

Correlation Between MMP-3, TIMP-3 Expression and Neuronal Apoptosis After Ischemia - Reperfusion Injury in Rates

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Abstract: Matrix metalloproteinases (MMPs) play a central role in many biological processes, such as embryogenesis, normal tissue remodeling, wound healing, and angiogenesis. How MMP3, TIMP3 expression and neuronal apoptosis (Fas, Fas-L and Bcl) may provide detrimental or beneficial actions during the injury and repair processes after cerebral ischemia-reperfusion injury in rates was studied. Adult rats underwent middle cerebral artery occlusion (MCAO) by the suture method. The expression of mRNA for TIMP-3, MMP-3, and neuronal apoptosis -were estimated in samples of Brain cortices of the ischemic penumbra (IPZ) and the core ischemic zone (ICZ) from 70 Rates with Ischemic control group (ICG), 10 sham-operated group (SOG) and 60 Ischemic β -sodium aescinate intervention group (I β G) using the reverse-transcriptase polymerase chain reaction with a synthetic multicompetitor standard. Also the amounts of neuronal apoptosis positive cells were evaluated by TUNEL assay. I β G expressed significantly TIMP-3, MMP-3 mRNA more than the ICG. Also in the ischemic zone Bcl-2 mRNA was strikingly increased whereas Fas and Fas-L mRNA was considerably decreased. At same time, the amount of apoptosis cells was maximally increased at 3 hours and was lowest decreased at 72 hours after reperfusion. Altogether, these results strongly suggest that inappropriate MMP-3 expression combined with increased TIMP-3, the ratio of apoptotic cells shows positive correlation with TIMP-3, negative correlation with MMP-3 and MMP-3, TIMP-3 Expression might modulate the process of type I and type II neuronal apoptosis through FAS pathway.

Key Words: Ischemia-reperfusion injury, neuronal apoptosis, matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase.

INTRODUCTION

Ischemical reperfusion injury induces a series of cytokine cascade reaction, which cause neurons necrosis or apoptosis. The cerebral ischemia tissue can induce the expression of death receptors, such as Fas and TNF- α . Once binding with the corresponding death inducing ligand, it will activate caspase protein cascade reaction and result in neuronal apoptosis ultimately^[1,2] Reperfusion of ischemic tissue after the infarct reduces ischemic damage. Several mechanisms have been proposed to explain the tissue damage associated with reperfusion of ischemic tissue. Reintroduction of oxygenated blood into the damaged region enhances the production of free radicals, recruits neutrophils and macrophages, and releases proteases^[3,4]. Proteases take

apart cellular structures in many neurological diseases. Neutral proteases, including cysteine, serine, and metalloproteinase (MP) gene families, have complex functions under normal and pathological conditions. Metalloproteinases (MPs), which include the matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase domain) protease gene families, act as sheddases in the removal of cell surface death receptors and death-inducing ligands^[5]. Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of matrixins that participate in controlling the local activities of MMPs in tissues^[6]. It has been confirmed that the intercommunion between the membrane death receptors and the death inducing ligand is an important

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process, which can induce cell apoptosis. MMP-3 and MMP-7 may degrade death receptors independently and inhibit neuronal apoptosis; while TIMP-3 can bind with MMP-3 to produce a complex, which can inhibit the hydrolyzation of MMP-3 and promote apoptosis^[7,8]. The present series of experiments were performed to determine whether there was differential expression of TIMP-3, MMP-3 and neural apoptosis in cerebral ischemia reperfusion zone.

MATERIALS AND METHODS

Ischemia-Reperfusion Protocol and Grouping: The study was approved by the University of Central South Animal Care Committee and conformed to the National Institutes of Health Guidelines for use of animals in research. Adult Wistar rats at 12-16 weeks of age weighing 250 to 320 g, were anesthetized with 1.5% halothane in 70% nitrous oxide and 30% oxygen. MCAO was done by insertion of an intraluminal nylon suture with a bulb on the end. Neck vessels were exposed through a midline incision, and branches of the right external carotid artery were isolated and ligated. A 6-0 silk suture was loosely tied around the external carotid artery stump. A 4-0-monofilament nylon suture was introduced into the external carotid and advanced into the MCA. A silk suture around the stump was tied down onto the thread with the end of the thread protruding slightly. Reperfusion was achieved by slowly pulling the thread back^[9].

A total of 120 Wistar rat were randomly assigned to three groups. 60 rates of IBG had undergone 3 hours of MCAO with reperfusion for 3, 6, 12, 24, 48,72(O3Rxh) hours and received an intraabdominal cavity (i.c.) injection of sodium aescinat (5mg/kg/24hour). SOG rats (10) dura was opened over the MCA, but the artery was not occluded. ICG rats had undergone 3 hours of MCAO (O3R0h), Ischemical 3hours with reperfusion for 3, 6, 12, 24, 48,72 hours (O3Rxh).

Analysis of Brain Tissue Injury: Two rates were selected randomly from each group. The brain was removed and frozen in 2-methylbutane cooled to 20°C for 20 minutes. A 3-mm tissue section was removed from a region approximately 3 mm away from the tip of the frontal lobe, incubated with 2% Triphenyltetrazolium chloride (TTC) solution at 37°C for 30 min and fixed in 4% paraformaldehyde (PFA) for 6h. The normal brain tissue presents red and ischemic presents pale.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analyzes:(1) the primers of TIMP-3, MMP-3, and β -actin were designed by an online primer design programm(e.g.,Primer3:

http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Fas, Fas-L and Bcl-2 were produced by Wetzel M and Ursula FM design [7,8] as shown in Table1. (2) After the rats were killed, the brains were removed and immediately frozen to -20°C for 15-20 mins, and the cortex would be denuded at the right side of brain, which is 7-11mm away from the olfactory bulb's most advanced end, then chose a upper 1/3 (ischemic penumbra zone) and a lower 2/3 (ischemic core zone) portions of the brain tissue from arrow-form splitto perisylvian separately [9], products are conserved in the liquid nitrogen. (3) The total RNA in brains was extracted according to manufacturer's protocol (U.S.A Gibco BRL Company); products were conserved in -20°C. (4) Reverse transcription reactions are performed with 5ul of total RNA and primer according to manufacturer's protocol (U.S.A MBI Company); products are conserved in -20°C (5) the suitest condition of PCR as shown in Table 1; (6) After electrophoresis, the gel shifted to Eagle Eye II picture-disposal system to measuring the light density of each product, and relative value of TIMP-3, MMP-3, Fas, Fas-L and Bcl-2.

Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL): TUNEL staining was carried out using the Apoptosis Detection Kit, (Beijing Zhongshan Biotechnoloy Engineering Co. Ltd.) in accordance to the protocol provided by the manufacturer. In short, slides were immersed in paraffin wax after which they were hydrated through alcohols of decreasing strength to 0.85% NaCl, and finally rinsed with PBS. The sections were fixed in a 4% methanol-free formaldehyde solution for 15 min and washed in PBS at room temperature. After this the slices were exposed to 20 μ g/ml Proteinase K for 15-20min at ambient temperature, rinsed twice (10min) with PBS, fixed in 4% methanol-free formaldehyde solution and incubated with POD for a 60 min at room temperature. Finally there was an incubation for 1h in a dark humidity chamber at 37°C in 100 μ l of DAB incubation buffer whereafter the reaction was stopped using 2 \times standard sodium citrate and rinsed with PBS. Apoptosis cells were visualized using a fluorescence microscope.

Statistics analysis: Data are expressed as mean \pm standard deviation ($\bar{x} \pm s$). Statistical analysis was performed by one-way ANOVA (For Comparisons among multiple groups), LSD-t or Games-Howell (for comparisons between multi-samples averages and one group average) and two-tailed Pearson or Spearman(analysis of the two variables). Analyses were performed using SPSS for windows statistical soft ware (version11.5).

Table1. Primer Sequences and Expected PCR Products Sizes of TIMP-3, MMP-3, Fas, Fas-L, Bcl-2 and β -actin

Primer	Primer Sequence	Product size (bp)	Reaction Condition
TIMP-3	Sense (227-245) 5'-AGA TGC CCC ATG CGC AGT A-3'	299	95°C5 min -[94°C45 s -54°C45 s -72°C50 s]x32 cycle -72°C7
	anti Sense (506-525) 5'-GTA CCC GAA ATT GGA GAG CA-3'		
MMP-3	min	426	95°C5 min -[94°C45 s -58°C45 s -72°C60 s]x34 cycle -72°C10
	Sense (1051-1070) 5'-CCG TCT CTT CCA TCC AAC AT-3'		
Fas	antiSense (1456-1476) 5'-AAA CCA GCT GTT GCT CTT CAA-3'	257	94°C3 min -[94°C30 s -53°C45 s -72°C60 s]x35 cycle -72°C7 min
	min		
Fas-L	Sense (174-192) 5'-CCG ACA ACA ACT GCT CAG A-3'	287	94°C4 min -[94°C60 s -60°C60 s -72°C60 s]x32 cycle -72°C7 min
	antiSense (412-430) 5'-GCA CCT GCA CTT GCT ATT C -3		
Bcl-2	Sense (298-319) 5'-ATA GAG CTG TGG CTA CCG GTG-3'	347	94°C3 min -[94°C30 s -53°C45 s -72°C45 s]x32 cycle -72°C7
	antiSense (563-584) 5'-CTC CAG AGA TCA AAG CAG TTC C-3'		
β -actin	Sense (294-312) 5'-TTA TAA GCT GTC ACA GAG G-3'	648	
	anti Sense (622-640) 5'-TGA AGA GTT CCT CCA CCA C-3'		
	min		
	Sense (400-419) 5'-GTA GCC ATC CAG GCT GTG TT-3'		
	anti Sense (1028-1047) 5'-CAG TGA GGC CAG GAT AGA GC-		

RESULTS

Animal's neural function default and TTC staining analysis: All groups present focal cerebral ischemic behaviors after waking up from anaesthesia, such as: left forelimb bends, or left forelimb downwards and cannot be lifted once withdrawing the neck; turn around towards outside; topple over to the left side while walking. Rats' brain tissues from SOG are dyed by TTC staining, the results show the tissues were all dyed unanimous red and no infarct tissues found. However in ICG and IBG found some pale infarct tissues, which sizes are inconsistent, at the right side brain, the part whose distance is 7-11 mm away from olfactory bulb is most distinct, while the left side brain still presents red. In ischemic groups, the zone of ischemia is reliable and steady.

RT-PCR: The time course of TIMP-3, MMP-3, Bcl-2 and Fas-L expression was assessed in tissue extracts prepared from ICG, SOG and IBG. In the SOG the mRNA expression of TIMP-3, MMP-3, Bcl-2 and Fas-L was detected, indicating its presence in normal tissues and Fac mRNA were absent. The results demonstrated that TIMP-3 mRNA was over expressed in ICG within 3 hours. After 3 hours of reperfusion TIMP-3 mRNA significantly increased. A second increase in mRNA was seen at 48 hours. Values of mRNA in the ICG were increased, but the rise at 3 hours and 48 hours in the IBG was more significant ($P < 0.01$). The expression of TIMP-3 mRNA in ICZ was present, but to a lesser degree than

IPZ ($P < 0.01$) and eventually peaked at 3 h. MMP-3 mRNA expression was pronounced (ie, minimal) in IBG at 0-12 hours but predominantly at the 24h time point after reperfusion compared with ICG ($P < 0.01$) as shown in Table2. MMP-3 mRNA expression is similar in both IPZ and ICZ zone. We assessed the relationship of (Fas, Fas-l, and Bcl-2) mRNA expression in IBG. The expression of, Fas and Fas-l mRNA was decreased but still higher than that of the ICG ($P < 0.01$), while Bcl-2 mRNA increased as shown in Table2.

Changes of Apoptosis Positive Neurons: Apoptosis cells distribute mainly in ischemic penumbra zone. There were a small number of apoptosis positive cells in sham-operated group. In the ischemic zone, the neuronal apoptosis positive cells was detected and peaked at 3 h after reperfusion. By 6 hours of reperfusion, the neuronal apoptosis positive cells were decreased, reaching minimal levels by 72 hours. Neuronal apoptosis positive cells were significantly increased IBG in compared with ICG ($P < 0.01$) as shown in Table2.

Relationship Between the Co-Expression of TIMP-3, MMP-3 and Apoptosis: In the IPZ, the expression of TIMP-3 mRNA shows positive correlation with the counts of apoptosis positive cells, while shows negative correlation with that of MMP-3 & Fas-L mRNA. The expression of MMP-3 shows positive correlation with that of Bcl-2 mRNA. In ICZ, the expression of TIMP-3 mRNA showed positive

Table 2: mRNA Expression of TIMP-3, MMP-3, Fas, Fas-L, Bcl-2 and counts of apoptosis positive neurons (A+N) after MCAO with Various Times of Ischemia and Reperfusion ($\bar{x} \pm s$)

Group			SOG	O3R0h	O3R3h	O3R6h	O3R12h	O3R24h	O3R48h	O3R72h
mRNA TIMP3	ICG	IPZ	0.70±0.07	0.89±0.03 ^{▲▲}	0.96±0.03 ^{▲▲}	0.89±0.07 ^{▲▲}	0.72±0.03	0.63±0.07	1.19±0.15 ^{▲▲}	0.85±0.06 ^{▲▲}
		ICZ	0.52±0.01	0.82±0.03 ^{▲▲}	0.89±0.07 ^{▲▲}	0.75±0.04 ^{▲▲}	0.66±0.08	0.69±0.12 [▲]	0.71±0.06 ^{▲▲}	0.68±0.07 [▲]
	IBG	IPZ			0.79±0.14 ^{**}	0.65±0.12 ^{**}	0.64±0.08	0.60±0.07	0.88±0.11 ^{**}	0.80±0.06 ^{**}
		ICZ			0.74±0.03 ^{**}	0.65±0.04	0.66±0.07	0.68±0.09	0.83±0.14 [*]	0.74±0.10
mRNA MMP3	ICG	IPZ	0.60±0.06	0.47±0.03	0.47±0.01	0.38±0.03 ^{▲▲}	0.56±0.02 ^{▲▲}	0.80±0.09 ^{▲▲}	0.49±0.05	0.36±0.07 ^{▲▲}
		ICZ	0.66±0.03	0.53±0.08	0.47±0.01 ^{▲▲}	0.43±0.01 ^{▲▲}	0.81±0.05	0.50±0.03 ^{▲▲}	0.51±0.04 ^{▲▲}	0.37±0.03 ^{▲▲}
	IBG	IPZ			0.46±0.06	0.57±0.04 ^{**}	0.43±0.03 ^{**}	0.44±0.03 ^{**}	0.45±0.04	0.71±0.04 ^{**}
		ICZ			0.48±0.04	0.75±0.05 ^{**}	0.66±0.03	0.53±0.01	0.64±0.04 [*]	0.55±0.05 [*]
mRNA FAS	ICG	IPZ	0.00±0.00	0.39±0.03 ^{▲▲}	0.50±0.01 ^{▲▲}	0.61±0.05 ^{▲▲}	0.55±0.03 ^{▲▲}	0.45±0.04 ^{▲▲}	0.00±0.00	0.00±0.00
		ICZ	0.00±0.00	0.42±0.03 ^{▲▲}	0.50±0.01 ^{▲▲}	0.47±0.03 ^{▲▲}	0.58±0.03 ^{▲▲}	0.48±0.04	0.00±0.00	0.00±0.00
	IBG	IPZ			0.30±0.03 ^{**}	0.42±0.02 ^{**}	0.43±0.02 ^{**}	0.40±0.03	0.00±0.00	0.00±0.00
		ICZ			0.38±0.03 [*]	0.40±0.03	0.46±0.05 [*]	0.43±0.13	0.00±0.00	0.00±0.00
mRNA FAS-L	ICG	IPZ	0.36±0.04	0.32±0.02	0.41±0.09	0.78±0.12 [▲]	0.42±0.06	0.46±0.03	0.79±0.05 ^{▲▲}	0.67±0.05 ^{▲▲}
		ICZ	0.33±0.02	0.31±0.02	0.30±0.01	0.63±0.10 [▲]	0.52±0.07 [▲]	0.40±0.02 [▲]	0.32±0.02	0.23±0.03
	IBG	IPZ			0.26±0.05	0.28±0.03 ^{**}	0.35±0.03	0.27±0.03 ^{**}	0.00±0.00 ^{**}	0.00±0.00 ^{**}
		ICZ			0.27±0.03	0.41±0.03	0.34±0.03 [*]	0.45±0.03	0.00±0.00 ^{**}	0.00±0.00 ^{**}
mRNA BCL-2	ICG	IPZ	0.83±0.06	0.82±0.04	0.73±0.04	0.62±0.04 ^{▲▲}	0.53±0.08 ^{▲▲}	0.83±0.18	1.00±0.06	0.80±0.07
		ICZ	0.84±0.03	0.81±0.05	0.60±0.07 ^{▲▲}	0.58±0.05 ^{▲▲}	0.53±0.09 ^{▲▲}	0.64±0.07 ^{▲▲}	0.92±0.11	0.77±0.05
	IBG	IPZ			0.67±0.04	0.75±0.04 [*]	0.76±0.05 [*]	0.64±0.04	0.75±0.07 [*]	1.04±0.08 [*]
		ICZ			0.87±0.04 ^{**}	0.93±0.06 ^{**}	1.02±0.11 ^{**}	0.67±0.07	1.06±0.14 ^{**}	0.85±0.05
A+N	ICG	IPZ	6.20±1.48	123.4±6.02 ^{▲▲}	206.4±19.0 ^{▲▲}	154.0±10.0 ^{▲▲}	129.6±4.04 ^{▲▲}	114.2±10.3 ^{▲▲}	107.0±4.06 ^{▲▲}	84.2±5.26 ^{▲▲}
		ICZ	6.40±1.14	65.0±4.85 ^{▲▲}	98.8±1.92 ^{▲▲}	82.8±8.47 ^{▲▲}	76.0±4.06 ^{▲▲}	60.0±5.15 ^{▲▲}	43.6±5.90 ^{▲▲}	32.6±6.69 ^{▲▲}
	IBG	IPZ			117.6±23.3 ^{**}	82.0±13.67 ^{**}	71.6±11.3 ^{**}	64.4±10.48 ^{**}	55.0±5.15 ^{**}	34.4±5.41 ^{**}
		ICZ			73.0±5.57 ^{**}	59.4±6.58 ^{**}	40.2±4.44 ^{**}	37.60±7.57 ^{**}	37.40±2.61 ^{**}	32.80±5.07 ^{**}

Each time point of ICG compared with SOG: ▲P<0.05, ▲▲P<0.01; time points of IBG compared with ICG: *P<0.05, **P<0.01.

Table3: Correlation between mRNA expression of TIMP-3, MMP-3, FAS, FAS-L, BCL-2 and the counts of apoptosis positive neurons

Variable index	TIMP-3 mRNA		MMP-3 mRNA		Counts of apoptosis cells	
	PZ (P value)	CZ (Pvalue)	PZ (P value)	CZ (Pvalue)	PZ (P value)	CZ (P value)
MMP-3mRNA	-0.307(0.010)	-0.244(0.04)				
FAS mRNA	0.394(0.000)	0.335(0.008)			0.492(0.000)	0.529(0.000)
FAS-L mRNA	0.283(0.027)				0.364(0.000)	0.280(0.001)
BCL-2 mRNA	0.332(0.005)		0.315(0.009)			
A+N	0.337(0.004)	0.366(0.001)	0.315(0.011)	0.294(0.024)		

correlation with the counts of apoptosis positive cells, and showed negative correlation with that of Fas-L mRNA. The expression of MMP-3 mRNA showed positive correlation with that of Bcl-2 mRNA. In ischemic zone, Fas & Fas-L mRNAs showed positive correlation with the account of apoptosis positive cell, while the expression of Bcl-2 mRNA showed negative correlation as shown in Table3.

DISCUSSION

We found marked TIMP-3 expression in neurons in ischemic zone. TIMP-3 alone appears insufficient to initiate cell death because it was seen in neuronal process in normal tissues. The present studies indicate that after focal ischemia there are marked differences in the expression of TIMP-3 and MMP-3 at the ischemic site. TIMP-3 showed expression as early as 0 hours after initiation of reperfusion, and maximal expression appeared at 3hours and 48 hours. MMP-3 was decreased at 0-12 hours and peaked at 24 hours. Our finding of the colocalization of TIMP-3 ischemic neurons suggests a role of TIMP-3 in cell death in the brain. Expression of the TIMP-3 mRNA was seen in both ischemic and normal tissue, while the RT-PCR showed an increase in protein only on the ischemic tissues. The increase in TIMP-3 protein at 3and 48 hours was consistent with induction. One possibility is that the TIMP-3 was bound to the MMPs that are increased by 24 hours after reperfusion, resulting in a higher molecular-weight complex. These results show that MMPs and TIMPs are dramatically affected by reperfusion but contribute in a complex manner to reperfusion injury. Matrix-degrading proteases are important in many normal and pathological processes, including motility of developing. Tissue inhibitor of metalloproteinase-3 was found in normal tissues, suggesting that other factors are needed along with TIMP-3 to promote cell death. Although further studies will be necessary to resolve the effect on the normal tissue, the results of the mRNA, provide convincing support for an increase in TIMP-3 on the ischemic zone. This is consistent with reports of TIMP-3 gene expression in brain injury and in Wobbler mice^[10,11]. Programmed cell death occurs by release of caspase-activating factors from mitochondria or by the stimulation of cell surface receptors that may or may not act through mitochondrial activation^[12]. These are referred to as the intrinsic and extrinsic pathways^[13]. Previous studies have suggested that TIMP-3 acts through cell surface receptors. The mechanism proposed is that TIMP-3 and inhibits MMPs that are

involved in the shedding of the DRs and DILs from the cell surface^[14,15]. Proposed sheddases include MMP-3, MMP-7, ADAM-10, and ADAM-17. Sheddases release either the DRs or the DILs from the cell surface, and, depending on the cell types and the substrate released, the sheddases can play a protective or damaging role^[16,17]. Death receptors include the members of the TNF superfamily, such as Fas. One study showed that inhibition of the sheddases -L, etc. All genes can promote apoptosis except Bcl-2, who plays a role in inhibit apoptosis. Inhibition mechanism includes that MMP-3 can hydrolyses cell's surface death receptor and death ligands directly which will suppress apoptosis, and also activating phospholipase A2 through Bcl-2 which would suppress Fas-induced apoptosis, and producing inhibition through type II apoptosis. . More important, that TIMP-3 and MMP-3 express by TIMP-3 stabilized TNF receptors on the cell surface, promoting cell death^[18]. Fas ligand and Fas receptors are associated with cell death in ischemic neurons. Mice lacking the Fas ligand gene have smaller strokes. In the same study^[19] it was shown that the anticancer drug, doxorubicin, induces the Fas ligand in cultured neurons and leads to apoptosis of the cells. We have studied the role of TIMP-3, MMP-3 and apoptosis gene in neuronal cell. We found that the occurrence of cell apoptosis after focal cerebral ischemia-reperfusion is an active death process controlled by multi-genes, which usually include: Bcl-2, Fas, Fas persistently in neurons and the expression activity of neuron surface. TIMP-3 & MMP-3 can modulate DRs' sensitivity and might regulate neuron apoptosis^[20].

CONCLUSION

Our results suggest that the balance between the MMP-3, TIMP-3 and apoptosis gene play a role in ischemic-reperfusion injury. Inhibition of the metalloproteinases altered the early damage to the brain tissue. MMP-3 and TIMP-3 might modulate the process of type I and type II neuronal apoptosis through FAS pathway. Sodium aescinat have beneficial effect on relation between TIMP-3, MMP-3 and neural apoptosis gene.

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