The *In Vitro* Fate of Rabbit Fetal Brain Cells after Acute *In Vivo* Hypoxia

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In the investigation of ischemia-induced brain damage, traditional methods using histopathology estimate brain cell death at a time remote from ischemic insult. These observations fail to take into account endogenous repair processes or ongoing injury cascades like apoptosis. The cells that are injured but not killed initially are the population most amenable to rescue. The hypothesis was that *in vivo* uterine ischemia–reperfusion would result in more cell death and apoptosis in fetal brain cells cultured *in vitro*. Near-term, 29 d gestation, pregnant New Zealand White rabbits were subjected to repetitive uterine ischemia for a cumulative time of 40 min ischemia and 20 min reperfusion. Immediately after uterine ischemia, the fetal brains were removed and dissociated into a cell suspension. The ischemic group had more cell death than non-ischemic controls

as assessed by Trypan Blue exclusion and propidium iodide (PI) uptake on a flow cytometer. Aliquots of cells were plated and cultured for 24 and 48 hr. The ischemic group had significantly more cell death (propidium iodide) than non-ischemic controls at 24 hr and significantly more apoptosis, as assessed by annexin-V binding in cells at 24 hr and caspase-3 activity at 48 hr. Fewer cells attached to the culture plates at 48 hr in the ischemia group. After uterine ischemia, certain fetal brain cells die immediately, and other cells undergo ongoing damage resulting in necrosis and apoptosis that is manifest later. This method offers insight into the fate of those cells and provides a tool for assessing interventions to decrease cell injury.

Key words: apoptosis; cell culture; cell death; fluorescence; flow cytometry; neurons; propidium iodide; mitochondria

Hypoxic–ischemic brain injury in children results in cerebral palsy, mental retardation, or learning disabilities (Robertson and Finer, 1985). Our laboratory is interested in the mechanisms of fetal brain injury after hypoxia–ischemia, especially those resulting from free radicals. A dilemma of investigating fetal hypoxic brain injury is that traditional methods using histopathology and fixed brain specimens to detect cell injury can only be used remote from the time of the insult (Rorke, 1992; Grafe, 1994). This poses problems for investigators because, first, free radicals have only a transient existence and are long gone by the time histopathological brain injury is manifest. Second, the fetal brain is in a dynamic state of growth, cell death, and repair (Oppenheim, 1991; Rabinowicz et al., 1996), and the postponed outcomes fail to take into account the dynamic state (Grafe, 1994). Third, injury cascades are initiated by hypoxia–ischemia that trigger apoptosis (Ferrer et al., 1994; Sidhu et al., 1997). Fourth, the fetuses undergo labor and birth, which are stressful events (Volpe, 1995) that result in cumulative injurious effect.

It is not clear what happens to brain cells *in vivo* that are injured but not killed by an insult. Do they suffer late death by apoptosis or necrosis, or do they recover? This study investigates cell fate immediately after injury to fetal brain but before the occurrence of repair processes, initiation of injury cascades, or birth. The focus of this study was to determine which cells are injured but not yet dead. Histological techniques immediately after an insult do not detect these cells and have difficulty detecting immediate cell death (Steinbach et al., 1999); only at later

stages do they detect cell death and apoptosis. By then, repair and injury cascades have modified these observations. Imaging studies and electrocorticography immediately after an insult reflect cellular injury but do not specify which cells are injured. This study therefore investigates cell fate in fetal brain by studying brain cells *in vitro*, immediately after an *in vivo* insult, using dissociated brain cells and flow cytometry. Flow cytometry allows the sensitive detection and rapid quantification of a large population of cells (Jaroszeski and Heller, 1998).

Apoptosis represents a defined cascade that includes loss of cell membrane polarity for phosphatidylserine, activation of caspase enzymes, and cleavage of DNA (Raff, 1998), but this is not detectable immediately after an insult (Joashi et al., 1999). To allow time to detect apoptosis, this study combines cell culture techniques with flow cytometry to detect cells that undergo apoptosis later.

The hypothesis was that *in vivo* hypoxia–reoxygenation would result in greater fetal brain cell death and apoptosis *in vitro*. A near-term model of repetitive uterine ischemia in pregnant 29 d New Zealand White rabbits (Tan et al., 1998) that mimicked the repetitive stress of labor was used. All dissociated brain cells were

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investigated immediately and in cell culture, at 24 and 48 hr. Furthermore, the ability of cells to attach to poly-L-lysine (Letourneau, 1975) was investigated as a measure of cellular function.

MATERIALS AND METHODS

Surgery preparation. *In vivo* global hypoxia–reoxygenation of fetuses was induced using repetitive uterine ischemia (cumulative 40 min) in 29 d gestation New Zealand white rabbits (Myrtle's Rabbits, Thompson Station, TN) as described previously (Tan et al., 1999). Briefly, the dams were anesthetized with intravenous fentanyl ($75 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$) and droperidol ($3.75 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$), and bag and mask ventilation were provided to maintain normal arterial pH (7.35–7.45), P_{CO_2} (32–45 torr), and P_{O_2} (70–100 torr). The dams then underwent spinal anesthesia by administration of 0.75% bupivacaine through a 25 gauge spinal needle in the L2–L5 intervertebral space. The fentanyl and droperidol dose was reduced by one-fifth to allow the dam to breathe spontaneously through a mask. Uterine ischemia was induced by inserting a 4F Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, CA) into the left femoral artery, advancing it 10 cm into the descending aorta to above the uterine and below the renal arteries, and inflating the balloon with 300 μl of saline. Reperfusion was accomplished by deflation of the balloon. Blood pressure monitoring of the right leg using a Doppler was performed to ensure continued ischemia or reperfusion. Immediately after ischemia–reperfusion (IR), the fetuses were removed via a hysterotomy, and the brains removed and placed in HBSS (Life Technologies, Rockville, MD).

Groups. The control group consisted of 16 fetuses born to seven dams not subjected to uterine ischemia. The IR group consisted of 10 fetuses born to four dams subjected to 20 cycles of 2 min uterine ischemia followed by 1 min reperfusion for a total of 40 min ischemia and 20 min reperfusion.

Brain cell suspension. The meninges were removed, and the cortex was placed in 0.025% trypsin and incubated on a rotating shaker at 37°C for 45 min. This concentration of trypsin was determined to be the lowest concentration of trypsin that would enable dissociation of cells without causing significant cell death. The brain suspension was spun at $300 \times g$ for 10 min, the trypsin was aspirated, and the cells were washed with HBSS before limited titration (30 times) in Neurobasal Media (Life Technologies). The brain suspension was passed through a sterile 70 μm filter to produce a single-cell suspension. Cellular number and viability were assessed with Trypan Blue exclusion.

The cellular suspension was diluted to 1×10^6 cells/ml and either plated for tissue culture, assessed immediately on the flow cytometer (Fig. 1), or spun at $300 \times g$ for 5 min and fixed in 1 ml of 1% paraformaldehyde in PBS for 24 hr.

For tissue culture, 1×10^6 cells per well (1 ml) were plated on poly-L-lysine (Sigma, St. Louis, MO)-coated plates in Neurobasal Media supplemented with B27 (Life Technologies), 50 $\mu\text{U}/\text{ml}$ penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 0.5 mM L-glutamine, and 25 mM glutamic acid.

Flow cytometry assessment. The flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA) was used to assess cells immediately or after 24 and 48 hr in culture. The flow cytometer sampling rate was 60 $\mu\text{l}/\text{min}$. Excitation used an argon laser, wavelength 488 nm. Fluorescence was measured at three wavelengths: $530 \pm 30 \text{ nm}$, $585 \pm 42 \text{ nm}$, and $>670 \text{ nm}$. For the immediate assessment, aliquots of 1×10^6 cells were spun at $300 \times g$ and resuspended in 1 ml PBS. Cell death was assessed after addition of 20 μg propidium iodide (PI) just before analysis (Fig. 1A). A second aliquot was used to assess mitochondrial function using Rhodamine 123 (Calbiochem, San Diego, CA); 5 μg was added, incubated at 37°C for 5 min, then 20 μg PI was added just before analysis on flow cytometer (Fig. 1B,C). The gate set for PI uptake was at 10^3 relative fluorescence intensity (RFI) because there was a definite demarcation at that point (Fig. 1A, High). Aliquots of fixed cells were stained with PI, and 90% of the cells stained above that gate. The intermediate PI fluorescence was defined as $<10^3$ RFI and above autofluorescence at 10^1 RFI. The gate set for rhodamine was at 10^2 RFI, above autofluorescence, with high and intermediate fluorescence at 10^3 and 10^2 RFI, respectively (Fig. 1B).

Apoptosis was assessed by three indicators. First, for annexin-V binding, annexin-V-FITC (Sigma), 0.25 μg , was added to 1×10^6 cells in 1 ml Neurobasal media, incubated at room temperature for 15 min, and spun at $300 \times g$ for 5 min, and the cell pellet was resuspended in 10 mM HEPES, 150 mM NaCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 4% BSA before analysis.

Figure 1. Flow cytometer dot plots using argon laser (Ex 488 nm and Em FL-1 = 530, FL-2 = 585, and FL-3 = $>670 \text{ nm}$) from one control animal and one IR animal. *A*, Fluorescence from addition of PI versus forward scatter. The PI fluorescence is subdivided into high (dead cells) and intermediate zones. *B*, Cells were exposed to Rhodamine 123, and the fluorescence was plotted versus forward scatter. The Rhodamine 123 fluorescence is subdivided into high and intermediate zones (intact mitochondrial function). *C*, To cells in *B*, PI was added and PI fluorescence was plotted versus Rhodamine 123 fluorescence. The intermediate zone is used as a marker for apoptosis (Ferlini et al., 1996).

Second, Caspase-3 activity was measured using the PhiPhiLux G1D2 kit (Calbiochem) (Komoriya et al., 2000) in a subset of animals and after 48 hr of culture. Briefly, cells were centrifuged at $300 \times g$ for 5 min at 4°C. The supernatant was removed, and 50 μl of peptide substrate (10 μM) was added to the pellet, then mixed by flicking the tube, and 10 μl of HEPES Buffer (120 mM), pH 7.4, was added. The open tubes were incubated in 5% CO_2 at 37°C for 20–60 min. The cells were washed with 1 ml of ice-cold flow cytometry dilution buffer (Calbiochem) and centrifuged at $300 \times g$ for 5 min at 4°C. The cells were resuspended in 1 ml ice-cold flow cytometry dilution buffer and analyzed on a flow cytometer.

Third, cells that had an intermediate fluorescence with rhodamine as well as PI were determined (Ferlini et al., 1996) (Fig. 1C, Intermediate).

Cell type. Cholera toxin FITC (Sigma) (1 $\mu\text{g}/\text{ml}$ added and incubated at 4°C for 30 min) or Tetanus Toxin C fragment (Neurotag Green; Roche, Indianapolis, IN) (10 $\mu\text{g}/\text{ml}$ added and incubated at 4°C for 45 min in the dark) were used to obtain an estimate of neuronal cell number, viability, and proportion. (Mirsky et al., 1978). Other cell markers were investigated after 24 hr of fixation in a subgroup of animals. Briefly, the cells were washed twice with 1 ml of PBS and then resuspended in 0.1% saponin (Sigma), 1% fetal bovine serum, and PBS. The antibodies were then added: mouse anti-O4 antibody (Chemicon, Temecula CA), 0.2 μg incubated at 4°C for 45 min in the dark, followed by 0.5 μg rat anti-mouse IgM FITC for 20 min for oligodendrocytes, or mouse glial fibrillary acid protein antibody (GFAP) (Roche), 0.5 μg followed by 0.2 μg goat anti-mouse IgG1 for 20 min for astrocytes.

Figure 2. Immediate cell death in the single-cell suspension was less in controls (white bars) than in IR (black bars) (* $p < 0.05$) as measured by Trypan Blue (TB) exclusion (A) (18 control, 10 IR fetuses) and propidium iodide (PI) (B) on the flow cytometer (18 control, 10 IR fetuses). C, Cell death, as measured by PI, at 24 and 48 hr, in the unattached cells was higher in IR compared with controls (13 control, 10 IR fetuses). D, Cell death, as measured by PI, at 24 and 48 hr in the attached cells (13 control, 10 IR fetuses).

Cell fate. The plates were incubated in 5% CO₂ at 37°C for 24 or 48 hr. At 24 and 48 hr, flow cytometer analysis was again conducted on both the unattached and attached cellular layers. The media was removed from the plates by gentle aspiration and then spun at 300 × *g* to pellet the unattached cells. The attached cells were detached from the plate by incubating with Versene (10 mM HEPES, pH 7.4, 0.2 gm/l EDTA) for 5 min and then washed in PBS. An absolute cell count was made, and both groups of cells were again assessed for cell death with PI; mitochondrial function was assessed with rhodamine and apoptosis.

The study was approved by the Animal Review Committee of the Evanston Northwestern Healthcare Research Institute. All animals received humane care in compliance with the *Guide for Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1985).

Statistical analysis used an unpaired *t* test with α -error set at $p < 0.05$. Data that were not normally distributed were analyzed by Kruskal-Wallis and Wilcoxon signed rank tests. Values are given as mean ± SEM.

RESULTS

Immediate cell death was significantly higher in IR than controls as measured by a 120% increase of Trypan Blue or a 125% increase of PI (Fig. 2). At 24 hr, cell death in the unattached cells was significantly higher in IR than controls, with a 73% increase in PI (Fig. 2).

Cellular function as measured by cell adherence was lower in IR. There were fewer adherent cells, at 24 and 48 hr, in IR than controls; the ratio of unattached to attached cells was 100% greater at 24 hr and 330% at 48 hr (Fig. 3). Mitochondrial function as assessed by cell staining with rhodamine was similar in both groups immediately (controls, 92 ± 3 IR, 87 ± 2% of total cells) and after 24 and 48 hr.

Measures of apoptosis, as measured by annexin-V binding and caspase-3 activity, were not different between IR and controls immediately after the insult. However, at 24 hr, annexin-V binding was significantly higher in IR than controls, in both the attached cells (170% increase) and unattached cells (230% increase) (Fig. 4). Annexin-V binding at 48 hr was significantly higher in IR than controls in the attached cells (110% increase) but not the unattached cells. Caspase-3 activity at 48 hr was significantly higher in IR than controls, in both the attached cells (77% increase) and unattached cells (120% increase) (Fig. 4).

When cells are injured, membrane permeability increases.

Figure 3. The ratio of the number of unattached/attached cells to poly-L-lysine-coated plate, at 24 and 48 hr, was higher in IR (black bars) compared with controls (white bars) (11 control, 10 IR fetuses) (* $p < 0.05$).

When cells that stained with high rhodamine and high PI (Fig. 1C, *High*) were investigated (indicating recent cell death), significantly more cells in IR stained immediately and at 24 hr (in unattached cells). With intermediate rhodamine and intermediate PI fluorescence (Fig. 1C, *Intermediate*), a significant increase was observed in IR unattached cells at 48 hr (IR 51 ± 5; controls 39 ± 2).

The number of neurons in the cell suspension as assessed by cholera toxin (control group 69 ± 5; IR group 47 ± 11) and tetanus toxin (control group 86 ± 4; IR group 86 ± 3) is similar in both groups. There also were no differences in O4 (control

Figure 4. Increased apoptosis at 24 and 48 hr in both the unattached and attached cell layers in IR (black bars) compared with controls (white bars) (* $p < 0.05$). A, Annexin-V binding in unattached cells at 24 and 48 hr (12 control, 10 IR fetuses). B, Annexin-V binding in attached cells at 24 and 48 hr. C, Caspase-3 activity in unattached cells at 48 hr (7 control, 7 IR fetuses). D, Caspase-3 activity in attached cells at 48 hr (5 control, 5 IR fetuses). Fewer attached cells were assessed because of failure of the assay in two animals in each group.

group 25 ± 2 ; IR group 15 ± 7) and GFAP (control group 6 ± 1 ; IR group 11 ± 5) staining between the groups.

DISCUSSION

The unique aspects of this study are, first, that after hypoxia-reoxygenation, 20–30% of the number of cells die immediately. Second, cells that are injured die later, by either necrosis or apoptosis. Third, the entire cortex was investigated immediately after the insult before repair or ongoing injury cascades could occur. This method of assessing brain injury, in an animal model of true global hypoxia-reoxygenation, provides a simple way to quantify the effects of an insult on the fetal brain. Culture of CNS cells typically focuses on cells that are alive and taken from animals that are not subjected to injury. In this study, all the cells that could be obtained from the brain were assessed. As expected, some cells died acutely after an insult (as assessed by Trypan Blue and PI), and some cells were injured (by the failure to attach). The acute cell death by Trypan Blue exclusion confirms the results that were published previously (Tan et al., 1998, 1999).

After hypoxic-ischemic injury, the fate of the cells that are not immediately killed is not clear. Some go on to recover, whereas others will suffer a late death either by apoptosis or necrosis (Ferrer et al., 1994; Sidhu et al., 1997; Colbourne et al., 1999). These are the cells that interventional strategies are most likely to succeed with (Cheng et al., 1998), because the treatment of the brain after hypoxia-ischemia is unlikely to be before the insult has occurred, so cells that are already dead cannot be revived. Hypoxia-ischemia in fetal rabbit brains resulted in increased late cell death and increased apoptosis.

The major advantages of this method of screening fetal brain injury after uterine ischemia are that it is relatively simple to do and it takes a short time; it occurs before any repair has had a chance to occur. The use of the flow cytometer allows a large number of cells to be assessed rapidly and a nonbiased quantification to be obtained (Jaroszeski and Heller, 1998). There are regions of the brain that have an increased vulnerability to hypoxia (Volpe, 1995), so our estimates of cell death and apoptosis will underestimate the damage to those regions, because they will be diluted by resistant regions. This method can be adapted with minor modifications to look at specific regions of the brain. The use of cell-specific antibodies may be used to look at insults in a cell-specific manner. Neuronal and oligodendrocyte numbers are consistent with those expected. Interestingly, the cells in culture lose the epitope for cholera toxin more than that of tetanus toxin, and this was not explained by an increase in type II astrocytes because the GFAP binding was not increased (data not shown).

Review of the literature indicates that the approach of using the flow cytometer to assess a heterogeneous population of cells from whole organs has been used previously in cardiac development (Prados et al., 1992) and chick embryo (Serna et al., 1998). To our knowledge, this is the first study that uses the flow cytometer to assess the whole fetal brain after an insult. The use of a flow cytometer assumes that a single-cell suspension is produced and is unbiased in cell type. The population of cells in the single-cell suspension is heterogeneous in size. The protocol uses gentle enzymatic digestion and mechanical disruption, which is similar to that used in the culture of neurons (Crawley, 1997). The control brains exhibited little cell death, which implies that the procedure itself does not kill the cells. The stress of disassociation may uncover some injured cells. By subjecting a control brain to the same procedure as a brain that has suffered an *in vivo* insult, some of the artifacts that occur because of the culture system are

removed in analysis. Few of the flow cytometer events were cell fragments because $>95\%$ stain with rhodamine, and when fixed, 90% stain highly with PI.

Poly-L-lysine in the concentration that we used provides a good substrate for cell adherence and is at a low concentration that should not be toxic to the cells (Yavin and Yavin, 1974; Letourneau, 1975; Goslin and Banker, 1998). Poly-L-lysine will eventually be digested by proteases from the cells, but this should not occur in the first 48 hr. This protocol could be modified, by using a different coating on the plates, to test different cell-adhesion molecules.

Apoptosis is part of normal development of the brain (Rabinowicz et al., 1996). It has been estimated at 0.86% in human brains, at 20 weeks gestation (Olano et al., 1996). In controls, $\sim 20\%$ of the cells were annexin-V positive, and the difference may be attributable to different methods of preparation or the different species involved. Triggers for apoptosis are related to growth factors, connections within the brain, and genetic and environmental factors. Hypoxic injury can result in an increase in apoptosis that is by many of these mechanisms. Apoptosis has been studied on the flow cytometer using rhodamine combined with the DNA marker ethidium bromide (Ferlini et al., 1996). In that study, a single-cell population was studied. Early apoptosis was marked by a slight increase in fluorescence caused by ethidium bromide and a slight decrease in fluorescence caused by rhodamine. In this study the whole cortex was used, so the cell population was heterogeneous in cell size and mitochondrial number. Changes in rhodamine fluorescence were harder to detect. Even then, IR in unattached cells at 48 hr had a significant increase of cells with intermediate rhodamine and PI fluorescence.

A unique aspect of this study is the investigation of dynamic cell changes after the extirpation of the brain at a specific time point. A spectrum of injury to fetal brain cells can thus be observed. As the cells progress toward cell disintegration, there is a greater decrease in cell adherence, loss of cell epitopes, and decrease in rhodamine uptake with an increase in PI uptake. By following these markers over time, injured cells may show injury remote from the insult.

If the fetus is left *in utero* after hypoxia-ischemia, then there will be some recovery. In the fetal brain, there is more cell division and growth to enhance this recovery. The fetal brain also has greater plasticity than the adult brain, with different parts of the brain able to compensate for the loss of other parts. This plasticity makes it all the more important to attempt to rescue cells that have not been killed by the hypoxic-ischemic insult. The challenge will be to identify those cells that are potentially salvageable immediately after the insult.

In conclusion, after an *in utero* hypoxic insult, there was a significant increase in cell death immediately, and greater cell death continued for 48 hr *in vitro*. There were fewer normally functional cells, as measured by attachment to poly-L-lysine-coated cell culture plates. There was an increase in apoptosis *in vitro* 24 and 48 hr after the *in utero* insult. We speculate that cells that undergo late cell death by apoptosis or necrosis are the ones that potential therapies should be targeted to.

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