Environmental Medicine, Vol. 46 No.1, 2, 2002 (page 86 ~ 88)

Overexpression of Calpastatin Ameliorates Functional Recovery from Ischemic Injury in the Rat Heart

Atsuo MAEKAWA,[1] Jong-Kook LEE,[2] Keiko MIWA,[2] Takashi NAGAYA[3]
Yuichi UEDA[1] and Itsuo KODAMA[2]
[1] Department of Cardio-Thoracic Surgery, in the field of Surgical Sciences
Program in Function Construction Medicine, Graduate School of Medicine
Nagoya University, Nagoya 466-8550, Japan
[2] Department of Circulation
[3] Department of Endocrinology and Metabolism
Research Institute of Environmental Medicine
Nagoya University, Nagoya 464-01, Japan

Abstract: Calpain, a Ca^{2+}-dependent cytosolic cystein protease, is as an important key protease involved in the ischemic reperfusion injury such as cardiac stunning. Myocardial stunning is a postischemic reversible contractile dysfunction of the heart. We hypothesized that overexpression of calpastatin, an endogenous calpain inhibitor, would attenuate the ischemia/reperfusion injury in the heart. Rat hearts were transfected with human calpastatin gene incorporated into adenovirus vectors. The hearts infected with adenovirus containing lacZ gene were used as controls. The hearts after transfection were heterotopically transplanted into the abdomens of recipient rats and excised on the fourth day after the surgery. The functional recovery from 30-min global ischemia was studied in the isolated hearts perfused in-vitro. The recovery of left ventricular developed pressure, max dP/dt, and min dP/dt during 60-min reperfusion was significantly greater in the calpastatin transfected group than control group heart by gene transfer. Adenovirus-mediated overexpression of calpastatin could be a novel biological storage to improve heart transplantation by minimizing ischemia/reperfusion injury.

Key words: gene therapy, ischemia/reperfusion injury, calpain, calpastatin, cardiac troponin I

A post-ischemic reversible contractile dysfunction of the heart has been termed stunning.[1)] Calpain, a Ca^{2+}-dependent cytosolic cystein protease, plays an important role in causing the contractile dysfunction in myocardial stunning, but precise molecular mechanism involved are not well understood. Recent studies have suggested that transient Ca^{2+} overload at the beginning of reperfusion after ischemia leads to the activation of calpain-I (m-calpain), which initiates a cascade of degradation of various proteins, in particular troponin I.[2,3)] Calpastatin is an endogeneous inhibitor of calpain. Overexpression of calpastatin would, therefore, have a protective action against the myocardial stunning following ischemia/reperfusion.

The present study was designed to this hypothesis. We examined functional recovery following ischemic insult in rat hearts overexpressed with human calpastatin by adenovirus-mediated gene transfer.

Materials and Methods

The present study was approved by the Committee of experimental animals, Research Institute of Environmental Medicine, Nagoya University.

Adenovirus

Plasmid containing human calpastatin cDNA was provided by Dr. Maki (School of Agriculture, Nagoya University). cDNA of calpastatin was subcloned into pHM6 (Roche Diagnostics), which express Hemagglutinin antigen of *Hemophilis* influenzae (HA epitope tag). Recombinant adenoviruses expressing calpastatin (AdCS) were constructed by the method described previously.[4)] Briefly, cDNA for calpastatin-HA-tag expression cassette was subcloned into a shuttle vector (pAC CMV)[5)] and cotransfected with pJM17[6)] into HEK293 cells. Homologous recombination between pAC CMV-CS and pJM17 resulted in a replication defective adenovirus. After propagation in HEK293 cells, the recombinant viruses were purified by two cycles of ultracentrifugation through a cesium chloride gradient. Purified viruses were stored at -80°C in a solution containing 135 mM NaCl, 5 mM KCl, 10 mM Tris-Cl (pH 7.4), 1 mM $MgCl_2$, 0.02% BSA and 10% glycerol. Adenovirus vector carrying LacZ, Ad*LacZ* (provided by Dr. Hikaru Ueno) was used as control.

Gene Transfection procedure

Gene transfer was performed to donor hearts as described before by Kypson *et al.*[7)] In brief, male Wistar rats (150–200

Reprint request to: Dr. Itsuo KODAMA at the above adress.

g) was anesthetized with pentobarbital (50 mg/kg, i.p.) and heparinized with 200 USP heparin (i.v.). The hearts isolated from donor rats were arrested with 15 ml GIK solution containing glucose (389 mM), $NaHCO_3$ (8.3 mM), and potassium (20 mM), and then 1ml of adenoviral solution (8×10^{11} particles of AdCS or AdLacZ) was injected via the coronary arteries.

Heterotopic heart transplantation

Heterotopic intra-abdominal heart transplantation was carried out in male Wister rats (200–250g) through the technique described by Ono *et al.*[8] The surgery was undertaken immediately after gene transfection. The donor ascending aorta and recipient abdominal aorta were connected by end-to-side anastomosis, and then, the donor pulmonary artery and recipient vena cava were connected by end-to-side anastomosis. These rats were sacrificed on the fourth day after the transplantation for in-vitro measurements of cardiac function.

In-vitro measurements of cardiac function

Transplanted hearts were rapidly excised and immediately perfused at a constant pressure (80 cmH_2O) on a Langendorff apparatus with modified Krebs-Henseleit buffer (118.0 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH2PO$_4$, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 11 mM glucose, 2.0 mM sodium pyruvate; gassed with 95% O_2 + 5% CO_2 to obtain pH 7.4 at 37°C). After stabilization, left ventricular developed pressure (LVDP), maximum dp/dt (max dp/dt), minimum dp/dt (min dp/dt), and heart rate (HR) were evaluated with the use of an intra-ventricular thin wall latex balloon through the left atrium. Left ventricular end-diastolic pressure was stabilized at 10 mmHg. Following global ischemia for 30 minutes at 37°C, the hearts were reperfused. All values are presented as means ± SEM. Statistical comparison was performed by Student's *t* test. P values < 0.05 were considered significant.

Results

Immunohistochemistry

An immunohistochemical analysis with anti-HA protein monoclonal antibody showed extensive overexpression of CS in the cytoplasm of cardiomyocytes in the hearts in the CS group. This overexpression was recognized in 50–60% of total cell population in the hearts of CS group.

Recovery of the left ventricle after ischemia/reperfusion

Table 1 summarizes baseline parameters of cardiac function before ischemia There were no significant differences

Table 1 Baseline function of hearts before ischemia

Treatment group	n	HR (beats/min)	LVDP (mmHg)	max dp/dt (mmHg/sec)	min dp/dt (mmHg/sec)
CON	5	256.5±13.7	85.8±5.4	1932.2±53.3	-1604.3±96.5
CS	5	271.8± 9.3	82.1±4.9	2048.4±45.3	-1646.2±52.8

Values are mean±standard error of the mean. HR: Heart rate; LVDP: left ventricular developed pressure; CON: Controle; CS Calpastatin. There was no significant difference in these parameters between the two groups before ischmia ($p<0.05$).

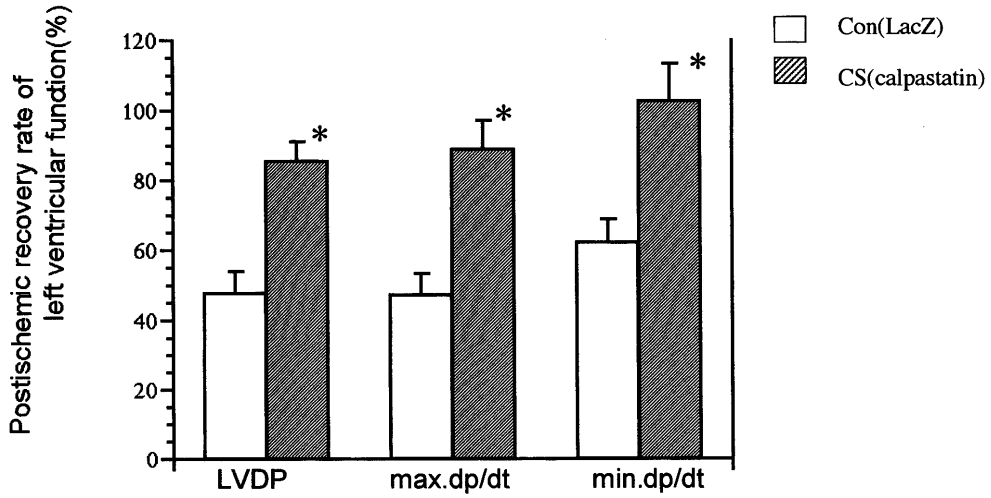

Fig. 1 Post-ischemic recovery rate of left ventricular functionin in control group (overexpressed with *LacZ*) and the CS group (overexpressed with calpastatin). LVDP: left ventricular developed pressure; max dp/dt: maximum dp/dt; min dp/dt: minimum dp/dt. Values represent mean ± SEM. *p < 0.05 vs control.

between CS and Control groups in any of these parameters. Figure 1 shows % recovery of LVDP, max dp/dt and min dp/dt at 60 min of reperfusion. The percentage recovery of there parameterse in CS group (n=5) was significantly higher than that in control group (n=5). In the controle group LVDP, max dp/dt and min dp/dt returned to 48%, 47% and 63% of the baseline. In the CS group, corresponding values were 86%, 89% and 103%, respectively.

Discussion

The present study revealed the beneficial effect of calpastatin overexpression, particularly on minimum dp/dt which reflects relaxation ability of heart (Fig 1). This result is compatible that overexpression of calpastatin might have protective effect on Tn I against ploteolysis by calpain. Calpain-Calpastatin system has been shown to be involved in apoptosis and necrosis of cardiac myocytes following ischemia/reperfusion.[2,9] An increase of calpastatin in cardiac myocytes was expected to have a protective action against ischemia/reperfusion injury through a prevention of calpain-dependent protein degradation, but the issue remains to be confirmed. Several studies have demonstrated selective TnI degradation under ischemia/reperfusion injury in heart.[10] Tn I is a component of troponin complex. The troponin complex is the regulatory element of the myofilament and mediates the calcium dependence of muscle contraction in heart. Degradation of Tn I by calpain, therefore, could play pivotal role in ischemia/reperfused heart, especially in the myocardial stunning characterized by an inhibition of calcium dependent contraction and relaxation. Because the phosphorylation of Tn I hastens relaxation, it is conceivable that proteolysis of Tn I impairs relaxation, which is hallmark of stunning.

Overexpression of calpastatin could be a proper gene to enhance tolerance to ischmia/reperfusion injury in heart.

References

1) Heyndrickx GR, Millard RW, McRitchie RJ, et al. Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. J Clin Invest 1975; 56: 978–985.
2) Gao WD, Liu Y, Mellgren R. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium: consequence of Ca^{2+}-depended ploteolysis? Circ Res 1996; 78: 455–466.
3) Gao WD, Atar D, Liu Y. Role of troponin I proteolysis in the pathogenesis of stunned myocardium. Circ Res 1997; 80: 393–399.
4) Hayashi Y, DePaoli AM, Burant CF, et al. Expression of a thyroid hormone responsive recombinant gene introduced into adult mice livers by replication defective adenovirus can be regulated by endogenous thyroid hormone receptor. J Biol Chem 1994; 269: 23872–23875.
5) Gomez-Foix AM, Coats WS, Baque S, et al. Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. J Biol Chem 1992; 267: 25129–25134.
6) McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology 1988; 163: 614–617.
7) Kypson AP, Peppel K, Akhter SA. Ex vivo Adenovirus-mediated gene transfer to the adult rat heart. J Thorac cardiovasc Surg 1998; 115: 623–30
8) Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac cardiovasc Surg 1969; 57: 225–229.
9) Papp Z, van der Velden J, Stienen G.J.M. Calpain-I induced alterations in the cytoskeletal structure and impaired mechanical properties of single myocytes of rat heart. Cardiovasc Res 2000; 45: 981–993.
10) McDonough JL, Arrell DK, Van Eyk JE. Troponin I Degradation and Covalent Complex Formation Accompanies Myocardial Ischemia/Reperfusion Injury. Circ Res 1999; 84: 9–20.

Received June 11, 2002; accepted June 26, 2002