● PERSPECTIVE

Regulation of apoptosis in the ischemic penumbra in the first day post-stroke

Stroke is one of leading causes of human disability and death. More than 17 million stroke incidences occur in the world each year. In ischemic stroke (70–80% of all strokes) cerebral vessel occlusion quickly, for few minutes causes oxygen and glucose depletion, ATP deficit, and tissue infarction. It is impossible to rescue neurons in the infarction core. However, the injury propagates to neighboring tissues and forms the transition zone (ischemic penumbra) where cells are damaged slower, for several hours. Protection of penumbra cells and restriction of infarction volume are the main goals of neurologists (Nakka et al., 2008; Chen et al., 2011; Puyal et al., 2013). Despite testing of numerous pro-survival drugs such as glutamate antagonists, blockers of Ca^{2+} channels, antioxidants, and apoptosis inhibitors, an effective anti-stroke neuroprotector that can rescue neurons within first post-stroke hours is not found yet. Even drugs that protect the ischemic animal brain or cultured neurons in the experiments were either ineffective or caused unacceptable adverse effects in humans (Nakka et al., 2008; Puyal et al., 2013). Therefore, further studies of neurodegeneration and neuroprotection mechanisms in the ischemic penumbra are needed. Unlike ischemic core, where cells die mostly through necrosis, apoptosis prevails in the penumbra (Ferrer, 2006; Radak et al., 2017; Uzdensky, 2019). The dichotomy between necrosis in the infarction core and apoptosis in the penumbra is not strict. Sometimes the signs of apoptotic cell death are revealed in the infarction core.

The primary results of rapid vessel occlusion in the ischemic core include cessation of glucose and oxygen delivery and suppression of ATP production. The following glycolysis activation causes lactate accumulation and tissue acidosis. The failure of Na^+/K^+- and Ca^{2+}-ATPases and the plasma membrane injury cause fall of ionic gradients, Na^+ and Ca^{2+} influx, and K^+ release. Extracellular K^+ depolarizes the neighboring cells. Water penetration causes cell and organelle swelling and edema. Massive glutamate release from damaged neurons activates NMDA receptors in depolarized neighboring cells. Ca^{2+} penetration stimulates excitotoxic cell death. Cytosolic Ca^{2+} activates proteinases, lipases, and nucleases that destruct cellular components. Ca^{2+}-activated calpain and cathepsins play the essential role in neurodegeneration. Intracellular Ca^{2+} also stimulates mitochondrial dysfunction, reactive oxygen species (ROS) generation, and NO production. The leakage of electrons from perturbed mitochondrial electron transport chain produces superoxide-anion (O_2^-) and other ROS. Ca^{2+}-activated neuronal NO synthase generates NO that reacts with O_2^- to produce peroxynitrite, a powerful oxidant. Severe oxidative stress includes lipid peroxidation, protein dysfunction, DNA damage and finally necrosis in the ischemic core, whereas weaker damage in the penumbra elicits predominately apoptosis (Nakka et al., 2008; Guo et al., 2011; Puyal et al., 2013; Radak et al., 2017).

Glutamate, K^+, ROS, NO, acidosis, and edema spread the injury and induce penumbra formation. The relatively long-living ROS (H_2O_2 and O_2^-) diffuse between organelles and cells. They activate both pro-survival and pro-apoptotic signaling cascades. The oxidative injury of endoplasmic reticulum and mitochondrial membranes stimulates release of stored Ca^{2+} and mitochondrial pro-apoptotic proteins such as cytochrome c, Smac/DIABLO, AIF, endonuclease G into the cytosol (Guo et al., 2011). The cell response to acute injury is initially performed by proteins present in the cell. However, if the injury is strong and present proteins are unable to cope with the primary lesion, additional proteins are synthesized. In the rodent brain, apoptotic proteins such as caspases 1, 3, 6, 7, 8, and 9, apoptosis inducing factor, Smac/DIABLO and HtrA2 are up-regulated in the ischemic penumbra during first 12 hours post-stroke (Ferrer, 2006). Apoptotic proteins and proteins that mediate their upregulation may represent potential targets for neuroprotective agents. However, the inhibitory effects of some tested caspase inhibitors were transient, and cell death progressed afterwards. Modulation of signaling proteins that control apoptosis may be more fruitful. The expression, phosphorylation and activation of different signaling proteins and transcription factors such as ERK1/2, MEK3/6, JNK, p38, c-Jun, c-Myc, ATF, CREB, Elk, STAT-1, STAT3, IκBα, PTEN, c-Fos, c-Jun, and Jun B were actually observed during first post-stoke hours in the ischemic penumbra (Ferrer, 2006; Uzdensky, 2019). The mechanisms of activation of some of them are known. For example, ROS target ASK1 (apoptosis signal-regulating kinase 1) that further activates proapoptotic MAP kinase JNK. In contrast, ERK1/2, another MAP kinase, maintains cell survival. Ca^{2+} and ROS-mediated signaling pathways stimulate prosurvival transcription factors such as nuclear factor-κB, cAMP response element-binding protein, and signal transducer and activator of transcription (Chen et al., 2011). However, the mechanisms of upregulation of other proteins are unknown.

To characterize the complex signaling responses in the penumbra tissue, multiple signaling proteins should be studied simultaneously. Current multiplex analytical methods based on gene and protein expression profiling provide such information. For example, using oligonucleotide-based microarrays to study RNA transcripts, the induction of immediate early genes *c-fos*, *c-jun*, *fra-1*, *c-myc*, and *HIF-1* and pro-apoptotic genes: *CASP3*, *GADD 34*, *GADD153*, and *E2F1* was shown in the ischemic rat brain (Lu et al., 2003). However, RNA profiles do not correlate well with protein profiles because of mRNA processing and degradation. The proteomic antibody microarrays contain the predetermined set of selected antibodies against hundreds of cellular proteins involved in signal transduction, apoptosis, vesicular transport, cytoskeleton, etc. They provide direct information on expression of several hundred selected signaling proteins in the ischemic brain.

This approach was used in the study of biochemical changes in the ischemic penumbra after photothrombotic stroke (PTS), an experimental model of ischemic stroke (Uzdensky, 2018), in the rat cerebral cortex. Several dozens of up-, or down-regulated proteins involved in pro- or anti-apoptotic processes were revealed at 1–24 hours after PTS (Demyanenko and Uzdensky, 2017). Two basic features of cell death regulation were observed in the PTS-induced penumbra (**Figure 1**):

– Both apoptotic and anti-apoptotic proteins are simultaneously over-expressed in the penumbra. The cell fate in the penumbra is determined by the balance between these opposite tendencies.

– Simultaneous and concerted up-regulation of various pro-apoptotic proteins that initiate, regulate, or execute the implementation of the apoptotic program. Their list includes:

a) Proteins that execute apoptosis: caspases 3, 6 and 7, Smac/DIABLO, and AIF;

b) Signaling proteins that initiate or regulate different pro-apoptotic pathways: Bcl-10, p38 and JNK, DYRK1A;

c) Transcription factors that control the expression of apoptotic proteins: E2F1, p53, c-Myc and GADD153;

d) Diverse multifunctional proteins that are normally involved in diverse cellular functions, but capable to induce apoptosis in specific situations: glutamate receptor NMDAR2a, neurotrophin receptor p75, Par4 (prostate apoptosis response 4), and glutamate decarboxylase GAD65/67 (Demyanenko and Uzdensky, 2017; Uzdensky, 2019). So, various apoptosis pathways in the ischemic penumbra are induced simultaneously from different initial points.

Althow signaling pathways and transcription factors that initiate and regulate expression of various pro- and anti-apoptotic proteins are incompletely known, one can assume some of them.

Transcription factor E2F1 is a key player that determine the cell fate. It controls expression of genes that regulate cell cycle and DNA synthesis and repair. E2F1 stimulates apoptosis when the cell cycle is suppressed. It induces expression of various pro-apoptotic proteins caspases 3, 7, 8 and 9, Smac/DIABLO, Apaf-1, ASK1, and p53. Its synthesis is controlled by p38 and c-Myc, which were also up-regulated in the PTS-induced penumbra. Apoptosis promoter p53 regulates transcription of hundreds target genes that regulate DNA repair, cell cycle arrest, and metabolism. It controls the expression of p21, MDM2 and caspase 6 that are up-regulated in the PTS-induced penumbra. p21 is up-regulated upon DNA damage. It inhibits p53-mediated apoptosis and arrests the cell cycle to allow DNA reparation. MDM2 stimulates p53 proteolysis. Over-expression of MDM2 mediated recovery of damaged neurons after stroke in rats. Transcription factor c-Myc regulates expression of 10-15% of all genes involved in regulation of energy metabolism, protein synthesis, cell cycle and apoptosis. It stimulates expression of p53 and E2F1. The up-regulation of c-Myc in the PTS-induced penumbra also potentiated apoptosis (Demyanenko and Uzdensky, 2017; Uzdensky, 2019).

Among PTS-induced multifunctional proteins that, except other functions, initiate apoptosis in specific situations, the level of Par4 increased by 1.5–2 times at 1–24 h in the PTS-induced penumbra. Its pro-apoptotic effect is associated with inhibition of nuclear factor-κB and down-regulation of Bcl-2, inhibitor of apoptosis, and calbindin. The ischemic induction of Par4, p53 and Bcl-2 family proteins are associated with mitochondrial dysfunction and release of pro-apoptotic proteins. The neurotrophin receptor p75 promotes neuronal apoptosis upon binding of neurotrophic factors. Its over-expression indicated the pro-apoptotic tendency in the penumbra.

Simultaneously, diverse anti-apoptotic proteins such as protein kinases ERK1/2 and Bα (Akt) were up-regulated in the PTS-induced penumbra. The anti-apoptotic activity of Akt is associated with inhibition of pro-apoptotic glycogen synthase kinase 3β (GSK-3β). The up-regulation of Akt and down-regulation of GSK-3β in the PTS-induced penumbra was anti-apoptotic. The up-regulation of estrogen and EGF receptors, that were also observed in the PTS-induced penumbra, stimulates Raf1/ERK and PI3-kinase/Akt pathways. The up-regulation of protein phosphatases 1α and MKP-1, which dephosphorylate cell proteins and stop their activation, was also involved in neuroprotection in PTS-induced penumbra (Demyanenko and Uzdensky, 2017; Uzdensky, 2019).

Ca^{2+}/calmodulin complex regulates cytoskeleton remodeling, gene expression, cell survival, release of neurotransmitters, learning and memory. Calmodulin-dependent kinase II phosphorylates and inhibits pro-apoptotic proteins NO synthase and GSK-3β. CaMKIV inhibits excitotoxicity through activation of the anti-apoptotic pathway PI3K/Akt, and phosphorylation of the transcription factor CREB, which controls expression of different anti-apoptotic genes.

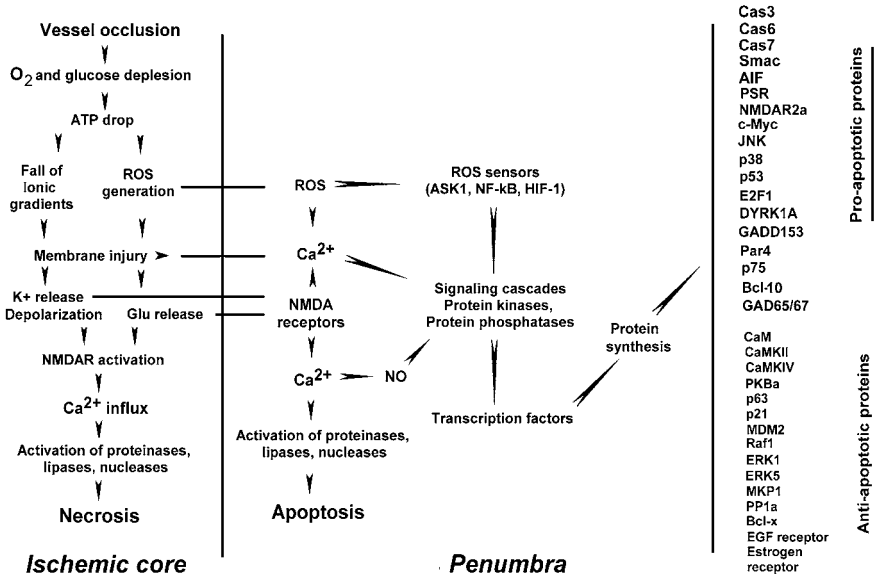

Figure 1 The scheme of the neurochemical processes in the brain after ischemic stroke leading to necrotic or apoptotic cell death and penumbra formation.

How do the primary injurious factors that spread from the infarction core (glutamate, K^+-mediated depolarization, ROS, NO, and acidosis) induce protein expression in the ischemic penumbra, and which signaling pathways and transcription factors regulate the expression of different pro- and anti-apoptotic genes is not well known. One can suggest that they first activate some sensor proteins such as Ca^{2+} and ROS sensors: Ca^{2+}/calmodulin-dependent proteins, ASK1, protein phosphatases and other thiol-containing enzymes, HIF1, and others. Then the stress-sensitive protein kinases such as JNK, p38, c-Myc stimulate transciption factors such as E2F1, p53, GADD153, CREB, nuclear factor-κB to induce expression of either pro-survival, or pro-apoptotic proteins.

Excessive intracellular Ca^{2+} can enter the neuronal nucleus and activate expression of specific genes depend on the Ca^{2+} penetration path. Ca^{2+} influx through synaptic MNDA receptors can induce calcium wave that propagates to the nucleus. Nuclear calcium stimulates the CaMKIV/CREB pathway that induces expression of genes involved in regulation of physiological functions: metabolism, synaptic transmission, and cell survival. In contrast, hyperactivation of extrasynaptic NMDA receptors induces pathogenic Ca^{2+} overload and expression of genes associated with apoptosis and neurodegeneration. In this case CREB is functionally inactivated by rapid dephosphorylation and following degradation. Activation of these receptors causes nuclear accumulation of histone deacetylases HDAC4 and HDAC5 that globally repress transcription (Bading, 2013).

ROS-sensitive transcription factors such as AP-1, HIF-1α, NF-κB, and STAT-1α trigger expression of genes associated either with neural dysfunction and death, or with cell survival (Chen et al., 2011). Their effects depend on the damage degree and cellular context. For example, during transient global ischemia, NF-κB is briefly activated in surviving neurons. However, prolonged injury induces production of death-associated proteins in neurons, which are destined to die. The pathway ATM/E2F1/p53 orchestrates neuronal apoptosis (Camins et al., 2007). Nitric oxide produced in ischemic cells influences transcription factors that regulate gene expression through the guanylate cyclase/cGMP/protein kinase G/CREB cascade. NO controls the activity of transcription factors NF-κB, N-Myc, c-Fos and c-Jun that regulate cell survival.

There are various problems to be addressed in future:

– Which signaling pathways and transcription factors control the expression of individual proteins that regulate cell survival and death in the ischemic penumbra?

– What cell types: neurons, glia, or vascular endothelium are most sensitive to ischemic damage? What is the role of intercellular interactions in the neurovascular units in penumbra?

– What is the dynamics and spatial distribution of processes involved in the responses of different penumbra cells to ischemia/reperfusion?

– Is multitarget therapy more promising for stroke treatment? What proteins and organelles should be affected, and what is the temporal sequence of their application?

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