

ORIGINAL**A novel peptide of endothelin family, 31 amino-acid length endothelin in patients with acute myocardial infarction**

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Abstract : Human chymase converts big endothelin (ET)-1 to 31-amino acid length ET-1 {ET-1(1-31)} that also possesses a potent vasoconstrictive action. In addition, ET-1(1-31) is an intermediate peptide, which is then readily transformed to mature ET-1 by the neutral endopeptidase 24-11. To investigate the relevance of pathophysiology of ET-1(1-31) *in vivo*, we have developed specific sandwich-type, enzyme-linked immunosorbent assay to measure the plasma concentration of ET-1(1-31) in healthy volunteers and patients with myocardial infarction. The plasma concentrations of ET-1(1-31) in healthy volunteers were 24.8 ± 5.2 pg/ml (n=11). ET-1(1-31) concentration in plasma was elevated in patients with acute myocardial infarction, and its elevation was several times higher and lasted longer than that of ET-1. In addition, tissue concentration of ET-1(1-31) in the myocardium from a patient with acute myocardial infarction was extremely high (12729.8 ± 2617.7 pg/mg protein). These results suggest that ET-1(1-31) may play some pathological roles in the remodeling, especially in sites where inflammatory cells produced a large amount of proteases, such as myocardial infarction. *J. Med. Invest.* 61 : 298-305, August, 2014

Keywords : endothelin-1(1-31), sandwich-ELISA, myocardial infarction, PTCA

INTRODUCTION

The peptides of endothelin (ET) family contain of 21 amino acids with three members, i.e. ETs-1, -2, -3 (1, 2). ETs are generated from the 38-amino acid precursors, big ETs, through cleavage of the Trp21-Val22 bond via the action of a membrane-bound metalloprotease, ET converting enzyme (ECE)-1 and -2 (3, 4). ETs are produced not only by endothelial cells, but also in various tissues, in

particular prepro-ET-1 mRNA is expressed widely in vascular endothelial cells, heart, brain, kidney, lung, uterus, and placenta (5). The circulating blood concentrations of ET-1 in healthy subjects have been reported to be very low as 1~3 pg/ml (6, 7), a finding suggesting exertion of their effects in a paracrine or autocrine manner in the local areas.

ET-1 is biologically active and acts as modulators of vasoconstriction, neurotransmission, cell proliferation, and hormone/cytokine production (8, 9). Thus, ET-1 is thought to play an important role in the regulation of circulatory system. The plasma ET-1 concentration was reported to be mildly elevated in patients with hyperlipidemia or atherosclerotic disease (10-12).

In our previous study, in addition to well-known

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ETs, ET-1(1-31) is generated following the specific cleavage of precursor big ET-1(1-38) at the Tyr31-Gly32 bond by human mast cell-derived chymase (13). Furthermore, it is an intermediate peptide which is transformed to ET-1 by the neutral endopeptidase (NEP) (14, 15). This ET intermediate peptide may be of clinical importance in conditions such as ischemic heart disease and acute coronary syndromes where there is increased generation of big ET-1(1-38) (16) and increased protease activities including chymase in tissues (17). In the following text, we use expression of ET-1(1-21) for mature ET-1 to avoid confusion with ET-1(1-31).

However, the pathophysiological and physiological role of ET-1(1-31) remains unclear. In this study, to investigate the relevance of pathophysiology and the concentration of ET-1(1-31) *in vivo*, we have used specific sandwich-type, enzyme-linked immunosorbent assay (ELISA) to measure the plasma concentration of ET-1(1-31) in healthy volunteers and patients with myocardial infarction with percutaneous transluminal coronary angioplasty (PTCA). In addition, we determined ET-1(1-31) concentration in heart tissue of patients with acute myocardial infarction.

METHODS AND MATERIALS

Blood Sampling

The patients (age ; 63 ± 11 years old, 9 males and 3 females) studied were the hospitalized patients with acute myocardial infarction with PTCA in Tokushima Red Cross Hospital, Tokushima prefecture, Japan. Blood samples of patients with acute myocardial infarction were collected before and the 1, 2, 3, and 7th day after the PTCA. Blood samples were obtained also from healthy volunteers (age ; 33 ± 13 years old, 6 males and 5 females) after an overnight fast.

The blood samples were collected and immediately transferred to a tube containing proteinase inhibitor (aprotinin 300 μ l, EDTA-2Na 20 mg), placed on ice for 10 minutes and plasma was immediately separated by centrifugation (3,000 rpm for 10 minutes at 4°C). Plasma samples were stored at -25°C until analysis.

The study was undertaken with the approval of the research ethics committee of Tokushima Red Cross Hospital and in accordance with the Declaration of Helsinki. Informed consent was obtained from all healthy volunteers and patients.

Human plasma sample preparation

Extraction of ET-1(1-31) in plasma was performed by absorption on Sep-Pak C18 cartridges (Waters, Milford, MA, U.S.A.). Sep-Pak C18 cartridges were preactivated by successive washes with 5 ml of acetic acid-ethanol (4% acetic acid, ethanol), 5 ml of methanol, 5 ml of Milli Q water and 5 ml of 4% acetic acid. Then 1 ml aliquot of the plasma sample was acidified with 3 ml of 4% acetic acid and applied to the column. The columns were washed with 1 ml of 4% acetic acid and 10 ml of Milli Q water, and the absorbed materials were eluted with 4 ml of acetic acid-ethanol. The eluate was dried under a continuous stream of nitrogen and the dried eluates were reconstituted with 1 ml ethanol, which were dried again for later analysis. The dried eluates were redissolved in 250 μ l of buffer A (0.02 M phosphate buffer, pH 7.0, 0.2% BSA, 0.4 M NaCl, 2 mM EDTA) and sonicated for 10 minutes. The reconstituted plasma samples were centrifuged at 12,000 rpm for 5 minutes in 4°C, which was taken for analysis.

A 1 ml of plasma was acidified with 250 μ l 2 N HCl kept for 30 minutes, neutralized by 250 μ l 2 N NaOH and then centrifuged at 10,000 rpm for 5 minutes. PBS(-) solution of twice volume was added to them after supernatant was loaded onto the column. The dissolved samples were applied to the ET-affinity column (IBL, Fujigaoka, Japan). The columns were washed with 20 ml of PBS(-), 5 ml of distilled water and the absorbed materials were eluted with 30% acetonitrile / 0.05% TFA. The eluate was dried using a SpeedVac concentrator and redissolved in 250 μ l of buffer A subjected the sandwich-ELISA.

Heart ET-1(1-31) concentration in a patient with myocardial infarction

The autopsy cardiac tissues from a patient with myocardial infarction were added to the cold 1 M acetic acid, pepstatin (10 ng/ml) solution about 10 times the amount of an organization. The samples were homogenized using Polytron homogenizer in ice and heat-treated it for 10 minutes at 100°C. After neutralized by 10 N NaOH, twice amounts of PBS(-) of the sample was added, and centrifuged in 4°C, 20,000 rpm for 30 minutes. The supernatant after centrifugality was purified by using affinity column, and it was considered as the sample for sandwich-ELISA.

Sandwich-ELISA

An EIA microtiter plate was coated with anti-ET mouse monoclonal antibody (1 µg/ml) (IBL, Fujioka, Japan) in 50 mmol/l carbonate buffer (50 µg/well), pH 9.6 at 4°C for overnight. The antigen was dissolved in buffer A (0.02 M phosphate buffer, pH 7.0 containing 0.2% BSA and 0.4 M NaCl and 2 mM EDTA) and mixed with biotinylation affinity purified ET-1(1-31) antibody (4.8 µg/ml) in buffer B (buffer A containing 10% skim milk) at the ratio of 1 : 1. The plates were allowed to stand at 4°C for overnight and after washing well by T-TBS buffer, and were blocked with 150 µl of blocking buffer (TBS containing 10% skim milk and 0.1% NaN₃) at 4°C for more than 6 hours. The wells were washed with T-TBS buffer, and then 50 µl /well of the sample were added to the plate at 4°C overnight. Biotinylated anti-ET-1(1-31) antibody (0.5 µg/ml, in 10 nmol/l phosphate buffer, containing 140 mmol/l NaCl and 1% BSA, pH 7.2) was added to each well and incubated at 4°C for 24 hours. After washing each well with T-TBS, the bound enzyme was reacted with En Vision+, peroxidase, anti-rabbit conjugated (2 µg/ml) (DAKO). Peroxidase activity was measured by using 3,3',5,5'-tetramethyl benzene dihydrochloride (Sigma, USA) as a substrate at 37°C, and the reactions were stopped by the addition of 2 M H₂SO₄ (50 µl/well). The density was determined by analysis of absorbance at 450 nm.

The cross-reactivity with a number of related compounds, such as ETs, vasopressin and bradykinin, was less than 0.01%.

RESULTS

Construction of the sandwich-ELISA for ET-1(1-31)

ET-1(1-31) peptide is prepared to the concentrations of 1, 5, 10, 20, 40, and 60 pg/well as an antigen, and the standard curve was established using the sandwich-ELISA. The standard curve with nearly a straight line could be obtained in a concentration dependent manner up to at least 1 pg/well, suggesting that we can measure quantitatively ET-1(1-31) up to 1 pg/well.

Establishment of the sampling method of ET-1(1-31) in blood

We added 20 ng/ml ET-1(1-31) into the serum of a pig, and collected the ET-1(1-31)-added sample.

We extracted ET-1(1-31) by using Sep-Pak C18 cartridges and affinity column. Then, we compared the recovery rate by these two methods. The sandwich-ELISA was performed by using ET-1(1-31) peptide (20 ng/ml) and the sample refined using each column as an antigen. The recovery rate of ET-1(1-31) peptide was the ratio with the measured value of the antigen obtained by each column. The recovery rate of ET-1(1-31) peptide obtained by the refining methods by Sep-Pak C18 cartridges was 12.9%, but affinity column improved the recovery rate very much, i.e., 99.2%. These results suggest that the refining process using affinity column was suitable for a sampling process of ET-1(1-31). Therefore, in the following experiments, the sample was prepared by affinity column.

Plasma concentration of ET-1(1-31) in healthy volunteers

The mean plasma concentrations of ET-1(1-31) in 11 healthy volunteers (6 men, 5 women, average age 33 ± 13 years old) with the sandwich-ELISA, was 24.8 ± 5.2 pg/ml. The mean value of male was 25.5 ± 3.1 pg/ml (average ages 32 ± 12 years old), and that of woman was 23.9 ± 7.3 pg/ml (average ages 34 ± 15 years old). Thus, there was no significant difference by sex in plasma concentrations of ET-1(1-31). There was no significant difference in plasma concentrations of ET-1(1-31) by ages either, i.e., those of 50's was 28.1 ± 4.8 pg/ml (n=3), and those of 20's 23.1 ± 5.3 pg/ml (n=7).

Plasma concentrations of ET-1(1-31) and ET-1(1-21) in patients with acute myocardial infarction

Fig. 1 shows the time course of plasma concentrations of ET-1(1-31) and ET-1(1-21) after acute myocardial infarction. Blood levels of ET-1(1-21) showed the normal value on admission, and after PTCA plasma concentrations rose promptly followed by rapid falling. Similarly with that of ET-1(1-21), the concentrations of ET-1(1-31) also showed rapid increase after PTCA and then decrease. The plasma concentration of ET-1(1-31) showed its peak at the 2nd day after the onset of myocardial infarction and remained at higher levels up to 7 days after the onset. Moreover, the plasma concentration of ET-1(1-31) always showed the 4~6 times higher value compared with ET-1(1-21) throughout the course. The concentrations of ET-1(1-31) in patients with myocardial infarction were 3~8 fold higher than that of healthy controls. Fig. 2 shows time course of the concentrations of ET-1(1-31) of each of the 12

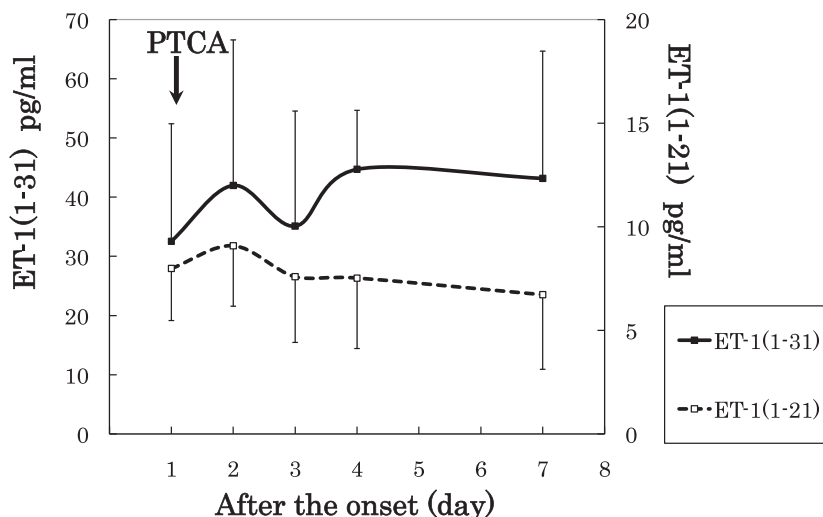


Fig. 1 Time course of the plasma concentrations of ET-1(1-31) and ET-1(1-21) in patients with acute myocardial infarction with PTCA measured by the sandwich-ELISA. Note that concentrations of ET-1(1-31) are higher than ET-1(1-21). ET-1(1-31) showed second peak and its elevation lasted longer than that of ET-1(1-21).

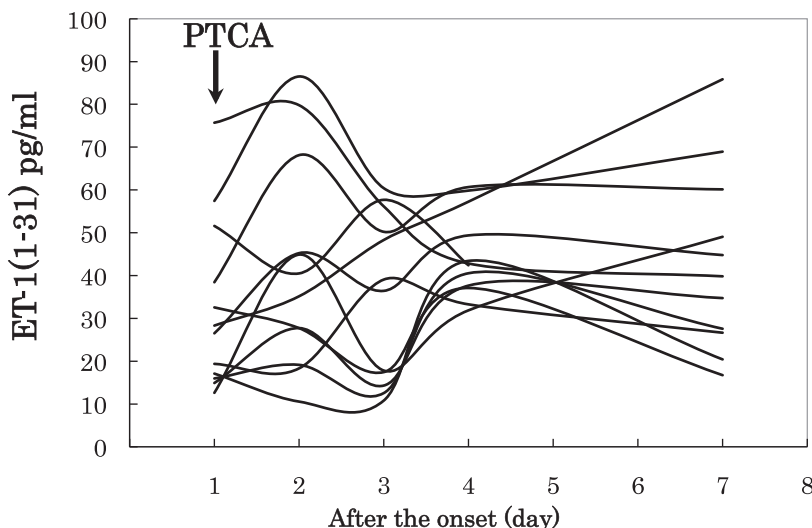


Fig. 2 The concentration of ET-1(1-31) in 12 patients with acute myocardial infarction after PTCA. The first peak of ET-1(1-31) levels appeared on the 2nd day after PTCA. Few patients showed gradual decrease after this peak, but most of the patients showed sustained elevation after the first peak after 3rd day.

patients with acute myocardial infarction with PTCA. Although the concentration and time courses were various, there was a tendency to show dual peaks. These results suggest that ET-1(1-31) may play some roles in acute myocardial infarction and its healing process.

Comparison between blood concentration of ET-1(1-31) and biochemical profile in the patients with myocardial infarction

As there were two patterns of ET-1(1-31) elevations, we compared the time courses of other parameters. Fig. 3 shows time course of the enzyme activity of CK, AST, LDH, and ET-1(1-21) within

ET-1(1-31) concentrations in 3 patients. All 3 patients showed that CK and AST rose markedly on the next day of PTCA, and it decreased promptly after the peak. LDH responded slowly compared to CK and AST, and maintained higher concentrations long time which was similar in time course with that of ET-1(1-31). In panel A, peak CK was 1365 IU/l suggesting the size of infarction was small. In this patient, ET-1(1-31) gradually decreased to normal values without second elevation and ET-1(1-31) showed a similar change with CK and AST. In panels B and C, in which the peak CK was higher than that of panel A, higher ET-1(1-31) levels was maintained for many days after the first peak, which was

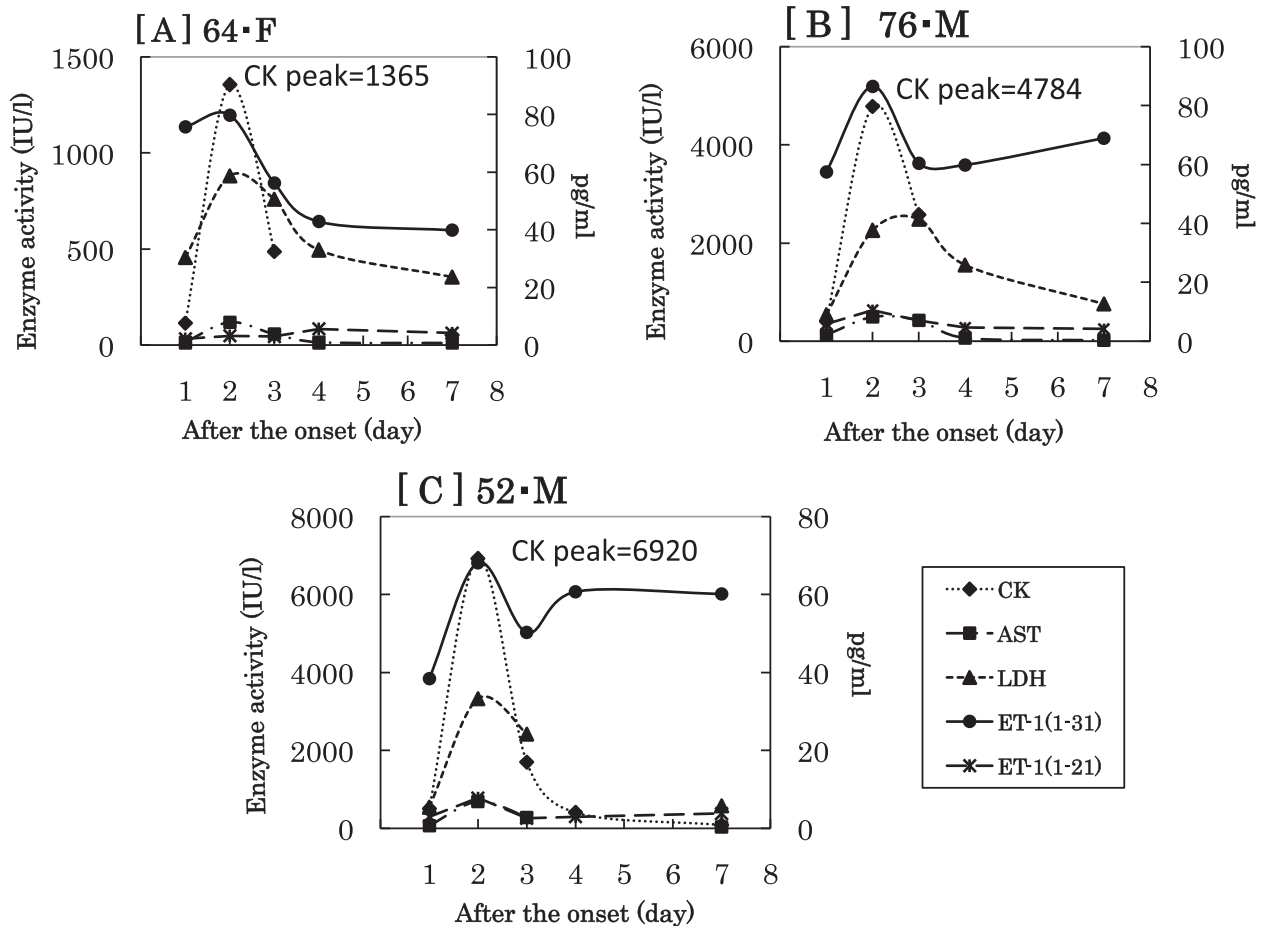


Fig. 3 The representative time course of ET-1(1-31), ET-1(1-21), the enzyme activity of CK, AST, and LDH changes in 3 patients with myocardial infarction. In small infarction (panel A), ET-1(1-21) and ET-1(31) decreased after the peak. In other 2 cases (Panels B and C), in which peak CK was higher, ET-1(1-31) showed sustained elevation after the first peak.

different in time course from that of ET-1(1-21), suggesting that ET-1(1-31) showed biphasic elevation in patients with larger infarction.

The ET-1(1-31) concentration of tissues in a myocardial infarction patient

ET-1(1-31) concentration of the heart (n=5) of the patient who died of myocardial infarction was measured with sandwich-ELISA, and the average value of ET-1(1-31) quantity per organization protein 1 mg was calculated. ET-1(1-31) concentrations of the heart with myocardial infarction showed extremely high values, i.e., 12729.8 ± 2617.7 pg/mg protein.

DISCUSSION

ET-1(1-31) is produced by several proteases in inflammatory sites and has similar action with ET-1(1-21) on vasoconstriction, neurotransmission, cell proliferation, and hormone/cytokine production.

Using a newly developed sandwich-type enzyme immunoassay for ET-1(1-31) with high sensitivity and specificity, we found that ET-1(1-31) exists at low concentrations in healthy volunteers, and was higher in patients with acute myocardial infarction than that of healthy volunteers. In addition, its elevation lasted longer, i.e. at least until 7 days after the onset, suggesting that ET-1(1-31) might have some pathological function after myocardial infarction.

In the previous reports (6, 7, 18-20), the plasma concentrations of big ET-1 and ET-1(1-21) analyzed by EIAs and radioimmunoassay was about 5.5 pg/ml and 1.5 pg/ml in healthy subjects, respectively. As for the plasma concentration of ET-1(1-31) in healthy subjects, Suzuki *et al.* (21) reported the value of 19.24 ± 5.70 pg/ml, and Leslie *et al.* 1.1 ± 0.1 pg/ml (22). In the present study, it was 24.8 ± 5.2 pg/ml, which is slightly higher than those of Suzuki *et al.* but much higher than those of Leslie *et al.* The discrepancy might be partly due to the difference of the column used for the purification of the antigen. In the present study, we established a

sandwich-ELISA that was purified antigen by ETs affinity column. The recovery rate of this affinity column was higher than Sep-Pak C18 cartridges that have been used in the previous studies. Thus, the differences in recovery of ETs might affect the measurements of the plasma concentration of ET-1(1-31).

The plasma concentration of ET-1(1-31) in normal subjects, several times higher than those reported in ET-1(1-21) or big ET-1, suggesting that ET-1(1-31) may have some pathophysiological function as it has similar action with ET-1(1-21), though slightly weaker than that of ET-1(1-21) (23-25). For gender differences, Miyauchi *et al.* have reported that men's plasma concentration is slightly higher than women's (26). Komatsumoto *et al.* (27) reported that there is a positive correlation between ET-1 and aging. In the present study, we found no significant difference between different genders or ages.

There have been no studies of circulating ET-1(1-31) in pathological conditions, although there are many studies of ET-1(1-21). Miyauchi *et al.* (14) and others (12, 16, 28-30) has been reported that plasma concentration of ET-1(1-21) in patients with acute myocardial infarction significantly rose after an attack and gradually decreased, and returned to the normal level in approximately two weeks. Furthermore, plasma concentrations of ET-1(1-21) rose after laparotomy and thoracotomy (31), coronary artery bypass grafting (32), and PTCA (33, 34), and these values were gradually normalized after surgery. In the present study, the peak values of CK, AST and those of first peak of ET-1(1-31) were observed at the same time after PTCA. In addition, ET-1(1-31) maintained higher concentration for a longer period, suggesting that ET-1(1-31) might relate to some pathophysiological function after myocardial infarction. The early peak of ETs as well as AST and CK seen after PTCA is due to reperfusion of damaged areas and washout by reperfusion. It is thought that the slightly higher concentrations at late phase might be due to participating in remodeling of blood vessel and the myocardium, as ET-1(1-31) also has functions similar to ET-1(1-21), although its function is weaker than that of ET-1(1-21).

It was considered that increased concentrations of circulating ET-1(1-21) are the result of production and release of ET-1(1-21). TGF- β and thrombin from platelets aggregated to the site of injury stimulate ETs production, and leakage or release of

ET-1(1-21) from injured endothelial cells by infarction or hemorrhage. In these areas, proteases are secreted abundantly, which cleave big ET into various forms of ETs, including ET-1(1-21) and ET-1(1-31). In agreement with these studies, we also found that the tissue concentration of ET-1(1-31) was extremely high in patients who died of myocardial infarction. ET-1(1-31) is the major bioactive peptide among the ET derivatives in human neutrophils (35). Furthermore, ET-1(1-31) possessed chemotactic activities toward neutrophils and monocytes (36) and stimulated eosinophil recruitment (37). Similarly with our study, ET-1(1-31) is more remarkable in the arteriosclerosis lesion than conventional ET-1(1-21), and strong expression is recognized with the inflammatory blood cells, suggesting that ET-1(1-31) participates in the development of the arteriosclerosis and myocardial remodeling with progress of inflammation (38). In addition, ET-1(1-31) is an intermediate peptide, which is then readily transformed to more potent ET-1(1-21) by the NEP, suggesting that ET-1(1-31) also plays an important role in providing a more potent peptide, ET-1(1-21).

Increase of the plasma concentration of ET-1(1-21) in heart failure correlates with the severity of heart failure, which has been reported to be useful in determining the prognosis (39, 40). It was thought that rise of ET-1(1-21) concentrations in the organic damages of vascular endothelium reflects the degree of vascular disease and the associated with remodeling. ET-1(1-31) equally, contributes to determine the severity of the conditions as well as diagnostic significance of a particular disease, suggesting that ET-1(1-31) might be an important marker for remodeling in postmyocardial infarction.

ET-1(1-31) is abundant not only in plasma but also in inflammatory lesions suggesting that this peptide plays in various pathological conditions. More recently, ET-1 receptor blockers, which also block ET-1(1-31) effects (23-25), became available in clinical medicine including pulmonary hypertension and myocardial infarction (41). The use of these drugs to inhibit ET-1(1-31) after myocardial infarction will help understanding of mechanism of ET-1(1-31) by future studies.

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