Plasmalemma Permeability and Necrotic Cell Death Phenotypes After Intracerebral Hemorrhage in Mice

Xiaoxia Zhu, MD*; Luyang Tao, MD, PhD*; Emiri Tejima-Mandeville, PhD; Jianhua Qiu, MD, PhD; Juyeon Park, BA; Kent Garber; Maria Ericsson, BS; Eng H. Lo, PhD; Michael J. Whalen, MD

Background and Purpose—Traumatic and ischemic brain injury induce plasmalemma permeability and necrosis; however, no studies have examined these aspects of cellular injury in intracerebral hemorrhage models.

Methods—In vivo propidium iodide (PI) and YOYO-1 were used to assess plasmalemma damage after collagenase-induced intracerebral hemorrhage in mice. Ex vivo aspartylglutamylvalylaspartic acid, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, and electron microscopy were used to assess the relationship between plasmalemma permeability and mode of cell death. Cell types vulnerable to plasmalemma damage were determined by immunohistochemistry.

Results—Plasmalemma permeability was first detected in the lesion at 1 to 3 hours and peaked at 48 to 72 hours. Neurons and IBA-1-positive cells with morphological features of monocytes were sensitive, whereas resident microglia and astrocytes were resistant to plasmalemma permeability. PI+ cells colocalized with fluorescent-labeled caspase substrates and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling beginning at 3 to 6 hours. At 48 hours, greater than half of injured cells were PI+/aspartylglutamylvalylaspartic acid− or PI+/terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling− suggesting necrosis, and <5% were PI−/terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling+ or PI−/aspartylglutamylvalylaspartic acid+. Electron microscopy confirmed ultrastructural features of necrosis at 24 hours after intracerebral hemorrhage, high mobility group box protein-1 was released from permeable cells, and mice deficient in receptor interacting protein kinase (RIPK) 3, a known necrosis trigger, had 50% less PI+ cells at 24 hours. Permeable cells remained in the brain for at least 24 hours with <10% spontaneous resealing.

Conclusions—Necrosis contributes to cell demise after intracerebral hemorrhage. Programmed necrosis and plasmalemma damage may represent novel therapeutic targets to prevent cell death or rescue injured cells after intracerebral hemorrhage. (Stroke. 2012;43:00-00.)

Key Words: apoptosis ■ inflammation ■ intracerebral hemorrhage ■ mice ■ necrosis ■ plasmalemma

Experimental models of intracerebral hemorrhage (ICH) and brain specimens from humans with ICH show delayed progressive cell death in hemorrhagic brain.1-5 Loss of plasmalemma integrity is a hallmark of cellular injury and death after renal and cerebral ischemia and traumatic brain injury (TBI).6-9 In experimental cerebral contusion, injured cells sustain plasmalemma damage early after injury and are degenerative by histochemical criteria, disappear from the injured brain within several days, and have biochemical and ultrastructural features of necrosis.8 In contrast, plasmalemma permeability after ischemic brain injury is delayed by several hours and peaks later than in cerebral contusion.6,7 Whether or not plasmalemma permeability is induced by ICH has not been previously reported. This is an important gap in the literature because plasma membrane permeability has been implicated as an initiating event in cell death as well as a trigger for the ensuing inflammatory response.10,11

Although numerous mechanisms of cell death including caspase-mediated apoptosis have been implicated in experimental ICH, surprisingly little study has been done to estimate the contribution of necrosis. One indirect way to assess necrosis is to interrogate plasmalemma function at the same time as caspase activity and DNA damage.6,7,9 Early necrosis produces rapid loss of membrane integrity, whereas membrane integrity is maintained until late stages of apoptotic cell death.6,12,13 Thus, early necrosis might show plasma-
lemna damage with or without DNA damage, whereas early apoptosis is associated with DNA damage with plasmalemma integrity maintained. Cells with plasmalemma permeability and DNA damage may be late necrotic, late apoptotic, or a mixed cell death phenotype initiated by concomitant activation of necrotic and apoptotic mechanisms.\textsuperscript{6,7} Acute ischemic and TBI induces plasmalemma permeability to propidium iodide (PI) through mechanisms that remain unknown.\textsuperscript{6–8} We and others have used PI and the green fluorescent dye YOYO-1 iodide in vivo to label injured cells after ischemic and TBI.\textsuperscript{6–8} These studies have demonstrated apoptotic-like (PI/terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling [TUNEL]+) and necrotic-like (PI+/TUNEL−) cell death phenotypes. Here, we used in vivo PI and YOYO-1 to identify cells with plasmalemma permeability and follow their spatial and temporal course after collagenase-induced ICH in mice. Using ex vivo TUNEL and caspase histochemistry, and ultrastructural analysis of injured cells, we tested the hypothesis that plasmalemma permeability is associated with markers of cell death and that a necrotic-like cell death phenotype is associated with ICH.

**Methods**

**Collagenase-Induced ICH in Mice**

All procedures and experimental protocols outlined subsequently were approved by the Massachusetts General Hospital Institution for Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male CD-1 mice ages 8 to 12 weeks weighing 25 to 35 g (Charles River, Wilmington, MA) and C57Bl/6 wild-type mice (Supplemental Table I; http://stroke.ahajournals.org) were used for all studies except for comparisons between receptor RIPK3 knockout (backcrossed over 14 times into CD-1 strain) and its wild-type littermates (C57Bl/6 wild-type mice; Supplemental Table I; http://stroke.ahajournals.org). Mice were anesthetized with isoflurane and euthanized at various times after ICH. Brains were removed and frozen in liquid nitrogen vapor and stored at −80°C. Alternatively, for IBA-1 immunohistochemistry, mice were transcardially perfused with 4% paraformaldehyde in 1% Osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4), pH 7.4, and DNA damage may be late necrotic, late apoptotic, or a mixed cell death phenotype initiated by concomitant activation of necrotic and apoptotic mechanisms.\textsuperscript{6,7} Acute ischemic and TBI induces plasmalemma permeability to propidium iodide (PI) through mechanisms that remain unknown.\textsuperscript{6–8} We and others have used PI and the green fluorescent dye YOYO-1 iodide in vivo to label injured cells after ischemic and TBI.\textsuperscript{6–8} These studies have demonstrated apoptotic-like (PI/terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling [TUNEL]+) and necrotic-like (PI+/TUNEL−) cell death phenotypes. Here, we used in vivo PI and YOYO-1 to identify cells with plasmalemma permeability and follow their spatial and temporal course after collagenase-induced ICH in mice. Using ex vivo TUNEL and caspase histochemistry, and ultrastructural analysis of injured cells, we tested the hypothesis that plasmalemma permeability is associated with markers of cell death and that a necrotic-like cell death phenotype is associated with ICH.

**Preparation of Brain Tissue and Detection of PI+ and YOYO-1+ Cells**

We detected cells with altered plasma membrane integrity using YOYO-1 and PI as previously described.\textsuperscript{8} YOYO-1 (Molecular Probes; 1 μg/g in 150 μL phosphate-buffered saline [PBS]) is a cell membrane impermeant cyanine nucleic acid stain with excitation–emission maxima at 491/509 nm that has been used to document plasmalemma permeability and resealing in vivo.\textsuperscript{9} YOYO-1 was administered intra-venously. In experiments designed to assess for spontaneous plasmalemma resealing, PI (1 μg/g mouse in 150 μL PBS; Sigma) was administered intravenously as well. For all other experiments, PI was administered by the intraperitoneal route. Mice were deeply anesthetized with isoflurane and euthanized at various times after ICH. Brains were removed and frozen in liquid nitrogen vapor and stored at −80°C. Brains sections (12 μm thick) were cut on a cryostat 150 to 200 μm apart from anterior to posterior lesion, placed on poly-L-lysine-coated glass slides, and stored at −80°C. Alternatively, for IBA-1 immunohistochemistry, mice were transcardially perfused with 4% paraformaldehyde and brains postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose, and cut on a cryostat as described previously.

Brain sections were photographed and analyzed by fluorescence microscopy (Nikon Eclipse T300 fluorescence microscope, Tokyo, Japan) using excitation and emission wavelengths for PI 568 and 585 nm and YOYO-1 490 nm and 520 nm, respectively. Operational definitions of cell death phenotypes were based on criteria from previous studies.\textsuperscript{6–9,14} The pure necrosis phenotype is defined as PI+/TUNEL−; pure apoptosis is defined as PI−/TUNEL+; and the mixed cell death phenotype is defined as PI+/TUNEL+. To show that PI and YOYO-1 label nucleated cells in the ICH model, some brain sections were counterstained with Hoechst 33342 (Thermo Scientific, Rockford, IL; 1:1000) for 15 seconds.

**Detection of In Situ Caspase Activity and TUNEL**

Carboxyfluorescein analogs of aspartylglutamylvalylaspartic acid fluoromethyl ketone (FAM-DEVDFMK, caspase-3 inhibitor), leucylglutamylthreonylglutamic acid fluoromethyl ketone (FAM-LETD-FMK, caspase-8 inhibitor), and leucylglutamylaspartic acid fluoromethyl ketone (FAM-LEHD-FMK, caspase-9 inhibitor) were reconstituted according to the instructions from the manufacturer (BioCarta), diluted 1:75 000 in PBS (pH 7.2), and reacted for 60 seconds with unfixed, frozen brain sections at room temperature. TUNEL was performed as previously described.\textsuperscript{12}

**Immunohistochemistry**

For immunohistochemical detection of high mobility group box protein-1 (HMGB1) or glial cell types labeled with PI or YOYO-1, paraformaldehyde fixed/perfused brain sections were washed in PBS and blocked in 2% normal goat serum and reacted with antimouse glial fibrillary acidic protein conjugated to Cy3 (1:300; Sigma), rabbit anti-HMGB1 (Abcam; Cambridge, MA) or antineuronal nuclei (1:300; Chemicon) conjugated to Cy3 (Amersham Biosciences). Antirabbit fluorescein isothiocyanate conjugated secondary antibodies (1:300; Jackson Immunologic labs) were used to detect IBA-1. Sections were washed, mounted and covered with 100% ethanol, covederslipped, and photographed using fluorescence microscopy.

**Quantitation of Cell Count Data**

YOYO-1+ and PI+ cells in CD1 mice and RIPK3 knockout and C57Bl/6 wild-type mice were counted in ×200 fields (1100 μm×1100 μm) randomly chosen from 4 to 5 brain sections separated at least 150 μm located within the lesion produced by ICH. Fields from the periphery (n=8) and core (n=8) of the hemorrhagic lesion (defined anatomically as the inner half of the lesion) were counted from the brain sections analyzed. For PI+ TUNEL+ and PI/caspase substrate cell counts, 6 fields per mouse were assessed from equal numbers of core and peripheral regions adjacent to the ICH or from 6 peripheral or 6 core regions for studies of core versus peripheral hemorrhagic regions. For quantitation of spontaneous resealing, all YOYO-1+ cells and YOYO-1+/PI− cells were counted in 8 to 10 ×200 fields randomly chosen in peripheral and core brain regions among 5 to 6 brain sections per mouse. Resealed cells were defined as YOYO-1+/PI− and nonresealed cells as YOYO-1+/PI+. Data for each mouse were the averages of the cell counts in the ×200 fields.

**Electron Microscopy**

Electron microscopy was performed as previously described with minor modifications.\textsuperscript{8} Mice were deeply anesthetized and transcardially perfused with 2% paraformaldehyde/2% glutaraldehyde (electron microscopy grade; Sigma) in PBS, pH 7.4. Brains were postfixed overnight and cryoprotected in sucrose and 4-mm brain sections were cut from the ICH region. For electron microscopy, brain tissue was cut on a microtome and sections were postfixed with a mixture of 1% Osmium tetroxide+1.5% potassium ferrocyanide (KFeCN6) for 30 minutes, washed in water, and stained in 1% aqueous uranyl acetate for 30 minutes followed by dehydration in alcohol and then infiltrated and embedded in TAAB Epon (Marivac Canada). Ultrathin sections (60–80 nm) were cut on a Reichert UltraCut-S microtome, stained with 0.2% lead citrate, and examined...
in a Tecnai G² Spirit BioTWIN Transmission electron microscope. Images were taken with a 2k AMT CCD camera.

**Statistical Analyses**

Data are mean±SEM. Cell count data were evaluated by analysis of variance followed by Bonferroni test. PI^+^ cells in RIPK3 knockout and wild-type mice were analyzed by rank sum. Motor data were analyzed by repeated-measures analysis of variance (group*time).

For all comparisons, \( P < 0.05 \) was regarded as significant.

**Results**

Intrastriatal collagenase injection produced ICH in ipsilateral striatum within 1 hour with blood–brain barrier damage and motor deficits (Supplemental Figure I). No injury was produced in contralateral hemispheres and injured cells (PI^+^, TUNEL^+^, DEVD+) were only detected in hemorrhagic brain regions in the ipsilateral hemisphere. Sham injury (injection of PBS instead of collagenase) produced no ICH lesion and only a few PI^+^ cells along the needle tract (data not shown).

Figure 1A shows representative photomicrographs of PI^+^ cells after collagenase-induced ICH. PI^+^ cells were detected as early as 1 hour and peaked between 48 and 72 hours (Figure 1B; \( P < 0.001 \) analysis of variance for all time points). At early time points, PI^+^ cells were mainly distributed within the periphery of the hemorrhagic lesion but at 24 to 48 hours, many PI^+^ cells were also present in core regions. PI^+^ cells exhibited at least 2 general labeling patterns with round, smooth nuclei consistent with necrotic-like morphology and condensed, fragmented nuclear labeling consistent with apoptosis, and all PI^+^ cells colocalized with Hoechst labeling, although not all Hoechst^+^ cells were PI^+^ (Figure 1B).

To determine identity of cell types sensitive to plasmalemma permeability, we administered YOYO-1 intravenously after ICH and subjected frozen brain tissue sections to ex vivo immunohistochemistry. No YOYO-1+/IBA-1^+^ microglia (Figure 2A) or YOYO-1+/glial fibrillary acidic protein^+^ astrocytes (Figure 2B) were detected at 6 hours after ICH; however, YOYO-1+/neuronal nuclei^+^ neurons frequently were detected (Figure 2C). By 24 hours, a few IBA-1^+^ microglia and a large number of spherical IBA-1^+^ cells that lacked the characteristic processes of brain microglia colocalized with YOYO-1 within hemorrhagic brain regions (Figure 2D). YOYO-1+/glial fibrillary acidic protein^+^ cells were not detected at 24 hours and YOYO-1+/neuronal nuclei^+^ neurons were robustly detected. At 48 and 72 hours, no glial fibrillary acidic protein^+^ cells had plasmalemma damage and almost all glial fibrillary acidic protein^+^ and IBA-1^+^ cells were strikingly localized around the outside the hemorrhagic lesion, a pattern that persisted for up to 14 days (Supplemental Figure II). Thus, within the first 24 to 72 hours, plasmalemma permeability was mainly detected in neurons and IBA-1^+^ cells with morphological features of monocytes, within the hemorrhagic lesion, whereas microglia and astrocytes (which localized mainly around the periphery of the lesion) were resistant at all times examined.

To determine relationships between plasmalemma damage and putative modes of cell death, we used in vivo PI labeling and ex vivo caspase substrate or TUNEL histochemistry. Figure 3

![Figure 1](http://stroke.ahajournals.org/)
Figure 2. Identification of permeable cell types after intracerebral hemorrhage (ICH). A–C, Detection of cell types with plasmalemma permeability to YOYO-1 iodide at 6 hours after ICH. A, Representative photomicrographs of YOYO-1+ cells (a), IBA-1+ (b), and overlay (c) showing no colocalization. B, Representative photomicrographs of YOYO-1+ cells (d), GFAP+ cells (e), and overlay (f) showing no colocalization. C, Representative photomicrographs of NeuN+ neurons (g) that colocalized with YOYO-1 (h) at 6 hours after ICH (i, overlay) suggesting that neurons are particularly sensitive to plasmalemma damage early after ICH. D, Detection of cell types with plasmalemma permeability to YOYO-1 at 24 hours after ICH. By 24 hours, YOYO-1+ cells (j) colocalized with IBA-1+ cells with morphological features of microglia (k) as shown in the overlay (l). YOYO-1+ cells (m) also colocalized with IBA-1+ cells with the morphological appearance of macrophages (n) at 24 hours (o, overlay). Scale bar, 10 μm for each panel. GFAP indicates glial fibrillary acidic protein; NeuN, neuronal nuclei.
shows typical examples of PI+, caspase substrate+, and TUNEL+ cells and their distribution at 48 hours after ICH, the peak time of plasmalemma permeability. Fluorescent-labeled DEVD-FMK detected activated caspases because positive staining was not observed using vehicle alone and was inhibited by pretreatment with nonlabeled ZVAD, a pan-caspase inhibitor (not shown). At 6 to 48 hours after ICH, PI+ cells often colocalized with caspase substrates (DEVD, LETD, and LEHD) or TUNEL (Figure 3A; LEHD and LEHD not shown). At 24 hours, PI/TUNEL cell count data in peripheral fields were PI+/TUNEL−, 40±8; PI−/TUNEL+7±1; PI+/TUNEL+52±7 cells/×200 field. At 48 hours, PI+/DEVD− and PI+/TUNEL− cells accounted for over half of all PI+ cells (Figure 3B), whereas PI−/DEVD+ and PI−/TUNEL+ cells were <5% each. At 48 hours, PI/TUNEL cell count data were remarkably similar in peripheral (PI+/TUNEL−, 59±6; PI−/ TUNEL+2±0; PI+/TUNEL+42±5 cells/×200 field) and core brain regions (PI+/TUNEL−45±3, PI−/TUNEL+2±1, PI+/TUNEL+39±4 cells/×200 field). At 24 and 48 hours, a pure necrotic death phenotype (PI+/TUNEL−) accounted for >40% and 50%, respectively, of PI+ cells.

To confirm the presence of necrotic cell death, we performed ultrastructural analyses of injured cells randomly selected from hemorrhagic brain at 24 hours after ICH, biochemical analysis of HMGB1 release, and quantitation of PI+ cells at 24 hours after ICH in RIPK3 knockout versus wild-type mice. Cells with morphological features of necrosis such as mitochondrial swelling, nuclear membrane disintegration, and characteristic nuclear karyorrhexis were observed in addition to classical apoptotic bodies associated with apoptotic cell death (Figure 4 and apoptotic bodies not shown). HMGB1 release was robust in cells within the hemorrhagic zone, and HMGB1 was strikingly absent from almost all YOYO-1+ cells at 6 hours (Supplemental Figure III). At 24 hours, no HMGB1 immunostaining was observed in the hemorrhage zone (not shown), and RIPK3 knockout mice had over 50% reduction in PI+ cells in hemorrhagic peripheral and core regions (Figure 5).

To assess survival time of injured cells, and whether permeable cells may repair their membrane damage after ICH, we administered YOYO-1 at 24 or 48 hours followed by PI at 48 or 72 hours, respectively, to label YOYO-1+ cells with persistent plasmalemma damage. Brain sections were analyzed for the presence of YOYO-1+ and PI+ cells. Most YOYO-1+ cells labeled at 24 hours (94.4%±2.0%) or 48 hours (98.7%±0.7%) also colabeled with PI at 48 or 72 hours, respectively, indicating only a minor prevalence of spontaneous membrane resealing (Figure 6 and 48 to 72 hours data not shown).

**Discussion**

To our knowledge, this is the first description of plasmalemma permeability and necrotic cell death in an ICH model. As previously reported in adult stroke and brain trauma models,6–8 in vivo PI and YOYO-1 labeling appears to be specific for cells with loss of membrane integrity in ICH, because PI colocalized with only a subset of Hoechst+ cells, and PI labeling was not observed in uninjured hemispheres or in...
PBS-injected mice (except along the needle tract). Moreover, PI labeling followed a discrete temporal course that peaked at 48 to 72 hours, and plasmalemma damage occurred in specific cell types (neurons and IBA1+ cells but not astrocytes) within the first 24 to 72 hours. PI and YOYO-1 labeling only occurred in nucleated cells as demonstrated by Hoechst colabeling, ruling out artificial labeling of erythrocytes, which lack a nucleus. These data suggest the validity of using in vivo PI and YOYO-1 to detect plasmalemma permeability in injured parenchymal cells as we and others have reported in other acute brain and renal injury models.6–9

The time course of plasmalemma damage in ICH is similar to that reported in murine ischemic stroke in which PI cells were first observed approximately 6 hours after middle cerebral artery occlusion and reperfusion.6 It is unlikely that direct membrane disruption by collagenase is a primary mechanism of plasmalemma damage because the peak of PI cells would be predicted to occur much earlier in this case. Robust blood–brain barrier damage is present from 1 to at least 48 hours in our collagenase ICH model (24-hour data shown in Supplemental Figure I). Thus, PI (640 Da) can easily gain entry to parenchymal cells through the damaged blood–brain barrier. The time course of plasmalemma permeability after experimental cerebral contusion, in which ICH contributes to tissue damage, was considerably faster with PI cells peaking at 1 to 6 hours after controlled cortical impact in vulnerable brain regions.8 Because we did not assess 4 to 6 days, it is possible that plasmalemma permeability might peak later than 72 hours after ICH. The differences in kinetics of plasmalemma damage in cerebral contusion (early) and collagenase-induced ICH (delayed) are not explained by delayed bleeding in ICH (collagenase injection produces a large hematoma by 1 hour) or by differences in amounts of fluorophore administration in controlled cortical impact8 and the current study.

Cell types vulnerable to plasmalemma damage within the first 24 hours were similar to those reported in neonatal and adult ischemic stroke models6,15 and in TBI.8 Neurons and rounded IBA1+ cells lacking processes were most vulnerable. The lack of plasmalemma damage in glial cells (almost all of which were detected outside the ICH lesion from 6 to 72 hours) and the low numbers of PI+ cells remaining at 7 days suggests that astrocytes and most microglia are relatively resistant to plasmalemma damage after collagenase ICH. Astrocyte resistance to plasmalemma damage was also reported in neonatal rat hypoxic–ischemic brain injury16 and in focal stroke in adult mice in which astrocytes exhibited delayed loss of plasmalemma and mitochondrial integrity and reduced ultrastructural damage compared with neurons.17 Neurons are highly vulnerable to early plasmalemma damage after stroke and TBI8–8 and ICH as suggested by the current study.

We found robust plasmalemma damage in IBA1+ cells within the ICH lesion at 24 hours. IBA-1+ cells could represent

Figure 4. Ultrastructural analyses of injured cells at 24 hours after intracerebral hemorrhage. a, Normal nucleus (N) and nuclear membrane (NM) of an intact neuron in the contralateral (uninjured) striatum showing intact cellular architecture. b, Swollen mitochondria (M), nuclear karyorrhexis, and dissolution of the nuclear membrane with mitochondria entry into the nucleus in a cell with necrotic morphology. c–d, Loss of nuclear membrane integrity consistent with necrosis (arrows indicate nuclear membrane). Scale bars: (a) 2 μm, (b) 3 μm, (c–d) 300 nm.

Figure 5. Decreased PI+ cells at 24 hours after intracerebral hemorrhage in mice deficient in receptor interacting protein kinase 3 (RIPK3 knockout). Upper panels, Representative photomicrographs depicting PI+ cells in hemorrhagic brain 24 hours after ICH in wild-type (WT) and RIPK3 knockout (KO) mice. Lower panel, RIPK3 KO mice had significantly decreased PI+ cells in peripheral, core, and combined peripheral and core ×200 fields at 24 hours after ICH. *P=0.008 vs WT. PI indicates propidium iodide; ICH, intracerebral hemorrhage.
activated microglia with loss of processes or bloodborne monocytes. Making this distinction is an important goal of future studies, because bloodborne monocytes play a protective role in preserving neurons and improving functional outcome after spinal cord and ischemic retinal injury, presumably by producing a milieu that limits the overall inflammatory response and subsequent neuronal death.18,19 Monocytes and microglia may facilitate resolution of ICH by phagocytosing erythrocytes and their toxic intracellular constituents, which drive neurodegeneration.20 On the other hand, inhibition of microglial transmigration and function reduced brain edema and inflammation early after experimental ICH.21 Thus, monocytes and microglia may exert beneficial or detrimental effects on outcome after ICH depending in part on timing after injury.

In the majority of injured cells, plasmalemma damage occurred before detectable activation of caspases and DNA damage (TUNEL), suggesting a significant contribution of necrosis to cell death after ICH.2 TUNEL and caspase substrate analyses alone cannot be used to rule in or out a specific cell death mode in central nervous system injury because TUNEL may label necrotic as well as apoptotic cells9 and caspases may be activated during necrotic cell death.10,11 The temporal sequence of plasmalemma damage followed by DNA damage and/or caspase activation is suggestive of necrosis in collagenase-induced ICH. This tentative conclusion is supported by ultrastructural analyses of a limited number of cells showing patterns of chromatin condensation, dissolution of nuclear and plasma membranes, and mitochondrial swelling characteristic of necrosis (Figure 4). We and others have demonstrated heterogeneous cell death phenotypes, including necrosis, in acute central nervous system injury paradigms using in vivo PI labeling7,8,14 and necrosis has also been reported in human brain samples obtained at autopsy from patients with ICH.22

HMGB1 is a nonhistone nuclear protein and prototype member of the alarmin family of damage-associated molecular proteins. HMGB1 is released from dead or dying cells when membrane integrity is compromised and interacts with specific receptors on immune cells as well as with other damage-associated molecular proteins such as liposaccharide, DNA, and cytokines to induce an inflammatory response.23 Thus, HMGB1 released from necrotic cells drives immune responses and promotes inflammation. In the current study, HMGB1 release was associated with loss of plasmalemma integrity in YOYO-1+ cells at 6 hours after ICH. Because HMGB1 release is associated with necrosis and subsequent inflammation after ischemic stroke,24,25 its release from YOYO-1+ cells in hemorrhagic brain suggests that HMGB1 may link necrotic cell death with the ensuing inflammatory response after ICH.

Using YOYO-1 pulse labeling, we found that >90% of YOYO-1+ cells labeled at 24 or 48 hours remained permeable to PI for at least 24 hours, suggesting limited spontaneous membrane repair. In contrast, membrane permeability in injured neurons may be reversible in mild fluid percussion and more severe controlled cortical impact TBI models.8,26 The current data suggest that plasmalemma damage may portend fatal outcome after ICH. This possibility is also supported by the observation that a significant percentage of PI+ cells colabeled with TUNEL and activated caspases, few PI+ cells were detected in hemorrhagic brain at 7 days (Figure 2), and by prior studies showing significant neuronal loss after ICH in rodents.27 Our data suggest at least a 72-hour window for rescue of significant numbers of PI+ cells after ICH, similar to that reported in controlled cortical impact.8 Necroptosis, a form of programmed necrosis mediated by RIPK1,28 is implicated in experimental stroke29 and TBI14 and in hemin-induced cell death in vitro.30 Thus, necrostatins (specific inhibitors of RIPK1)30 may have use in preventing ICH-induced cell death. In the current study, RIPK3 knockout mice had approximately 50% reduction in PI+ cells at 24 hours compared with wild-type, providing powerful proof of principle that plasmalemma permeability can be manipulated and that RIPK3 could mediate programmed necrosis (necroptosis) after ICH.31

Other mechanisms of collagenase-induced ICH that may lead to plasmalemma damage include hemoglobin- and iron-induced oxidative stress, which may directly induce membrane damage and permeability.32 Interestingly, hemoglobin-induced cell death in primary rat cortical neurons involved caspase activation, but membrane damage and cell death were inhibited by an antioxidant and not by caspase inhibitors.33 These data lend support to the idea that necrosis and caspase
mechanisms each contribute to ICH-induced cell death. Our findings also suggest that pharmacological membrane resealing may be an effective strategy to rescue injured cells or prevent bystander injury from release of damage-associated molecular pathogens from permeabilized cells. Intracerebroventricular administration of Poloxamer P188, a prototype membrane-sealing agent, reduced acute cell death after ICH in rats, but membrane resealing by P188 was not reported in that study. Data from the current study provide a framework for novel therapeutic strategies to limit cell death and improve outcome after ICH in patients, for whom no specific therapy is currently available.

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Disclosures
None.

References
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Supplemental Table 1: Summary of Experiments

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<tr>
<td>PI+ cell survival, resealing</td>
<td>24-48 and 48-72 h</td>
<td>4-5/group</td>
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<sup>a</sup>: For quantitation of labeled cells at 24 h (n = 8) and 48 h (n = 4)

PI+, Propidium iodide-positive; IHC, Immunohistochemistry; GFAP, glial fibrillary acidic protein; IBA-1, ionized calcium binding adaptor molecule 1; NeuN, neuronal nuclear marker; TUNEL, terminal deoxynucleotidyl transferase mediated nick end labeling.
Supplemental Figure 1. (A) Intracerebral hemorrhage produced by collagenase injection. (B) Blood brain barrier damage assessed by Evans blue fluorescence in hemorrhagic brain. C, core; P, periphery; Nl, normal brain. (C) Motor deficits assessed by the wire grip score after collagenase intracerebral hemorrhage. p < 0.01 for group.
Supplemental Figure 2: Localization of IBA-1+ cells (microglia/monocytes) and GFAP+ cells (astrocytes) after intracerebral hemorrhage. (a) IBA-1+ microglia and (b) GFAP+ astrocytes surround the periphery of the hemorrhagic lesion (L) as early as 6 h. At 72 h IBA-1+ cells (c) and GFAP+ cells (d) reside exclusively around the outer limits of the hemorrhagic lesion. GFAP+ Astrocytes surround the lesion at 7 (e), and some are observed entering the lesion core at 14 d (f). Scale bars: a, b 10 um; c, d 40 um; e, 5 um; f, 100 um
Supplemental Figure 3: HMGB1 is released after intracerebral hemorrhage. Mice were subjected to collagenase ICH and cells with plasmalemma damage were labeled with YOYO-1 (green) at 5-6 h. Mice were killed at 6 h and assessed for HMGB1 (red) release by immunostaining. (a-d) Lack of HMGB1 immunostaining in ipsilateral compared to contralateral striatum (a, c) is not fully explained by loss of cell density as evidenced by Hoechst staining (blue) (b, d). YOYO-1+ cells rarely colocalized with HMGB1, indicating release from necrotic cells (arrows in e, f). Scale bars, a-d, 100 um; e-f, 40 um.