with DNase I substantially decreased the presence of broken cells in V−/PI+ cells (∼ 5–10%) by 34.4 and 54.0% using 50 and 200 μg/mL DNase I, respectively, but it also significantly decreased the population of V+/PI+ cells by 31.1 and 77.0%, respectively (21). Accordingly, we could expect that ∼ 3–5% of isolated cells were virtually damaged cells originating from cellular isolation procedures. We therefore used papain and DNase I prior to mechanical dissociation for brain tissues. Our results with

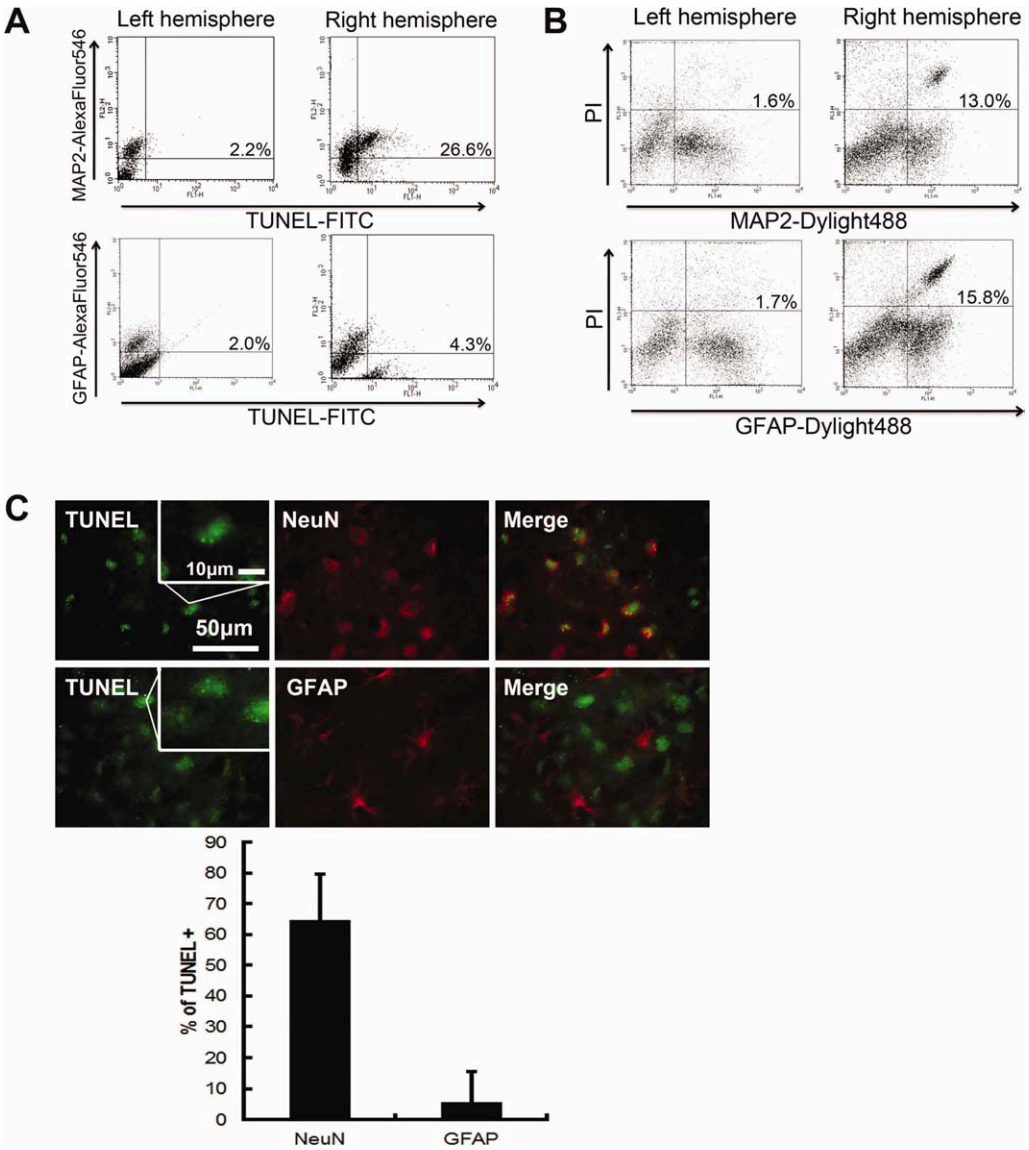

Figure 5. FC and histologic analyses for relative vulnerability of neurons and glia to ischemic necrotic and apoptotic cell deaths. **A**: At 72 h after reperfusion, 77.6% of cells positively labeled with TUNEL were neuronal origin, as determined by MAP-1 immunohistochemistry. Only 12.1% of cells positively stained with TUNEL exhibited immunopositive reactions for GFAP. **B**: The susceptibility for necrotic cell deaths was, however, compatible between neurons and glia. **C**: This relative vulnerability of neurons and glia to ischemic necrotic and apoptotic cell deaths, determined by flow cytometry was in consistent with those determined by histologic analysis for the TUNEL-labeled brain sections. $n = 6$ per group. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

relatively low percentages (∼ 5–10%) of annexin V−/PI+ cells over time in both the ischemic right and the contralateral, intact hemispheres supported that a combination use of papain and DNase I before mechanical dissociation substantially improved the removal of mechanically damaged cells during subsequent cell isolation whereas it improved the preservation of injured V+/PI+ cells caused by ischemia-reperfusion damage.

Following cerebral ischemia, TUNEL-positive cells appeared in the ischemic brain as early as 12 h after reperfusion and were maximally identified at 3 days postinsult (22,23). It was, however, observed that some TUNEL-positive cells showed characteristics of necrosis by their weak diffuse cytoplasmic staining without nuclear condensation or apoptotic bodies (24,25). Consistently, we detected very low levels of annexin V+/PI− cells in both ischemic and the contralateral hemispheres in the FC analysis. A significant population of annexin V+/PI+ cells was, however, found as early as 3 h, and this signaling remained strongly detectable up to 72 h postinsult. Thus, it was evident that only a small proportion of damaged neural cells had exclusive membranous PS extrusion (i.e., without permeability change), and most of the annexin V-positive cells became stainable as a consequence of increased membrane permeability either caused by primary necrotic

damage or being in a stage of late apoptosis undergoing secondary necrotic changes.

In the study, we have observed that a high percentage of Percoll might have exaggerated cellular membranous permeability and, thus, increased cell loss of the ischemic brain tissues during the process of dissociation, thereby resulting in underestimation of the FC analysis for ischemic brain pathology (26). In contrast, ischemic brain tissues isolated by 20% Percoll and stained with annexin V and PI showed a similar degree of brain damage to that obtained from the conventional image analysis for the Nissl stained-brain sections. This observation supported that ischemia-reperfusion have induced increased membranous permeability of neural cells which were vulnerable to hyperosmotic pressure generated by high percentages of Percoll. In particular, high concentrations of Percoll should be avoided in the application of flow cytometry for outcome measures of the ischemic neurons, since neuronal cells are more susceptible to apoptotic cell damage caused by ischemia-reperfusion, compared to glia. Additional studies are, however, needed to verify the detailed mechanisms observed here, but this observation may be worth further attention to avoid error interpretation in FC analysis (26).

Conclusions

We have observed that FC analysis for ischemic brain tissues isolated by 20% Percoll and labeled with annexin V and PI yielded compatible results of ischemic brain pathology, as compared to that obtained from the Nissl and the TUNEL stained-brain sections. Our results support the application of FC to determine the extent and degree of progression of ischemic brain damage, as well as relative susceptibility of different phenotypic neural cells to apoptotic and necrotic cell death following ischemic stroke. We highlighted the use of papain as digestive enzyme in combination with Percoll at a low concentration to facilitate the removal of tissue debris and to improve membrane integrity preservation for the injured neural cells.

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