Basic Investigation

The Effect of Lumbrokinase on P-selectin and E-selectin in Cerebral Ischemia Model of Rat

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Purpose: To find the effect of lumbrokinase (LK) on P-selectin and E-selectin in ischemic rats. Methods: Male healthy Spragur-Dawley rats weighing 180-220 g (n=90) were divided into 4 groups: (1) normal control group (n=5), (2) sham-operated group (n=35), (3) ischemic group (n=35), (4) LK group (n=15). LK 10mg/kg (2000UK activity of LK) was given by intraperitoneal injection in the LK group 30 minutes before experiment. Same volume of normal saline was given in the sham-operated group and ischemic group. The ischemic model was made by modified Haruo Nagasawa's method. Immunohistochemistry was used to observe the P-selectin and E-selectin positive cells in the ischemic region. Results: P-selectin and E-selectin positive cells in ischemic regions were observed in the ischemic group, and the peak of expression was at 6 hours and 12 hours, respectively. The similar changes were not observed in normal control group. There were only a few positive cells in the sham-operated group. In LK group, the P-selectin and E-selectin positive cells were significantly less than those in the ischemic group ($P<0.05$ at 3 hours after the onset, $P<0.01$ at 6 hours and $P<0.01$ at 12 hours, respectively). Conclusions: LK might significantly decrease the immunoreactions of P-selectin and E-selectin in ischemic lesion.

P-selectin and E-selectin are two substances of adhesion glycoprotein family which mediate leukocyte-endothelial cells and leukocyte-platelet adhesive interactions[1]. P-selectin which exists in the Weibel-Palade bodies of the vascular endothelium and the -granules of the platelet is rapidly translocated to the cell surface after stimulation with thrombin, histamine, or other mediators[2]. In contrast, the expression of E-selectin is induced by cytokines, such as tumor necrosis factor or interleukin-1 [3]. Either P-selectin or E-selectin may have a dominant function in promoting leukocyte adhesion to the endothelium and subsequent recruitment into the inflammatory tissue. They play an important role in mediating the inflammatory damage. Up-regulation of P-selectin and E-selectin are also induced by focal cerebral ischemia at early stage, and are believed to play a significant role in the initial rolling interaction of leukocytes on activated endothelium in ischemic cerebral diseases[1]. Lumbrokinase (LK) is extracted from angleworm which is called Di Long (**地龙** Lumbricus) in Chinese traditional medicine. This study was aimed to find the effect of LK on P-selectin and E-selectin in rats.

Materials and Methods

1. Preparation of animal model

Male healthy Spragur-Dawley rats weighing from180 to 220 g (n=90) were used in this study, which were

provided by Experimental Animal Center of PLA General Hospital. Rats were anesthetized with 10% chloral hydrate solution, 350 mg/kg, by intraperitoneal injection. The ischemic model was made by modified Haruo Nagasawa's method[4]. The animal experiment was performed in the room temperature.

The rats were divided into 4 groups. 1) Normal control group (n=5), no any operation was performed. 2) Sham-operated group (n=35), the animal's skin and tissues were opened via a ventral midline incision to expose and carefully isolate left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA), then incision was closed. The animals were randomly divided into seven subgroups (n=5, each) according to the time of sacrifice (0.5, 1.0, 3.0, 6.0, 12.0, 24.0 and 48.0 hours after operation, respectively). 3) Ischemic group (n=35), the rats went the same procedures as in the sham-operated group, except that ECA was ligated with a 6-0 silk suture around the mobilized ECA stump at the crotch of CCA, the same silk suture placed around the CCA adjacent to the crotch, a monofilament nylon-wire of diameter 0.185mm inserted into ICA through a puncture of CCA. The nylon suture was then gently advanced about 18 mm, and reached along the distal segment of ICA to the origin of middle cerebral artery (MCA) to block blood flow into MCA，and then CCA ligated. The ischemic group was also randomly divided into seven subgroups (n=5, each) as the sham-operated group. 4) LK group (n=15), the rats went the same procedures as in the ischemic group. The animals in the LK group were randomly divided into 3 subgroups (n=5,each) according to the time of sacrifice (3.0, 6.0, 12.0 hours after operation, respectively). LK 10mg/kg (2000UK activity of LK, LK was kindly supplied by Bai Ao Pharm. Co, Ltd.) was given by intraperitoneal injection in the LK group 30 minutes before experiment, while same volume of normal saline was given in the sham-operated group and ischemic group.

2. Preparation of specimens

The rat was anesthetized with 10% chloral hydrate solution, 350mg/kg, by intraperitoneal injection. The thorax was rapidly opened with a transcardial perfusion by 100ml of 0.9% sodium chloride for rinsing blood. The brain was rapidly removed and stored in liquid nitrogen.

Specimen was taken out from liquid nitrogen and kept in freezer for recovering temperature to -20℃ gradually. Specimen was embedded in Tissue-Tek OCT compound. Coronal brain sections were cut from optic chiasm by Cryout1800 freezing sectioner. Sections were cut with 100 micron of compartment. Three sections were continuously cut for each group, 10 micron per section.

3. Immunohistochemistry for P-selectin and E-selectin expression

The sections were stained by immunohistochemistry for P-selectin, E-selectin and control (0.01M PBS instead of the primary antibody).

Frozen sections were come to room temperature and air dry for 30 minutes. Sections were fixed in cold acetone (4℃) for 10 minutes, washed in 0.01M PBS three times for 5 minutes each, incubated with 3% hydrogen peroxide diluted in PBS at room temperature for 10 minutes to quench endogenous peroxidase activity, washed in distilled water for 5 minutes and 0.01M PBS twice for 5 minutes each, and stained by ABC staining systems.

4. Procedures of ABC staining systems

1) Specimens were incubated with 1.5% normal blocking serum (Beijing Zhong Shan Biotechnology Co., Ltd) for 10 minutes at room temperature. The blocking serum was removed from slides without washing.

2) Specimens were incubated with primary antibodies (P-selectin 1:50, E-selectin 1:20, Santa Cruz Biotechnology, Inc.) or PBS (control) for 2 hours at 37℃, then closed in water box overnight at 4℃. Primary antibodies were removed from slides and specimens washed with three changes of PBS for 5 minutes each.

3) Specimens were incubated with biotinylated secondary antibody (Beijing Zhong Shan Biotechnology Co., Ltd) for 30 minutes at room temperature. The secondary antibody was removed and specimens were washed with three changes of PBS for 5 minutes each.

4) Specimens were incubated with avidin biotin enzyme reagent (Beijing Zhong Shan Biotechnology Co., Ltd) for 30 minutes at room temperature. The reagent was removed and specimens were washed with three changes of PBS for 5 minutes each.

5) Specimens were incubated with DAB (Beijing Zhong Shan Biotechnology Co., Ltd) in dark environment at room temperature, and color was observed under light microscopy until desired stain intensity developed. Specimens were washed in deionized water for 5 minutes.

6) Specimens were dehydrated step by step through alcohols and xylene. The excess xylene was immediately wiped off and 1-2 drops of neutral colophony was added, specimens were covered with a glass coverslip and observed under light microscopy.

5. Statistics

The numbers of P-selectin and E-selectin immunoreactive cells (positive cells) in a vision field were counted under light microscope at 200 × magnification. The mean of the positive cells were calculated in 10 vision fields per slide as the result of the slide.

The data was treated with SPSS 10.0. The values were presented as ($\bar{x} \pm s$). Analysis of variance was performed to compare the means across the groups. The student's test was performed for determining differences between ischemic and LK groups. If P value was less than 0.05 or 0.01, the difference was considered to be significant or very significant.

Results

No P-selectin and E-selectin positive cells were detected in normal control group.

The values of P-selectin positive cells in the different groups were shown in Tab.1. The morphological changes of P-selectin positive cells were shown in Fig.1-3.

As Table 1 shown, P-selectin immunoreactivity in ischemic lesion was detected as early as 30 minutes after the onset of cerebral ischemia and the positive cells were gradually increased with the ischemic time went along, the peak of expression at 6 hours after the onset, and then gradually decreased. However, no changes were observed in normal control group and the changes were indistinct in sham-operated group. In LK group, the numbers of the positive cells were significantly less than those in the ischemic group at 3 hours (P<0.05), at 6 hours (P<0.01) and at 12 hours (P<0.01), respectively, after operation.

Table 1. The number of P-selectin positive cells in ischemic lesion in rats.

Group	The time of cerebral ischemia (hours)						
	0.5	1.0	3.0	6.0	12.0	24.0	48.0
Sham-operated	6.7±0.4	3.8±0.5	4.5±0.7	9.3±1.2	4.9±0.4	8.4±1.6	6.9±1.1
Ischemic	8.2±1.5	42.7±8.4	73.5±10.3	145.8±19.9	64.6±8.5	22.3±3.1	17.5±2.7
LK			51.7±11.6*	83.1±13.5**	35.2±6.2**		

LK group vs ischemic group * P<0.05, ** P<0.01

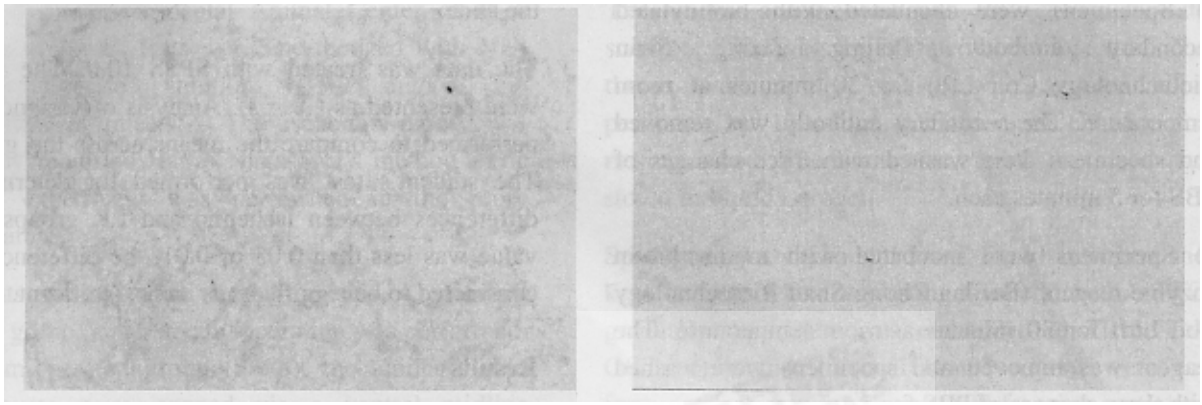

Fig.1 P-selectin (6 hours), sham-operated group, 200×.

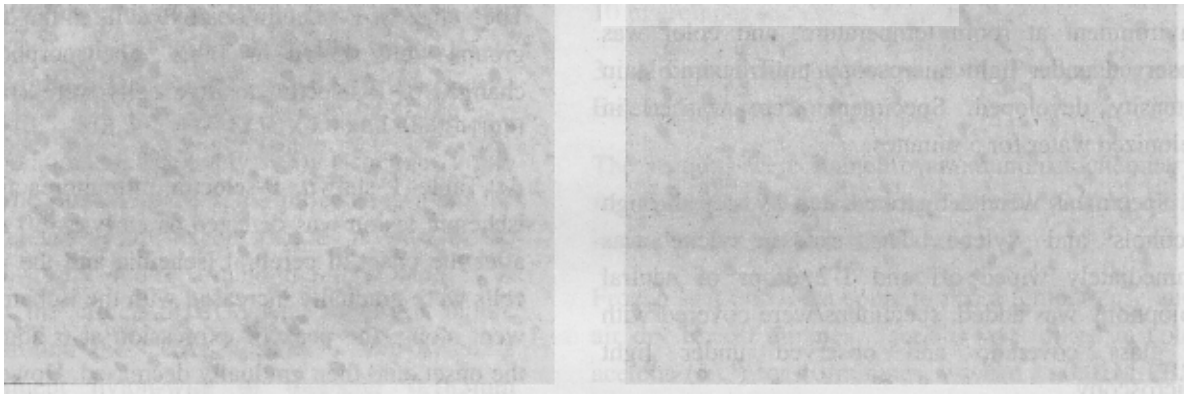

Fig.2 P-selectin (6 hours), ischemic group, 200×.

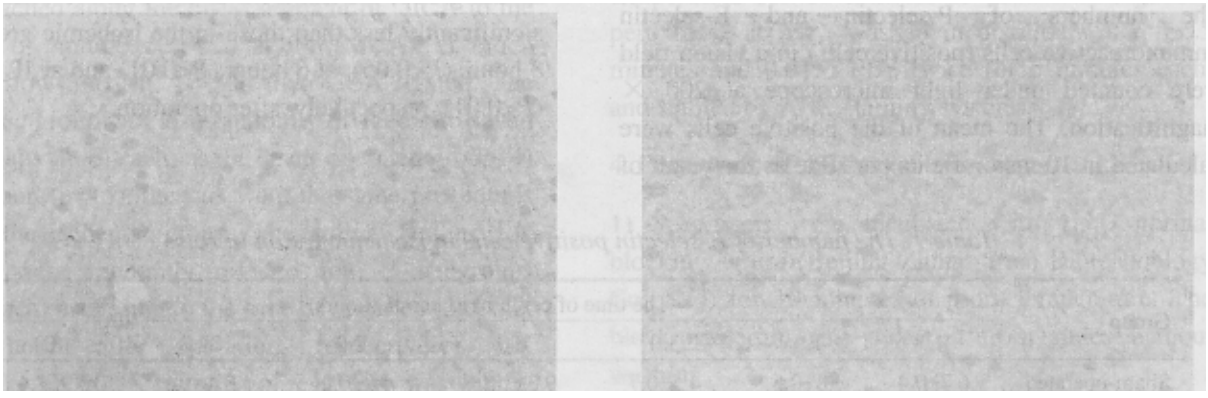

Fig.3 P-selectin (6 hours), LK group, 200×.

Fig.4 E-selectin (12 hours),sham-operated group, 200×.

Fig.5 E-selectin (12 hours), ischemic group, 200×.

Fig.6 E-selectin (12 hours), LK group, 200×.

The values of E-selectin positive cells in the different groups were shown in Tab.2, and the morphological changes of E-selectin positive cells shown in Fig.4-6.

Table 2. The number of E-selectin positive cells in ischemic lesion in rats of different groups

Group	The time of cerebral ischemia (hours)						
	0.5	1.0	3.0	6.0	12.0	24.0	48.0
Sham-operated	4.1±0.6	3.7±0.4	3.2±0.4	6.5±0.8	6.7±0.7	4.5±0.6	4.9±0.5
Ischemic	6.1±1.0	5.3±0.6	29.4±5.1	68.4±9.7	83.5±12.2	42.6±7.8	47.4±8.3
LK			21.8±3.5*	43.7±5.8**	55.4±7.3**		

LK group vs ischemic group * $P<0.05$, ** $P<0.01$

As table 2 shown, expression of E-selectin was slightly detected at 0.5 and 1 hour after the onset of cerebral ischemia. The positive cells were significantly increased at 3 hours after the onset, and the peak reached at 12 hours, and then decreased, in the ischemic group. The changes were very indistinct in sham-operated group. In LK group, the positive cells were significantly less than those in the ischemic group ($P<0.05$ at 3 hours after the onset, $P<0.01$ at 6 hours and $P<0.01$ at 12 hours, respectively).

Discussions

Selectins which include L-selectin, P-selectin, E-selectin, and also designated CD62 antigens, comprise a family of carbohydrate-binding proteins involved in mediating cellular interactions with leukocytes. L-selectin and designated LECAM-1 or CD62L are expressed on the majority of B and naive T cells, the most monocytes, neutrophils and eosinophils. L-selectin joins the injury of transient focal cerebral ischemia, but inhibiting L-selectin does not reduce the injury in animal model[5]. P-selectin (also designated GMP-140 or CD62P) is mainly expressed on activated platelets and endothelial cells, and E-selectin (also designated ELMA-1 or CD62E) expressed on endothelial cells. Zhang R et al[1] observed three models of focal cerebral ischemia, embolic, thrombotic and suture model, in rats, and found that the temporal profiles of up-regulation of P-selectin and E-selectin were similar in these models. In our experiment, the results showed that the P-selectin and E-selectin positive cells increased in ischemic cerebral lesion and the peaks developed at 6 and 12 hours after onset of ischemia, respectively, indicating that the P-selectin and E-selectin take part in the process of ischemic lesion, which is accordance with previous reports[1,3,5,6].

Dry angleworm is one of Chinese traditional drugs which are used to treat ischemic vascular diseases with good results[8]. The results of our previous studies also revealed that it might effectively decrease the death rate and ameliorate signs in cerebral ischemic gerbils. It is considered to be one of the effective drug for treating ischemic vascular diseases[8]. LK is a component extracted from angleworm, and is used to treat ischemic cardio- and cerebro- vascular diseases in China. It was shown by present study that LK given before ischemia might significantly decrease the levels of P-selectin and E-selectin and the decrease of P-selectin and E-selectin immunoreactivity lasted until 12 hours after the drug given. The results might explain why the drug was effective in model of ischemic stroke reported previously.

As early intervention after onset of acute cerebral ischemia，reperfusion is essential to minimize brain cell injury, although reperfusion itself may cause additional injury so-called reperfusion injury. The inflammatory reaction characterized in interaction of a part of early leukocyte with endothelium may contribute to the additional injury to blood vessels and surrounding brain tissue after the reperfusion.

The selectin family of adhesion molecules mediate the initial, rolling and tethering of leukocytes on endothelium. P-selectin is rapidly expressed on ischemic endothelium in the brain blood vessel[6]. Furuya K et al[7] reported that administration of a murine antibody preparation to rats brought out the production of host antibodies against the protein, activation of circulating neutrophils, complement activation, and sustained microvascular activation. Authors administered murine anti-rat ICAM-1 antibody, and found that the treatment did not significantly reduce infarct size, although it could inhibit neutrophil trafficking. And it was found that E-selectin, endothelial P-selectin, and ICAM-1 were up-regulated in animals treated with murine anti-rat ICAM-1 antibody. The observations provided several possible mechanisms for central nervous system related clinical deterioration in acute ischemic stroke. Ruehl ML et al[6] reported that blocking the selectin-mediated tethering step might limit the inflammatory component of reperfusion injury in the brain. Fucoidin, a competitive inhibitor of P-selectin and L-selectin, has been reported to decrease leukocyte accumulation during reperfusion of other organs. Ruehl ML et al[6] studied the effect of both leukocyte and endothelial selectin after inhibition with the selectin inhibitor, fucoidin 25 mg/kg, after cerebral ischemia and reperfusion, and found that selectin blockade significantly reduced cerebral infarction size and improved neurological function. In addition, a trend toward decreased cerebral edema was demonstrated by selectin inhibition. They considered that the treatment with a selectin anti-inflammatory agent had protective action on focal stroke and reperfusion. LK might significantly decrease the immunoreactions of P-selectin and E-selectin in our model. Therefore, it may be reasonably proposed that LK may ameliorate ischemic brain injury by down-regulation of P-selectin and E-selectin expression in ischemic brain tissue, although the mechanism of the down-regulation has kept to be not understood.

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