Ischemic Stroke Brain Sends Indirect Cell Death Signals to the Heart

Hiroto Ishikawa, MD, PhD; Naoki Tajiri, PT, PhD; Julie Vasconcellos, BS; Yuji Kaneko, PhD; Osamu Mimura, MD, PhD; Mari Dezawa, MD, PhD; Cesar V. Borlongan, PhD

Background and Purpose—Ischemic stroke is a leading cause of mortality and morbidity in the world and may be associated with cardiac myocyte vulnerability. However, it remains uncertain how an ischemic brain contributes to cardiac alternations. Here, we used experimental stroke models to reveal the pathological effects of the ischemic brain on the heart.

Methods—For the in vitro study, primary rat neuronal cells were subjected to 90-minute oxygen–glucose deprivation (OGD). Two hours after OGD, the supernatant was collected and cryopreserved until further biological assays. Primary rat cardiac myocytes were exposed to ischemic–reperfusion injury and subsequently to the supernatant derived from either the OGD or non–OGD-exposed primary rat neuronal cells for 2, 6, 24, or 48 hours. Thereafter, we measured cell viability and mitochondrial activity in rat cardiac myocytes. For the in vivo study, we subjected adult rats to transient middle cerebral artery occlusion, and their brains and hearts were harvested for immunohistochemical analyses at 3 months later.

Results—The supernatant from the OGD, but not the non–OGD-exposed primary rat neuronal cells, caused significant reduction in cell viability and mitochondrial activity in rat cardiac myocytes. Ischemic stroke animals displayed phenotypic expression of necrosis, apoptosis, and autophagy in their hearts, which paralleled the detection of these same cell death markers in their brains.

Conclusions—Ischemic stroke was accompanied by cardiac myocyte death, indicating a close pathological link between brain and heart. These results suggest a vigilant assessment of the heart condition in stroke patients, likely requiring the need to treat systemic cardiac symptoms after an ischemic brain episode. (Stroke. 2013;44:3175-3182.)

Key Words: apoptosis ◼ autophagy ◼ brain ischemia ◼ myocytes, cardiac ◼ necrosis

Ischemic heart and cerebrovascular disease are the first and second leading causes of death in the world. The United States spends $206.8 billion for cardiac disease and $53.9 billion for ischemic stroke, including expenses for healthcare services, medications, and loss of productivity. The risk factors of cardiovascular or cerebrovascular diseases involve environmental and genetic entities, most notably high blood cholesterol levels, high blood pressure, diabetes mellitus, obesity, and history of cardiovascular diseases.

In the clinic, most deaths following ischemic stroke are a direct result of neurological damage. Second to neurologically linked fatalities are deaths caused by cardiac failure. Of note, 2% to 6% of deaths are of cardiac origins in the 3 months following ischemic stroke. Although this percentage of cardiac cell death declines after the early stage, data show that those who have ischemic stroke are more likely to present with cardiac death than age-matched nonstroke victims, with the former exhibiting abnormal rhythms in ECG, as well as large changes in cardiac enzyme and plasma catecholamines. Cardiac enzymes are most closely associated with elevated troponin and creatine phosphokinase levels, which become evident when cardiac cells are under stress and dying. Increased catecholamine levels are associated with high blood pressure and tachycardia and are present also during stress.

For years, the correlation between cerebrovascular incidents had been ascribed primarily to overlapping risk factors. However, damage to the insular cortex has been shown to produce a high incidence of cardiac death compared with other brain regions, in that up to 88% of patients with insular cortical stroke present with cardiac symptoms in the following weeks after stroke. The role of the insular cortex in sympathetic and parasympathetic nervous system control has been implicated in the observed cardiac alterations. Cardiac autonomic tone is controlled by the insular cortex, and with the loss of this regulatory function after stroke, cardiac compromise is more likely to ensue. Disagreement remains on whether a specific region of the insular cortex or as a whole differentially causes cardiac myocyte death. Indeed, insular cortex damage is rarely seen without injury to other structures in the brain when middle cerebral artery occlusion (MCAo) is induced. Therefore,
the direct involvement of the insular cortex in cardiovascular disorders following stroke is still not well established.

In the present study, we explored the relationship between neuronal cell death and cardiac myocyte compromise using both in vitro and in vivo stroke models. For the in vitro study, we used the oxygen–glucose deprivation (OGD) condition in primary rat neuronal cells (PRNCs) and used the supernatant to explore cellular changes in rat cardiac myocytes (RCMs) following ischemic–reperfusion (I/R) injury. For the in vivo study, we induced transient MCAo in adult rats and performed immunohistochemical analyses on the brains and hearts of stroke rats to reveal different cell death markers. We hypothesized that the ischemic brain compromises cardiac myocytes through secretion of cell death factors.

**Material and Methods**

**In Vitro Study**

**Cell Culture**

PRNCs were obtained from BrainBits. As per the manufacturer’s protocol, cells (4 x 10^5 cells/well) were suspended in 200 μL supplemented neurobasal medium containing 2 mM L-glutamine and 2% B27 in the absence of antibiotics and grown in Poly-L-Lysine-coated 96-well (BD Biosciences) at 37°C in humidified atmosphere containing 5% carbon dioxide. PRNCs were grown until reaching ≥70% cell confluence. We immunocytochemically determined that PRNCs expressed the vesicular glutamate transporter-1. Thereafter, PRNCs were subjected to OGD condition as described below.

RCMs were obtained from Lonza. As per the manufacturer’s protocol, cells (1.5 x 10^5 cells/well) were suspended in 200 μL supplemented rat cardiac myocyte basal medium (Lonza) containing rat cardiac growth medium SingleQuots and incubated for 4 hours in nitrocellulose-coated 96-well at 37°C in humidified atmosphere containing 5% carbon dioxide. At day 1 in vitro (DIV1), 80% of medium was removed from the cells and treated with prewarmed rat cardiac myocyte basal medium containing 200 μmol/L BrdU for 4 hours. At DIV3, 50% of the medium was removed from the cells and changed to a fresh rat cardiac myocyte basal medium containing 200 μmol/L BrdU. Thereafter, at DIV5, RCMs were subjected to further experiments as described below.

**OGD in PRNCs**

At DIV5, PRNCs were subjected to 90 minutes OGD condition as described previously. Briefly, the neuronal cells were initially exposed to OGD medium (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1 mM NaH2PO4, 26.2 mM NaHCO3, 0.01 mM glucose, 1.8 mM CaCl, pH 7.4), placed in an anaerobic chamber (PlasLabs) containing 95% nitrogen and 5% carbon dioxide for 15 minutes at 37°C, and finally the chamber was sealed and incubated for 90 minutes at 37°C (hypoxic-ischemic condition). After the hypoxic-ischemic condition, the culture was reintroduced to the normoxic condition containing 5% carbon dioxide for 2 hours resembling a reperfusion. Thereafter, the supernatant was collected from the culture and subjected to the following experiments described below.

**I/R Injury in RCMs**

At DIV5, RCMs were subjected to I/R injury using OGD condition. After I/R injury, RCMs were incubated with DMEM or the supernatant from PRNCs in normoxic condition. To determine the exposure period that was most toxic, we preset the reperfusion at different time points as follows: Group A: 2 hours incubation with DMEM without I/R injury as a control; Group B: 2 hours reperfusion with DMEM; Group C: 6 hours reperfusion with DMEM; Group D: 24 hours reperfusion with DMEM; Group E: 48 hours reperfusion with DMEM; Group F: 2 hours incubation with the supernatant from primary rat neuronal cells (PRNCs) without I/R injury; Group G: 2 hours reperfusion with the supernatant from PRNCs; Group H: 6 hours reperfusion with the supernatant; Group I: 24 hours reperfusion with the supernatant; and Group J: 48 hours reperfusion with the supernatant, which are summarized in the Table. Thereafter, RCMs were subjected to further analyses.

**Immunocytochemistry**

To confirm that the OGD condition produced neuronal death in PRNCs, immunohistochemistry was performed. After OGD condition, PRNCs were rinsed twice in PBS and fixed in 4% paraformaldehyde fixative for 5 minutes at room temperature. After the fixation, cells were rinsed in PBS and PBS containing TWEEN-20 (ab64247; abcam) (PBST) twice each. They were blocked by 5% goat serum (50062Z; Invitrogen) for 30 minutes at room temperature. They were incubated with first antibodies; an anti–tumor necrosis factor (TNF)-α antibody (mouse monoclonal [ab1793]; abcam) for necrosis, anti-active Caspase 3 antibody (rabbit polyclonal [ab13847]; abcam) for apoptosis, anti–Fas Ligand antibody (rabbit polyclonal [ab15285]; abcam) for apoptosis, and anti-MAP1LC3A antibody (rabbit polyclonal [ab64123]; abcam) for autophagy, with 5% serum and 0.2% triton X-100 (Fischer Scientific, Pittsburgh, PA) for 1 hour at room temperature. After primary antibody incubation, they were rinsed twice in PBST again. Next, they were incubated in secondary antibodies; goat anti–mouse IgG Alexa Fluor 488 conjugate (Invitrogen) and goat anti–rabbit IgG Alexa Fluor 594 conjugate (Invitrogen) for 1 hour at room temperature. After incubation with secondary antibodies, they were rinsed in PBST and PBS twice each. Thereafter, they

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**Table. Summary of Experimental Groups and Immunohistochemical Results of Cell Death Markers in the In Vitro Study**

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury</td>
<td>No</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
<td>I/R</td>
</tr>
<tr>
<td>Medium</td>
<td>DMEM</td>
<td>DMEM</td>
<td>DMEM</td>
<td>DMEM</td>
<td>DMEM</td>
<td>Supernatant from PRNCs</td>
<td>Supernatant from PRNCs</td>
<td>Supernatant from PRNCs</td>
<td>Supernatant from PRNCs</td>
<td>Supernatant from PRNCs</td>
</tr>
<tr>
<td>Reperfusion time (h)</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>24</td>
<td>48</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>TNF-α</td>
<td>11.0±7.0%</td>
<td>27.5±13.5%</td>
<td>35.8±22.6%</td>
<td>32.8±14.0%</td>
<td>45.2±19.3%</td>
<td>15.3±14.2%</td>
<td>36.4±19.9%</td>
<td>38.6±24.9%</td>
<td>51.4±31.7%</td>
<td>60.2±30.1%</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>27.9±12.3%</td>
<td>32.6±17.1%</td>
<td>53.1±11.4%</td>
<td>59.8±16.7%</td>
<td>73.5±12.0%</td>
<td>27.5±11.7%</td>
<td>34.7±10.1%</td>
<td>69.8±10.4%</td>
<td>71.3±8.4%</td>
<td>89.4±6.6%</td>
</tr>
<tr>
<td>Fas Ligand</td>
<td>35.4±9.0%</td>
<td>44.3±17.6%</td>
<td>61.0±12.9%</td>
<td>59.6±15.1%</td>
<td>76.1±16.5%</td>
<td>42.4±19.9%</td>
<td>55.6±18.7%</td>
<td>59.4±15.6%</td>
<td>73.0±17.6%</td>
<td>87.2±7.7%</td>
</tr>
<tr>
<td>MAP1LC3A</td>
<td>30.7±8.7%</td>
<td>27.2±8.3%</td>
<td>51.7±15.2%</td>
<td>86.5±11.2%</td>
<td>82.9±10.4%</td>
<td>42.0±18.6%</td>
<td>59.9±31.6%</td>
<td>73.3±13.4%</td>
<td>85.5±12.4%</td>
<td>95.2±3.3%</td>
</tr>
</tbody>
</table>

* Rat cardiac myocytes were cultured for 5 days and then subjected to ischemic–reperfusion (I/R) injury. Group A: 2 hours incubation with DMEM without I/R injury as a control, Group B: 2 hours reperfusion with DMEM, Group C: 6 hours reperfusion with DMEM, Group D: 24 hours reperfusion with DMEM, Group E: 48 hours reperfusion with DMEM, Group F: 2 hours incubation with the supernatant from primary rat neuronal cells (PRNCs) without I/R injury, Group G: 2 hours reperfusion with the supernatant from PRNCs, Group H: 6 hours reperfusion with the supernatant, Group I: 24 hours reperfusion with the supernatant, and Group J: 48 hours reperfusion with the supernatant. TNF-α indicates tumor necrosis factor-α.
were incubated with Hoechst 33342 (Sigma) for 10 minutes at room temperature for nucleus staining and rinsed twice in PBS.

Next, to reveal the toxicity of the supernatant harvested from PRNCs on RCMs, RCMs were subjected to I/R injury (as described above), then subsequently exposed to the supernatant from OGD- or non-OGD-exposed PRNCs and processed for immunocytochemical analyses of cell death.

**Cell Viability Assay**

Measurement of RCMs viability was performed using fluorescent live/dead cell assay. A 2-color fluorescence cell viability assay was performed using calcein-AM (Invitrogen) that revealed an intense uniform green fluorescence in live cells and ethidium homodimer (EthD-1, Invitrogen) characterized by red fluorescence bound to the nuclei of damaged cells. Following IR injury, the cells were incubated with 2 μmol/L calcein-AM and 4 μmol/L EthD-1 for 45 minutes at room temperature in darkness. After washing with PBS, the green fluorescence of the live cells was measured by the Gemini EX fluorescence plate reader (Molecular Device), with excitation at 485 nm and emission at 538 nm. To calibrate the cell viability precisely, the values were standardized from florescence intensity.

**Mitochondrial Activity Assay**

Changes in 3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyltetrazolium bromide (MTT) as revealed by cellular dehydrogenases has been used as a measure of mitochondrial activity. MTT assay was performed according to the manufacturer’s protocol (Roche) as previously described. After I/R injury, the RCMs were incubated with 0.5 mg/mL MTT 37°C and 5% CO2 and incubated with lysis buffer overnight in a humidified atmosphere at 37°C and 5% CO2. The optical density of the supernatant harvested from OGD- or non-OGD-exposed PRNCs on RCMs, RCMs were subjected to I/R injury (as described above), then subsequently exposed to the supernatant from OGD- or non-OGD-exposed PRNCs and processed for immunocytochemical analyses of cell death.

**In Vivo Study**

**Stroke Surgery**

Stroke surgery was performed using the transient MCAo model as previously described. Animals were anesthetized with 1% to 2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask. Body temperature was maintained at 37°C ±0.3°C during the surgical procedures. A midline skin incision was made in the neck with subsequent exploration of the right common carotid artery, the external carotid artery, and internal carotid artery. A 4-0 monofilament nylon suture (27–28 mm) was advanced from the common carotid artery bifurcation until it blocked the origin of the MCA. Animals were allowed to recover from anesthesia during MCAo. After 60 minutes of transient MCAo, animals were reanesthetized and reperfused by withdrawal of the nylon thread. The animals were then closed and allowed to recover from anesthesia. We have previously standardized the MCAo model, with stroke animals showing 280% reduction in regional cerebral blood flow during the occlusion period as determined by laser Doppler (Perimed). To further ensure similar degree of stroke insults, physiological parameters including PaO2, PaCO2, and plasma pH measurements were monitored, and we found no significant differences in our stroke animals. We initially used 10 rats for the stroke surgery and subsequently included 8 rats that reached the criterion of 80% cerebral blood flow reduction during occlusion. Age-matched animals that were exposed to sham surgery served as controls. However, during the initial conduct of analyses, only trace levels or nondetectable signals of all cell death markers were obtained from these sham animals, thus subsequent analyses (reported here) focused on stroke animals.

**Histology and Immunohistochemistry**

Rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde fixative at 3 months after MCAo. Brains and hearts were harvested and postfixed in the same fixative for 24 hours, followed by 30% sucrose in PBS for 1 week. Frozen sections were then cut at 30 μm in a cryostat and stored at −20°C. To demonstrate cell death, immunohistochemical investigations were performed. Frozen sections were incubated overnight at 4°C with first antibodies tested for in vitro study. After rinsing 3 times in PBS, sections were incubated for 2 hours at room temperature in the same secondary antibodies as noted above for immunocytochemistry and with Hoechst33342 (Sigma) as counterstain. The sections were washed again 3 times in PBS and mounted on glass slides using mounting medium. Control studies included exclusion of primary antibody substituted with 10% goat serum in PBS. No immunoreactivity was observed in these controls.

**Evaluation of Immunostained Cells**

To evaluate the number of immunopositive cells, we semiquantitatively defined cells as follows: no positive cells (no cells/1 visual filed); (−), low (1–3 cells): (+), medium (4–9 cells): (++) (10 cells): (+++).

**Statistical Analysis**

Analysis of variance and post hoc t test were used for statistical analyses. All data were presented as mean±SEM. In all analyses, P<0.05 was considered statistically significant.

**Results**

**PRNCs and RCMs Are Vulnerable to Cell Death After I/R Injury**

PRNCs after OGD displayed significant cell death. Necrotic cells (TNF-α-positive cells) were widespread under OGD (20.3±9.0%), but limited under normal condition (8.2±4.7%) (P<0.05). Apoptotic Caspase 3-positive cells were rampant in PRNCs under OGD (78.4±19.1%) and higher than PRNCs in normal condition (43.1±9.8%) (P<0.05). Fas Ligand–positive apoptotic cells were markedly increased in OGD (77.7±7.0%) compared with normal condition (14.6±4.9%) (P<0.01). In addition, autophagic MAP1LC3A-positive cells were significantly higher in OGD (86.3±6.4%) than normal condition (39.4±8.3%) (P<0.01) (Figure 1).

For the analyses of autophagy-related cell death, the number of cells with MAP1LC3A immunoreactivity reached over 80% and peaked at 24 hours after I/R injury. Necrotic cells (TNF-α-positive cells) were widespread under OGD (86.3±6.4%), but limited under normal condition (43.1±9.8%) (P<0.05). Fas Ligand–positive apoptotic cells were markedly increased in OGD (77.7±7.0%) compared with normal condition (14.6±4.9%) (P<0.01). In addition, autophagic MAP1LC3A-positive cells were significantly higher in OGD (86.3±6.4%) than normal condition (39.4±8.3%) (P<0.01) (Figure 1).

In immunocytochemical analyses of RCMs, all cell death markers were significantly increased after I/R injury compared with normal condition (summarized in Table). Analyses of necrosis revealed TNF-α immunoreactivity was increased and reached over 40% at 48 hours after I/R injury. Analyses of apoptosis showed the number of cells expressing Caspase 3 and Fas Ligand immunoreactivity were increased time dependently and reached over 70% at 48 hours after I/R injury. In the analyses of autophagy-related cell death, the number of cells with MAP1LC3A immunoreactivity reached over 80% and peaked at 24 hours after I/R injury (Figure 2).

**Supernatant From PRNCs Is Toxic to RCMs**

Next, we used the supernatant from OGD-exposed PRNCs as a medium in RCMs culture after I/R injury to examine whether the supernatant produced an exacerbating toxic effect on the cardiac myocytes. In immunocytochemical analyses of RCMs with the OGD supernatant, all cell death markers were detected (summarized in Table). Analyses of necrosis revealed TNF-α immunoreactivity in the OGD supernatant groups (Group I and J) was significantly higher at 24 and 48 hours after I/R injury compared with the non-OGD groups (Group D and E) (P<0.05), with >60% necrotic cells detected.
at 48 hours after I/R injury. Analyses of apoptosis demonstrated time-dependent increments in Caspase 3 and Fas Ligand immunoreactivity. Significant increments in Caspase 3 immunoreactivity started as early as 6 hours after I/R injury, whereas Fas Ligand exhibited significant increments not until 24 hours after I/R injury ($P<0.05$). Both Caspase 3 and Fas Ligand in the OGD supernatant groups reached over 80% immunoreactivity at 48 hours after I/R injury. In the analyses of autophagy-related cell death, significant elevation of MAP1LC3A immunoreactivity was detected as early as 2 hours after I/R injury ($P<0.05$) and reached over 90% immunoreactivity at 48 hours after I/R injury (Figure 2).

In analyzing cell viability and mitochondrial activity in RCMs, I/R injury alone without the supernatant from OGD-exposed PRNCs (DMEM treatment group) did not lead to cardiac myocyte cell death, even up to 48 hours after I/R injury. With the addition of the supernatant from OGD-exposed PRNCs, calcein assay showed that RCM viability significantly decreased at 24 hours after I/R injury compared with normoxic condition (control) or versus non-OGD (DMEM) treated groups ($P<0.01$). In the MTT assay, the relative mitochondrial reductase activity of RCMs was also significantly reduced with the addition of the supernatant from OGD-exposed PRNCs at 48 hours after I/R injury ($P<0.05$) (Figure 3).

Both Brain and Heart in Chronic Ischemic Stroke Rats Exhibit Cell Death

Immunohistochemical results revealed cell death in the ischemic stroke brain and heart. In the brain analyses, all cell death markers were positive in ipsilateral side; TNF-α-positive and Fas Ligand–positive cells were mostly found in the ipsilateral side, while Caspase 3 and MAP1LC3A were positive in both hemispheres, the contralateral side immunoreactivity was less intense than ipsilateral side (Figure 4). In the heart analyses, all cell death markers were detected in cardiac myocytes; low number of TNF-α-positive and Fas Ligand–positive cells (+), moderate number of Caspase 3-positive cells (++), and high number of MAP1LC3A-positive cells (+++) were detected in the heart after ischemic stroke. The immunoreactivity of the same cell death markers in brains and hearts from sham animals was less intense than those from stroke animals (Figure 4).

Discussion

In the present study, we explored whether ischemic stroke conferred cell death signals to the heart. The in vitro study showed that the supernatant from ischemic (ie, OGD exposed) PRNCs rendered toxic effects on RCMs. Similarly, the in vivo study showed that ischemic stroke animals exhibited RCM cell death. Altogether these results suggest a close pathological link between ischemic stroke and cardiac arrest.
Heart failure and ischemic brain stroke are major causes of mortality and disability around the world. Sometimes, the failure of these 2 organs occurs simultaneously, resulting in a more severe condition. The present study focused on cardiac myocyte death following ischemic stroke, using experimental in vitro and in vivo stroke models. Cardiac arrest leading to brain damage has been previously reported. Here, we show that ischemic stroke could also lead to cardiac alterations.

How does the ischemic stroke disrupt heart function? A normal, nonpathogenic heart autonomously beats while the brain controls heart rate via adrenergic pathways affected by exercises and emotions. Previous clinical studies have shown that the damaged brain, as a consequence of ischemic stroke, is not able to control the heart rhythms suggesting that the heart compromise may be a direct result of ischemic stroke. The insular cortex in the brain is most closely linked to the heart rhythm control, as documented in laboratory and clinical studies, indicating a close interaction between heart alterations and insular cortex damage following ischemic stroke. Interestingly, an ischemic event increases inflammation in atherosclerotic plaques and extramedullary monocytopenia via progenitor cells released from the bone marrow stromal niche, resulting in plaque rupture. However, heart compromise following ischemic stroke unrelated to insular cortical damage has also been reported, supporting the notion of an indirect control of heart alterations by the ischemic brain. Accordingly, a key rationale in the present study is to explore this indirect cell death pathway between the brain and heart.

An unexpected finding of this study is that the direct I/R injury to the RCMs (without OGD supernatant from PRNCs, only DMEM; Figure 3) actually did not cause cardiac myocyte death (see calcein and MTT assays), which might reflect a compensatory protective mechanism during the acute stage of injury (0–48 hours). The addition of OGD supernatant was shown here to trigger the massive cell death to the heart, suggesting that the indirect pathway of ischemic stroke regulating cardiac cell death plays an important role in cardiac failure which until now has not been fully recognized. Other potentially parallel pathways linking stroke and cardiac cell death also need to be considered including systemic inflammatory responses, neuroendocrine-mediated myocardial suppression, and changes in circulating endothelial progenitor cells.

Immunocytochemical analyses of PRNCs after OGD and RCMs after I/R injury showed that all cell death markers were elevated after I/R injury and treatment with supernatant harvested from primary rat neuronal cells (PRNCs) exposed to oxygen-glucose deprivation (OGD). Cell death markers, including tumor necrosis factor-α (TNF-α), Caspase 3, Fas Ligand, and MAP1LC3A, were elevated after I/R injury and treatment with supernatant harvested from PRNCs exposed to OGD compared with the addition of supernatant from non-OGD-exposed PRNCs. Scale bar represents 50 μm.
positive. Moreover, RCMs cultured with the supernatant from OGD-exposed PRNCs showed significant time-dependent increase of positive cells against all cell death markers compared with the control. Consistent RCM alteration was recognized in all cell death markers by 24 hours after I/R injury. Of note, MAP1LC3A positivity was the most sensitive cell death marker altered in RCMs which was significantly increased even in the early phase (2 and 6 hours) after I/R injury, suggesting that the ischemic stroke robustly affected the autophagic pathway over the apoptotic and necrotic cell death mechanisms, in rendering toxic effects on the heart.

RCM viability as revealed by calcein assay supported the immunocytochemical results. By 24 hours of reperfusion, the treatment groups exhibited significantly lower viability than the other groups. In the mitochondrial activity assay, the group exposed to 48 hours of reperfusion exhibited the lowest viability compared with all groups.

In vivo results revealed all cell death markers were detectable in both the brain and heart. Interestingly, Caspase 3 (apoptosis marker) and MAP1LC3A (autophagy marker) are positive in contralateral side of brain, suggesting that the ischemic cell death in the ipsilateral side of brain (damaged brain area) may spread to the contralateral side of brain (intact area).

The converging results between the present in vitro and in vivo studies support the notion of an indirect pathological pathway of cell death originating from the brain to the heart. In particular, that the supernatant from the PRNCs after OGD produced toxic effects on RCMs suggests that soluble factors secreted by the ischemic brain cells may convey cell death signals to cardiac myocytes. It is likely that extractions of tissues (albeit, soluble factors) from stroke brain may render toxicity to the heart. The cell death cascade associated with ischemic stroke regulating the cardiac cell death suggests that the damaged or dead neuronal cells likely started to secrete soluble toxic factors at the early stage of ischemic insult, then the soluble factors subsequently reached the heart via the circulatory system at the later stage of ischemic injury. The present study thus offers a complementary indirect pathological link between ischemic stroke and cardiac failure, adding to the reported direct insular cortex regulation of heart rhythms.7,31

The toxic molecule’s identity secreted by the ischemic brain remains to be determined, and subsequently, the specific mechanism is still unknown. With soluble toxic factors, adverse effects to the other vital organs in addition to the heart may be recognized and warrant further investigations. The routine clinical procedure is to check for any brain dysfunction following heart compromise. The present study suggests that a closer examination of heart function should be considered as part of the diagnosis following ischemic stroke.

The present study examined the effects of ischemic stroke on heart alterations, which parallels a series of studies demonstrating that cardiac arrest may lead to or accompany ischemic stroke.5,9,20,32,33 Altogether, these 2 lines of investigations implicate an overlapping cell death signaling pathway between ischemic stroke and cardiac arrest. The understanding of the molecular, cellular, and anatomic changes that occur in stroke and cardiac failure may reveal novel treatment strategies that will aid the clinical outcomes of patients suffering from these 2 maladies.
Acknowledgments
Dr Ishikawa, J. Vasconcellos, and Dr Borlongan conceived the research theme and the experimental design and wrote the first draft of the manuscript. Dr Ishikawa, Dr Tajiri, J. Vasconcellos, and Dr Borlongan performed the experiments. All the authors edited and approved the final manuscript.

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Disclosures
None.

References

Figure 4. Immunohistochemical analyses of brain and heart in ischemic stroke rats. In the brain, necrotic, apoptotic, and autophagic cells were mostly detected in the ischemic stroke ipsilateral hemisphere. Whereas Caspase 3 and MAP1LC3A were observed in both hemispheres, the contralateral side immunoreactivity was less intense than ipsilateral side. The heart from the ischemic stroke animal also displayed all cell death markers. The immunoreactivity in sham animals was less intense than in stroke animals. Scale bar represents 50 μm. TNF-α indicates tumor necrosis factor-α.

TNF alpha | Caspase 3 | Fas ligand | MAP1LC3A
---|---|---|---
Contralateral side | | | |
Ischemic stroke | + | + | +
Sham Surgery | + | + | +
Heart | + | + | +
Ipsilateral side | | | |
Ipsilateral side | +++ | +++ | +++ | +++

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