90-2314-B-002 -387 - 杜永光 國立臺灣大學醫學院外科 90/08/01至91/07/31

實驗性蜘蛛膜下腔出血後腦血管攣縮所引起之細胞凋亡——
第二部份：細胞凋亡之分子生物學證據

Apoptosis in cerebral vasospasm after subarachnoid hemorrhage.

Part II: Molecular biological evidence of apoptosis.

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Introduction

Subarachnoid hemorrhage (SAH) after the rupture of intracranial aneurysm is a common problem observed in aged patient. In Taiwan, it is estimated that about 3,000 cases of SAH occurs per year (1). This number only consists 6-7% of the total number of patients with cerebrovascular disease, however, the prognosis of SAH is the worst among all the patients with cerebrovascular disease (2).

Re-bleeding and vasospasm are two major consequences of SAH to affect its prognosis. After the initial bleeding, there are many different approaches to prevent re-bleeding. On the contrary, the real mechanism of vasospasm after SAH is not completely known yet, thus, there is no good method to treat vasospasm (3).

Apoptosis or programmed cell death is different from the ordinary cell death or necrosis. The whole process of apoptosis is controlled by the operation of genes (4-10). Apoptosis is generally exists in physiological or pathological changes of various organs. In normal physiological condition, this phenomenon exists in tissues with both rapid or slow cell regeneration (11) and is thought to have an important role in tissue growth and differentiation. Apoptosis is also observed in some immunological reaction (12) and endocrinal reaction (13). In pathological condition, dysfunction of apoptotic process may lead to problems like neoplasm or acquired autoimmune diseases (14). Among the diseases of various organs, apoptosis is most frequently occurs in the pathological changes of central nervous system such as degenerative diseases, ischemic change, and intoxication (15).

Material and Methods

General Protocol

Forty-eight male New Zealand rabbits, weighing 3.5-4 kg and aged 20-24 weeks were used in this study. The animals were divided into 2 experimental groups and 2 control groups with sham operation (12 each). Two bleeding model (39) were applied to create subarachnoid hemorrhage near the basilar artery. Vasospasm was documented and graded by angiography. All the animals were sacrificed at 7 days after the second bleeding (or corresponding date in control groups). Brain tissue and the basilar artery with its branches were removed to check the molecular biological evidence of apoptosis. The endothelium and the muscular layer of the basilar artery as well as the brain tissue were removed and homogenized separately to extract their DNA. Specimen obtained from one group of the animals, the degrees of DNA fragmentation were analyzed with agarose electrophoresis. Specimen obtained from the other group of the animals underwent TUNEL stain to exam the degree of *in situ* DNA fragmentation the cells of different tissues. The differences of degrees of

apoptosis in the control animals and the experiment animals were analyzed. The correlation of the degree of vasospasm and the degree of apoptosis in each animal is also analyzed.

Induction of vasospasm

Rabbits were anesthetized with 3.5% halothane and then maintained with N_2O and O_2. Surface warming with heating pad was applied to keep anal temperature at 37C.

A midline incision was made at the neck of the animals to isolate the subclavian arteries and the vertebral arteries on both sides. In order to achieve an accurate measurement of the diameter of the basilar artery, vertebral artery was ligated on one side. Thus, the effect of laminar flow in the basilar artery can be avoided while injecting the contrast medium from the opposite vertebral artery. Then a PE-50 tubing was inserted into the unligated vertebral artery and fixed to a three-way connector. This tubing can be used repetitively for multiple sessions of angiography during the experiment.

Then the animals were rotated to the sphinx position and their head were fixed with stereotactic frame. A 25-gauge butterfly needle was inserted to the foramen magnum to withdraw 2 ml cerebrospinal fluid. Same amount of autologus blood from the arterial catheter is then injected into the cisternal magnum and the animals were kept on 45-degree head down position for 30-60 minutes. Cisternal injection of autologus blood was repeated once at 24 hours interval.

In sham operation group, same procedure was performed except that after the withdrawing, 2ml of cerebrospinal was re-injected back to the cisterna magnum.

Cerebral angiography and measurement of vasospasm

After the insertion of vertebral catheter, 3ml of urographin was injecdted via this catheter and x-ray flim was taken on the same time. The film was magnified and put on a view box to measure the diameter of the basilar artery. Diameter of the basilar artery was measured at three points: the upper end of the basilar aretery (A), the lower end of the basilar artery (B), and the narrowest point of the basilar artery (C). The percentage of vasospasm is expressed as:

$$\% \text{ of vasospasm} = \{1-[C/(A+B)/2]\} \times 100\%$$

The first angiography and measurement was done before the injection of blood into cisterna magnum, the second angiography and measurement was performed at 7 days after the bleeding.

The animals were sacrificed after the second angiography. The animals were anesthetized with pentobarbital again. A PE-50 catheter was inserted into the left

atrium after thoracotomy for cardiac perfusion. Perfusion was started with 200ml hepartinized saline and followed by 100ml 10% buffered formalin phosphate. The brain was removed after perfusion and fixed in 10% buffered formalin phosphate again. These brain specimens were then divided into two groups. Group A underwent agarose gel electrophoresis to exam DNA laddering and group B underwent TUNEL stain for *in situ* analysis of apoptosis.

The brains were sliced into 2mm coronal sections. The sections before and after the anterior commisure were then embedded in paraffin and cut into 5 μ thin slices. These slices were stained with hematoxylin and eosin, then with Apop Tag Kit special stain to identify DNA fragmentation and apoptotic bodies. The basilar artery was removed and underwent same procedure. Slices stained with Apop Tag Kit were examed under 200x light microscope connected with image analyzer to calculate the number of DNA fragment and apoptotic body.

Agarose gel electrophoresis

For identification of DNA fragmentation ladder pattern in apoptosis, agarose gel electrophoresis is used in this study. Under dissecting microscope, the endothelium and muscular layer of the basilar artery as well as brain tissue adjacent to the basilar artery and the posterior circulation were carefully dissected for electrophoresis. Brain tissue specimens, 5x5x5 mm each, were taken from the middle portion of the pons, both hemisphere of the cerebellum and both sides of the occipital lobes. These specimens were homogenized under dry ice cooling and diluted with 0.3M NaCl. Then, 2.5 times of ethanol in volume was added and the test tube was put on ice for an hour to allow precipitation of cellular fragment. DNA was then separated with ultracentrifuge and washed in 70% ethanol. DNA concentration was determined with spectrophotometry after dissolve the samples in TE buffer.

Stepwise agarose gradient gel (0.7%-1.2%) containing 0.4ug/ml ethtidium bromide was used for electrophoresis. Samples with 3ug DNA and 1ug 1000bp marker were put into each slot of the agarose gradient gel and underwent electrophoresis with 100V electric currencefor 90 minutes. The gel was then transfer to florescence box to be exposed under ultraviolet light and the distribution of DNA on the film was documented with Polaroid camera.

In situ analysis of apoptosis

In situ analysis of apoptosis was performed by teminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method.

The brain specimens from group B were sliced after fixed with formalin and embedded in paraffin. The brain slices were washed with distil water and placed in

20ug/ml proteinase K solution under room temperature for 15 minutes to dissolve protein. The slices were washed again for 4 times, 2 minute each time and placed in 2% phosphate buffered saline for 5 minutes to eliminate endogeneous peroxidase activity. Then, the slices were washed again and placed into TdT buffer (containing 30mM/L triz-HCl buffer, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride).

After these procedures, solutions containing deoxynucleotidyl transferase and biotinlylated dUTP was dropped on the top of these slices and the slices were transfer to 37C oven for 60 minutes. These slices were put into solution containing 300 mM NaCl and 30mM sodium citrate for 15 minutes to terminate the reaction. The slices were washed and 2% bovine serum albumin was put on top of the slices for 10 minutes and washed again. After putting these slices in phosphate buffered saline for 5 minutes, streptaridin peroxidase was put on top of the slices for 10 minutes and emended into phosphate buffer saline for 5 minutes.

Finally, the slices were stain with 3,3'-diaminobenzidine tetrahydrochloride under room temperature for 30 minutes. After these procedures, same slices were stained again with H-E stain for counter stain.

Statistical analysis

The brain slices were examinated under 300X optic microscope to count the number of TUNEL-positive apoptotic cell. This microscope was linked to a computed imaging analyzer. The apoptotic cells in each high power field were automatically identified and counted. The number of apoptotic cell in 20 different high power fields was averaged to represent the total apoptotic cell number in each respective studied animal.

The differences of number of apoptotic cells between experiment animals and animals with sham operation was analyzed with unpaired Student-t test. The correlation among the degree of vasospasm and the number of apoptotic cells in all the studied animals was analyzed with ANOVA.

Results

The average degree of vasospasm was 68 + 23 % (32% reduction of the vessel diameter) in experimental group and 97 + 12% (3 % reduction of the vessel diameter) in sham operation group ($p< 0.05$). The average apoptotic cells in experimental group were 8.4 + 2.2 cells / high power field (Fig. 1) and 0.0 + 0.2cells / high power field in sham operation group (Fig. 2). The agarose gel electrophoresis revealed laddering pattern of DNA fragmentation in all the endothelium samples of the experimental animals (12/12, 100%). The laddering pattern of DNA fragmentation is positive in 83% (10/12) of the pontine sample, 66% (8/12) of the left cerebellar hemisphere

sample, 58% (7/12) of the right cerebellar hemisphere sample, and 33% (4/12) of both occipital lobe samples from the experimental group (Fig. 3). In sham operation group, no laddering pattern has been detected from all the samples of the endothelium of basilar artery, the pons, the cerebellar hemispheres, and the occipital lobes (Fig. 4). The correlation of the degree of vasospasm and the count of apoptotic cells after TUNEL stain in each individual animal in the experimental group was analyzed with ANOVA and revealed a good correlation between these two variants ($p<0.05$, $r = 0.59$).

Discussion

Among all the studies of apoptosis, apoptosis after cerebral hypoxia and ischemia is the most extensively studied topic. In morphological studies, dense chromosome cluster has been observed at the early stage of apoptosis (16). Then the cell nucleus lost its integrity, the cytoplasma becomes dense but the cell membrane is intact (17). Vacuolarization of the cytoplasma is also an important feature of apoptosis in cerebral ischemia (16). In most studies, no change of mitochondria has been observed (8, 11). In the later stage of apoptosis, apoptotic body is formed inside the cytoplasma (5, 14). These morphological changes are different from the changes after cell necrosis. The time sequence of these morphological changes are different in various tissues and generally it takes one to seven days to complete these changes in brain cells after ischemia (17). Current studies indicate that in the process of apoptosis, although many molecular biological changes can be detected, yet these morphological changes are more reliable for defining apoptosis. In our previous study, we have demonstrated that cytoplasma vacuolarization and apoptotic body are observed in the basilar artery and pontine parenchymal cells of animals subjected to SAH (18).

The molecular biological changes in apoptosis are mainly the cleavage of DNA to cause the products of gene degradation. Agarose gel electrophoresis is very useful for identifying these DNA degradation products to verify the existence of apoptosis (13). In the present study, we have demonstrated that DNA laddering pattern is observed in endothelial cells of the basilar artery in all the experimental animals but not in any of the sham operated animals. This finding strongly indicate that apoptosis is an important feature of cellular damage in vasospasm after SAH. Different degrees of DNA laddering are also seen in various brain tissue related to ischemic change of vasospasm.

Fragmentations of DNA can also be detected by in situ terminal transferase-mediate dUTP nick-end labeling (TUNEL) method. In the present study, TUNEL stain positive apoptotic cells are observed in all the animals with SAH. This finding also demonstrated a good correlation with the degree of vasospasm of the

basilar artery of the animals. These results provide a reliable evidence of existence of apoptosis in vasospasm after SAH.

In conclusion, the present study verified that apoptosis is an important feature of vasospasm after SAH by the observation of DNA cleavage in agarose gel electrophresis and the production of apoptotic cell by TUNEL stain.

Legends:

Fig.1. Laddering pattern of DNA in agarose gel electrophoresis of the basilar artery endothelial sample of the animal subjected to SAH.

Fig.2. Laddering pattern of DNA in agarose gel electrophoresis of the pontine parenchymal sample of the animal subjected to SAH.

Fig 3. TUNEL stain of the brain slices (at the region of pons) from animals subjected to SAH showing TUNEL stain positive apoptotic cells.

Fig 4. TUNEL stain of the brain slices (at the region of pons) from control animals showing no apoptotic cells.

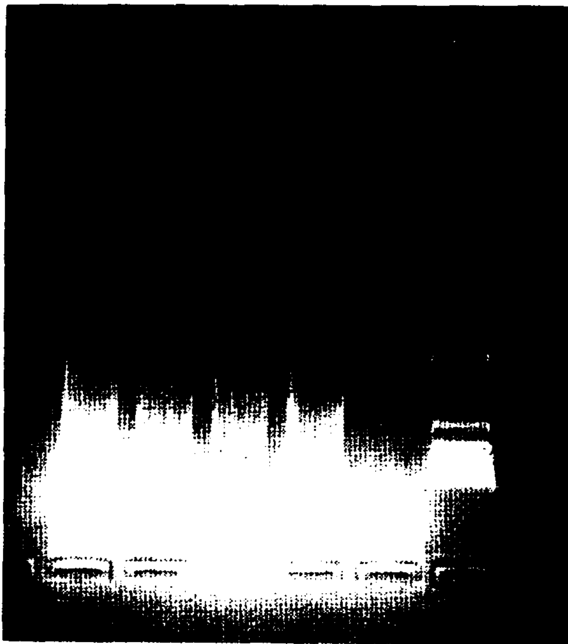

Fig 1

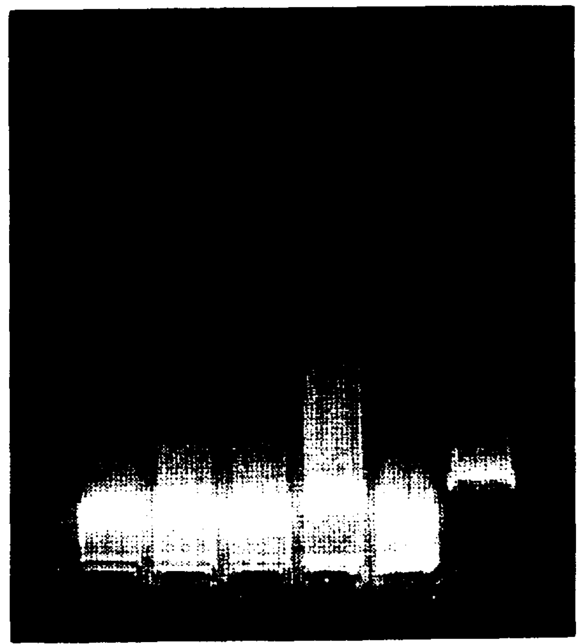

Fig 2

Fig 3

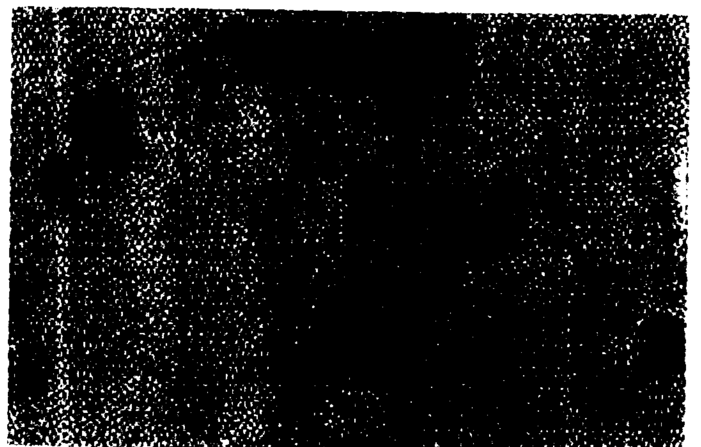

Fig 4

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