Identification of Differentially Expressed Genes in Rat Hippocampus After Transient Global Cerebral Ischemia Using Subtractive cDNA Cloning Based on Polymerase Chain Reaction

Naoki Yokota, MD, PhD; Masato Uchijima, PhD; Shigeru Nishizawa, MD, PhD; Hiroki Namba, MD, PhD; Yukio Koide, MD, PhD

Background and Purpose—The purpose of this study is to identify new molecules that play important roles in the phenomena that occur in the hippocampus after transient global cerebral ischemia, as clues to better understanding of the mechanisms.

Methods—A subtractive cDNA library was established by suppression subtractive hybridization of rat hippocampal tissues after transient global cerebral ischemia. With differential screening of the library, upregulated fragments were identified. The mRNA expression levels of selected genes were measured with semiquantitative reverse transcriptase polymerase chain reaction (PCR).

Results—Among more than 100 isolated fragments, approximately half were determined to be identical to known sequences. The rest showed high homology to known sequences, and only 2 did not exhibit homology to any known sequences. The expression of 5 genes identified in this study increased in 24 hours after ischemia to a level twice as high as that in sham-operated controls. These included furin, prosaposin, synaptotagmin IV, heat shock protein 105, and the neutral and basic amino acid transporter (NBAT). The increases in the mRNA expression levels of the genes except NBAT, as revealed by semiquantitative reverse transcription PCR, were statistically significant at both 6 and 24 hours after ischemia.

Conclusions—Genes isolated are thought to be associated with production of proteins necessary for degeneration, neuroprotection, and reconstruction of neurons. How the expression of these genes relates to functional changes after ischemia remains to be determined. PCR-based subtractive cDNA cloning is demonstrated to be a useful tool for analyzing in vivo gene expression in animal ischemia models. (Stroke. 2001;32:168-174.)

Key Words: cerebral ischemia, global ■ cerebral ischemia, transient ■ cloning ■ DNA, complementary ■ hippocampus ■ polymerase chain reaction

Cerebral ischemia is caused by reduced blood supply to the brain tissue. Global (forebrain) ischemia can be produced by transient cardiac arrest. Severe global ischemia has been known to be followed by death of some neurons in the basal ganglia and the cerebral cortex, especially in the CA1 pyramidal neurons of the hippocampus, where selective and delayed degeneration has been reported to occur within days after the event.1 These phenomena, such as delayed neuronal death, neuroprotection, and reconstruction, are the results of molecular events that reflect changes in both gene transcription and in posttranscriptional and translational regulatory processes.2

Recently, the molecular events underlying these phenomena occurring after brain ischemia have been successfully elucidated.3,4 Within several minutes after ischemia, the so-called early immediate genes, such as c-fos, c-jun, and zif268, are expressed,5 which then trigger the expression of several other genes through activator protein-1 and cAMP response element.6 Thereafter, heat shock proteins (HSPs) (in particular, HSP70) and several cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-6, monocyte chemottractant peptide-1) are expressed.7 These cytokines induce the expression of adhesion molecules (intercellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1, P-secretin) in the cerebral vasculature, which initiates inflammatory reactions. In addition, these cytokines also activate the expression of some inflammation-related genes such as...
inductive nitric oxide synthase and cyclooxygenase-2. At this stage, apoptosis-related genes, such as p53 and bax, are expressed, and DNA fragmentation and cell death, which is designated as programmed cell death, occur. Despite these advancements in the understanding of molecular mechanisms underlying the events that occur after brain ischemia, the search for other as yet unidentified genes that may also play important roles in these phenomena is still in progress.

The aim of this study is to identify specific molecular pathways involved in the phenomena, such as delayed neuronal cell death, protection against ischemic damage, and tissue regeneration, that occur in the hippocampus under the pathophysiologial condition of prolonged ischemia.

Materials and Methods
Transient forebrain ischemia was produced by the 4-vessel occlusion method in rats, and differentially expressed genes in the hippocampus after ischemia were isolated with the use of suppressive subtractive hybridization as a polymerase chain reaction (PCR)-based subtractive cDNA cloning.

Induction of Transient Global Ischemia and Preparation of Tissue Specimens
All experiments were performed according to the Rules of Animal Experimentation and the Guide for the Care and Use of Laboratory Animals of Hamamatsu University School of Medicine. Transient forebrain ischemia for 10 minutes was induced by the 4-vessel occlusion method. On the day before the induction of ischemia, adult male Wistar rats (weight, 220 to 280 g; purchased from Nippon SLC, Japan) were anesthetized with pentobarbital, and reversible clamps were placed loosely around the common carotid arteries of both sides, without occluding the vessels. The vertebral arteries were permanently occluded by electrocautery at the first cervical vertebra. A No. 0 surgical silk thread was passed through the neck, excluding the carotid arteries, vagal nerves, jugular veins, and esophagus. Sham-operated control animals were also administered anesthesia; skin incisions were made and they were subjected to the carotid manipulations, but ischemia was not induced. On the following day, forebrain ischemia was induced by tightening the carotid artery clamps and the external suture encircling the neck muscles for 10 minutes. The body temperature was maintained at 37°C to 37.5°C during and after the ischemia by a heating lamp connected to a rectal thermistor. At the end of 10 minutes, the carotid artery clamps were released, and the neck suture was cut to allow recirculation to the brain. The ischemic insult caused loss of the righting reflex and the thermistor. At the end of 10 minutes, the carotid artery clasps were placed loosely around the common carotid arteries of the 10 sham-operated rats. The 10 sham-operated rats were killed for each of the following time points: before ischemia and 6, 24, and 72 hours after ischemia.

RNA Preparation and Double-Stranded cDNA Synthesis
Total RNA was prepared by the guanidinium acid phenol method. mRNA was isolated with oligo(dT) 

Establishment of Subtractive cDNA Library Using Linker-Ligated PCR
Subtractive cDNA library was established by suppression subtractive hybridization, essentially as described by Diatchenko et al. Briefly, dscDNA was digested with the blunt-ended restriction enzyme Rsal. Test dscDNA was synthesized from the hippocampal tissues of the 10 rats subjected to ischemia (24 hours after ischemia) and driver dscDNA from those of the 10 sham-operated rats. The digested test dscDNA was ligated to adapter 1 (5′-GTAATACGACTCTATAG-GGGTCCTCGAGCGGCCGCGAGGT-3′, 3′-CCCGTCATCA-5′) and adapter 2 (5′-TGTAGCGTGAAGACGACAGAAATAGGCTGCGGAGGGCCTCGAGCGGCCGCGAGGT-3′, 3′-CCCTCAGAACAGCCACGATA-5′) in separate reactions. After heat denaturation (1.5 minutes, 98°C), the tester cDNAs ligated with either adapter 1 or 2 were mixed with an excess of driver cDNA and hybridized in 50 mmol/L HEPES, pH 8.3/0.5 mol/L NaCl/0.2 mmol/L EDTA for 10 hours at 68°C. After the first hybridizations, these 2 samples were mixed, and the heat-denatured driver cDNA was added to the same hybridization buffer. The hybridization was allowed to proceed for an additional 10 hours at 68°C. The final hybridization mixture was diluted in dilution buffer (20 mmol/L HEPES, pH 8.3/50 mmol/L NaCl/0.2 mmol/L EDTA), heated at 72°C for 7 minutes, and stored at −20°C. PCR amplification was performed with the use of the Advantage cDNA PCR Core Kit (CLONTECH). The subtracted cDNA was diluted, and the first PCR was performed with the PCR primers P1 (5′-GTAATACGACTCTATAGGAGGCC-3′) and P2 (5′-TGTAGCGTGAAGACGACAGAAATAGGCTGCGGAGGGCCTCGAGCGGCCGCGAGGT-3′) under the following protocol: 75°C for 7 minutes, followed by 30 cycles at 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 150 seconds, and final extension at 68°C for 15 minutes. The amplified product was diluted, and secondary PCR was performed under the same conditions for 10 cycles using the nested primers P1N1 (5′-TGACGCTGAGCAGAAATAGGCTGCGGAGGGCCTCGAGCGGCCGCGAGGT-3′) and P2N2 (5′-TGTAGCGTGAAGACGACAGAAATAGGCTGCGGAGGGCCTCGAGCGGCCGCGAGGT-3′). Products from the secondary PCR were inserted into pBluescript II (Stratagene) at the EcoRI site and transformed into competent cells, Escherichia coli DH5a (TOYOBO).

Isolation of Uregulated Fragments and Their Identification: Differential Screening
Randomly selected bacterial colonies were grown in 100 μL of Luria-Bertani medium with ampicillin in 96-well plates at 37°C overnight. These clones were amplified under the same conditions for 20 cycles with the nested primers 1 and 2 in Multplate 96 (MJ Research). The PCR products were spotted onto Hybond-N (Amersham Pharmacia Biotech) and denatured twice in 0.5N NaOH on Whatman 3MM filter paper for 2 minutes and then neutralized by dipping in Tris-HCl (pH 7.5) for 2 minutes. The DNAs were fixed to the filter by irradiated ultraviolet light.

Forty microliters of forward- and reverse-subtracted secondary PCR products were completely digested with Rsal, Khol, and Smol restriction enzymes. The digests were electrophoresed on a 2% agarose/ethidium bromide gel, and the low-molecular-weight band was cut to remove the adapter sequence. cDNAs were recovered and purified using the GENE CLEAN Kit (BIO 101) and labeled with (α-32P) ATP (ICN Biomedicals) using the Random Primed DNA Labeling Kit (Roche Diagnostics).

The filters were air-dried and prehybridized in Rapid Hibi solution (Amersham Pharmacia Biotech) for 30 minutes, and hybridization was allowed to proceed at 65°C for 3 hours after the addition of 106 cpm/mL of 32P-labeled cDNA probes prepared from the rat hippocampus of either sham-operated or ischemia-induced rats 24 hours after the ischemia induction. The filters were washed twice with 2× SSC and 0.1× SSC at 50°C, exposed to an imaging plate, and analyzed by the BAS 1000 imaging analyzer system (Fuji Photo Film).

The clones positive for the subtracted probe and negative for the reverse-subtracted one were selected, and plasmids were prepared. The method for the preparation of the plasmid DNA was described previously by Sambrook et al. The DNAs were purified with the use of the GENE CLEAN Kit (BIO 101) and sequenced by the dideoxy chain termination method with the Dye Terminator Cycle.
Sequencing FS Ready Reaction Kit and the DNA autosequencer 373A/GeneScanTM (PE Biosystem). The sequences were analyzed by the DNASIS program (Hitachi), and a homology search was performed against the sequences in the DDBJ (version 1.60) database with the use of the BLAST program.

Semiquantitative RT-PCR for Analyses of mRNA Expression

mRNA was prepared as described above. Two micrograms of the total RNAs prepared from the rat hippocampus of ischemia-induced rats 24 hours after ischemia induction and the sham-operated rats at several time points (before ischemia and 6 hours, 24 hours, 72 hours, and 7 days after ischemia), as described, was used as the template for synthesizing the cDNAs. cDNAs were synthesized with the oligo(dT)12–18 primers with the use of the SuporScript II cDNA Synthesis Kit (Roche Diagnostics). The reaction mixture was incubated at 42°C for 2 hours, and the reaction was terminated by heating at 70°C for 20 minutes. The mixture was diluted in 100 mL of Tris-EDTA (pH 8.0) before the PCR amplification was performed. PCR amplification was performed by adding a 2.5-μL aliquot of each cDNA sample to 25 μL of the reaction mixture with the use of the Advantage cDNA PCR Core Kit (CLONTECH). The gene-specific primers used in this study were designed on the basis of the sequences of the isolated fragments, as shown in Table 1. The following amplification protocol was used: denaturation at 95°C for 30 seconds, annealing at each temperature (as shown in Table 1) for 30 seconds, and extension at 72°C for 2.5 minutes. After PCR, 10 μL of each of the PCR products was electrophoresed, separated on a 8% acrylamide gel, and stained with ethidium bromide. Densitometric analyses were performed in tagged image file format with the use of the ATTO densitometer (AE-6900 MF, ATTO), and the relative peak area was expressed in densitometric units. For each sample, the number of densitometric units in the bands was standardized to that in the control (G3PDH) band. The rate of amplification with the primer sets used here was exponential for each cycle determined, and the pattern of amplification remained constant through different runs.

Postischemic relative values were obtained by comparison of preischemic values. The data were expressed as mean±SEM and analyzed by 2-tailed unpaired t test. A 95% confidence level was considered to denote statistical significance.

Results

Approximately 2000 clones were differentially screened, >100 positive clones were selected, and the plasmids were purified and sequenced. Therefore, the percentage of the upregulated clones in the subtracted library was approximately 5%. Among the fragments isolated, approximately half of the fragments were found to be identical to known rat sequences (as shown in Table 2) by homology search against the sequences in a database (DDBJ; DNA Data Bank of Japan). The rest of the fragments were highly homologous to known mouse, rat, and human sequences, and only 2 fragment did not exhibit homology to any known sequences. Rat growth hormone (GH) (J00739, 6 times), BC1 RNA (M16113, 4 times), mouse 28S ribosomal RNA (X00525, 6 times), and rat clone AA818403 (unknown function, 3 times) showed multiple hits. However, the structures and functions of many of the rat or mouse genes hit in the homology search are still unknown or known only to a small extent.

For the identified genes, the clones, such as prosaposin, pentaxine, furin, synaptotagmin IV, NBAT, cell adhesion kinase (CAK), cytochrome C, HSP105, GH, and BC1 RNA, which were identified as cDNA sequences, were selected. Background clones, such as housekeeping genes, and clones that had already been revealed to play roles in the ischemic phenomena of the central nervous system by previous studies were eliminated. In GH and BC1 RNA, single bands of PCR products were not acquired in the fragments, and therefore the

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Sequence (5'-3')</th>
<th>Temperature,* °C</th>
<th>Length,† bp</th>
<th>Cycle‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furin</td>
<td>Sense AGACCTGATGATGGAGGAAGG</td>
<td>56</td>
<td>189</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Antisense GTGTCATTGATCATCTCGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prosaposin</td>
<td>Sense TAGTCTCTATTTAAGCTGACA</td>
<td>56</td>
<td>224</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Antisense ACTTCTGATGATTAAGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBAT</td>
<td>Sense AGTTTATGCAAGTTCTGAG</td>
<td>54</td>
<td>191</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Antisense TTATCATCTCTGTTGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptotagmin IV</td>
<td>Sense ATTAGTGAACCAAGATAG</td>
<td>50</td>
<td>225</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Antisense AACTCTAACAATCACCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP105</td>
<td>Sense AGTTGGTTAATCGATAG</td>
<td>50</td>
<td>188</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Antisense TTTGCTAAAACCTAAGGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>Sense ACCACAGTCATGCTATCAC</td>
<td>50</td>
<td>500</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Antisense TCACCACCGTGTGCTGTA</td>
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</tr>
</tbody>
</table>

*Annealing temperature. †Length of PCR products. ‡Number of PCR cycles.
The mRNA differential display method was first reported by Liang and Pardee\textsuperscript{25} and is widely used as an efficient method for isolating differentially expressed genes in neural tissue.\textsuperscript{21,22,27} Although this method is potentially a quick method for identifying differentially expressed genes, it is associated with a high incidence of false-positives and redundant gene identification, poor reproducibility, biased gene display, and lack of functional information about the cloned cDNA.\textsuperscript{11} Therefore, we chose the method of suppression subtractive hybridization, which is based on the suppression PCR effect,\textsuperscript{28} to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non-target DNA amplification.\textsuperscript{11} However, this method also has some disadvantages. It requires multiple intensive steps compared with the mRNA differential display method. The average cDNA size is small (0.1 to 2 kb) because of blunt-ended restricted enzyme digestion (although it is sufficient for a homology search against the sequence in a database using the BLAST program). Excessive PCR cycles result in a dramatic increase in background and redundant gene identification (data not shown). Furthermore, some differential screening steps are needed to minimize the background before proceeding to further analyses. Analyses of timely and spatial expression of the molecules that play important roles after the ischemic phenomena are essential for understanding the underlying mechanisms. However, compared with the cell culture system, the quantity of acquired mRNA in in vivo study is quite small. Therefore, we used whole hippocampi for the analyses. Despite these disadvantages, the PCR-based subtractive cDNA cloning technique provides an efficient tool for analyzing the phenomena in the rat hippocampus in a transient global ischemia model. Using this technique and the in vivo transient global cerebral ischemia model, we have identified 5 new genes (furin, prosaposin, synaptotagmin IV, HSP105, and NBAT) involved in the mechanisms underlying the ischemia-induced phenomena in the rat hippocampus.

Furin is a member of the mammalian prohormone convertases (PCs), which are expressed in the central and peripheral nervous systems in both neurons and glial cells.\textsuperscript{29} The neurotrophins, including nerve growth factor, brain-derived neurotrophic factors, and neurotrophin-3, are synthesized as large inactive precursors, which are then enzymatically cleaved within the ArgXLys/ArgArg site to produce the respective active forms. Furin has been shown to exhibit this cleavage activity. Kainic acid–induced seizures have been reported to be associated with increased expression of the PCs furin and PC1 in the mouse hippocampus in a differential manner.\textsuperscript{30} Many neuroprotective peptides may require post-translational processing. They play a role in either protecting neurons from injury or in assisting them to recover from it. These enzymes are not uniformly distributed throughout the hippocampus, which might explain why there are differences
in the vulnerability of hippocampal neurons to ischemic damage.

Prosaposin, a 66-kDa glycoprotein, is the precursor of saposins A, B, C, and D, which act as cofactors in the hydrolysis of sphingolipids by lysosomal hydrolases.\textsuperscript{31} Prosaposin was identified as a neurotrophic factor,\textsuperscript{32} and subsequently a neurotrophic sequence was identified in the amino terminal portion of the saposin C domain.\textsuperscript{33} Prosaposin has an atrophic effect on some types of neurons, such as newborn cerebellar granular cells, and its neuroprotective actions are similar to those of insulin-like growth factor-1 rather than those of brain-derived neurotrophic factors. Prosaposin may play a role in cerebellar development, including the programmed cell death of cerebellar neurons.\textsuperscript{34} It is suggested that, together with furin, prosaposin might protect neurons in the hippocampus from ischemic damage.

The Ca\textsuperscript{2+}-dependent release of neurotransmitters and neuropeptides from presynaptic nerve terminals is the central event in synaptic neurotransmission. Recently, accumulated genetic and electrophysiological evidence has suggested that synaptotagmin I plays an integral role in synaptic vesicle fusion and neurotransmitter release.\textsuperscript{35–37} Although at least 9 other members of the synaptotagmin gene family have been identified, the roles of these genes in synaptic vesicle function are not yet well understood. Synaptotagmins are synaptic vesicle proteins, which are thought to play a role in depolarization-induced, calcium-mediated exocytosis and neurotransmitter release. Subtractive library construction and differential screening were used to identify a cDNA of synaptotagmin IV from a cell-specific immediate early gene induced in rat PC12 pheochromocytoma cells. Kainic acid–induced seizures in rats are followed by the accumulation of synaptotagmin IV in the hippocampus and piriform cortex. It has been also suggested that the synaptotagmin IV gene might provide a direct link between depolarization-induced neuronal gene expression and the subsequent modulation of synaptic structures and functions in the ischemic hippocampus.\textsuperscript{38}

HSP105 belongs to one of the HSP families, which have been classified into several families according to their apparent molecular mass: high-molecular-mass HSPs (HSP90, HSP70, HSP60, HSP47) and low-molecular-mass HSPs. HSP70, HSP60, and HSP90 have been studied extensively. These proteins interact with other proteins to mediate protein folding and unfolding and assembly and disassembly of proteins as molecular chaperones.\textsuperscript{39–41} Murine HSP105 was recently cloned and was revealed to be highly expressed in the brain. In the mouse, HSP105, in addition to apg-1 and apg-2, has been identified as a member of the HSP110 family.\textsuperscript{42} These studies indicate that the HSP110 family is significantly large and is a diverged relative of the HSP70 family with unique sequence components. It was recently demonstrated that the mRNA expression of all members of the HSP110 family was induced by brain ischemia/reperfusion in a rat transient forebrain ischemia model.\textsuperscript{27,43} HSP105 was also identified in oligodendrocytes by the differential...
display method, and increased mRNA expression for this protein was noted to be induced by complement activation.\textsuperscript{21} 

NBAT was cloned from the kidney and intestine of the rat and rabbit by expression cloning and screening cDNA libraries as a Na\textsuperscript+-independent transporter of cationic and neutral amino acids\textsuperscript{34,40} and is localized to enteroid cells and enteric neurons. Recently, NBAT expression was detected in the medulla and spinal cord, localized predominantly in axonal terminals, and was associated with large, dense core vesicles and discrete segments of the plasma membrane. It was suggested that NBAT subserved a role as a vesicular or plasmalemmal transporter in monoamine-containing cells, including chromaffin cells and autonomic neurons.\textsuperscript{47}

Taken together, on the basis of the knowledge of their own functions, these isolated genes were suggested to be involved in the molecular process of phenomena such as neural degenerative, neuroprotective, or reconstructive phenomena, which occur after global ischemia in the rat hippocampus. Further analyses on timely and spatial expression of the molecules and further clarification of their roles in molecular mechanisms underlying ischemic phenomena should be accomplished. This study may be the first to provide in vivo evidence of involvement of neuroprotective molecules, such as furin or prosaposin, in the phenomena occurring in the ischemic hippocampus. It is also suggested from this study that PCR-based subtractive cDNA cloning is a powerful tool for isolating transcriptionally regulated genes that play important roles in ischemic phenomena.

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References

Cerebral ischemia induces the sequence of molecular events that lead to the activation of many genes which produce agents involved in the processes of delayed cell death neuroprotection and reconstruction. Some of these molecular events have been well established. It is known that within minutes after the onset of ischemia, the so-called early immediate genes are expressed; this is followed by the expression of genes that lead to the generation of heat-shock proteins and cytokines; thereafter, apoptosis-related genes are expressed and mediate cell death.

Undoubtedly, other unidentified genes are expressed and are important in these processes. In the preceding article, Yokota and colleagues used the sophisticated techniques of PCR-based subtractive cDNA cloning to identify previously unknown genes that might be expressed in cerebral ischemia. They found 5 such genes in the first 24 hours after ischemia. These included furin, prosaposin, synaptotagmin IV, and heat-shock protein 105, as well as the neutral and basic amino acid transporter. Furin is a mammalian prohormone convertase involved in the generation of neurotrophins; prosaposin is a glycoprotein precursor of saponins, which are cofactors in the hydrolysis of sphingolipids; synaptotagmins are synaptic vesicle proteins that may play a role in exocytosis and neurotransmitter release; heat-shock proteins mediate protein folding and unfolding and play a role in neuroprotection. Finally, the neutral and basic amino acid transporter is involved in vesicular transport of various substances, including monoamines.

This is a fertile field for investigation. Undoubtedly, additional genes may be identified that are important in the events that follow brain ischemia. Additional work will also be necessary to show how these newly identified genes expressed after ischemia are involved in the complex processes of cellular death and tissue regeneration.

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