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Blockade of cell adhesion by a small molecule selectin antagonist attenuates myocardial ischemia/reperfusion injury

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Abstract

Reperfusion injury is related closely to inflammatory reactions such as the activation and accumulation of neutrophils. We investigated the efficacy of a novel small molecule selectin antagonist (bimosiamose) in a rat model of transient left coronary artery occlusion (30 min) and reperfusion (24 h). Treatment with bimosiamose (25 mg/kg, intravenously at reperfusion) showed a significant reduction in infarction area/area at risk of approximately 41% compared to vehicle control ($P=0.01$) and preserved the left ventricular function. The accumulation of polymorphonuclear neutrophils at the site of area at risk was decreased significantly, accompanied by 78% reduction of the myeloperoxidase activity. Parallel-plate flow chamber analysis revealed that bimosiamose showed a significant inhibition in rolling (62%, $P<0.001$) and adhesion (38%, $P<0.05$) of HL-60 cells to activated human umbilical vein endothelial cells compared with vehicle control. This study demonstrates for the first time that bimosiamose, a novel small molecule selectin antagonist, attenuates significantly ischemia/reperfusion injury.

Keywords: Reperfusion injury; Myocardial infarction; Selectin; Cell adhesion; Echocardiography

1. Introduction

One of the most important early mechanisms of myocardial reperfusion injury is related to neutrophil attachment to the vascular endothelium with subsequent infiltration into the damaged myocardium (Hansen, 1995). Polymorphonuclear neutrophils activation causes inflammation (Entman et al., 1991), endothelial cell dysfunction (Murohara et al., 1994), and cellular necrosis (Braunwald and Kloner, 1985). The adhesion process is initiated with polymorphonuclear neutrophils rolling primarily mediated by P-selectin and E-selectin on the vascular endothelial surface and shedding of L-selectin upon neutrophil activation (Carlos and Harlan, 1994). After rolling, polymorphonuclear neutrophils adhere to endothelium more firmly and infiltrate into tissue (Hansen, 1995). Through this series of neutrophil activation, rolling and transition to firm adhesion, polymorphonuclear neutrophils accumulate in the vascular lumen and cause plugging of the microvasculature (Engler et al., 1983). Furthermore, polymorphonuclear neutrophils infiltrate into myocardium and release a variety of cell-activating and cytotoxic mediators (Neumann et al., 1997), resulting in impaired coronary reperfusion. Therefore, prevention of neutrophil accumulation is quite an effective strategy for attenuation of myocardial reperfusion injury.

Each selectin recognizes carbohydrate-containing structures. Previous studies demonstrated that sialyl Lewis^x (SLe^x), Lewis^x or Lewis^a-containing carbohydrates are major ligands of the selectin family (Foxall et al., 1992). SLe^x antagonists can be used as an inhibitor of selectin binding to their ligand. Actually, it has been reported that a monoclonal antibody against SLe^x, SLe^x analog and SLe^x-containing oligosaccharide attenuated myocardial reperfusion injury in vivo (Buerke et al., 1994; Lefer et al., 1994; Seko et al., 1996b).

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In the present study, we used a small molecule non-oligosaccharide (bimosiamose) in a well-established model of rat myocardial ischemia/reperfusion. Bimosiamose inhibits E-, P-, and L-selectin binding to their ligand, SLe^x, generally and is more potent than SLe^x in vitro study (Kogan et al., 1998). The main purpose of this study was to determine the effects of bimosiamose on myocardial reperfusion injury, physiological cardiac function, and adherence of polymorphonuclear neutrophils to the vascular endothelium under physiological flow condition.

2. Materials and methods

2.1. Surgical procedures

Eight to ten-week-old male Sprague–Dawley rats (250 to 300 g weight) were used in this experiment. Rats were anesthetized with 40 mg/kg sodium pentobarbital intraperitoneally (i.p.) immediately before operation. Rats were intubated orally with a polyethylene tube for artificial respiration (SN-480-7, Shinano, Tokyo, Japan). The left anterior descending coronary artery was visualized using a microscopy and ligated with 6–0 silk suture. Myocardial ischemia was confirmed by epicardial cyanosis and wall asynergy. After 30 min of coronary artery occlusion, reperfusion was made by loosening the suture and verified visually. The chest wall and the skin were then closed with 3–0 silk suture. Animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.2. Reagents

The structure of bimosiamose (TBC-1269) elucidates two mannoses and two carboxylic acid groups in a configuration to mimic the dimeric structure of SLe^x (Fig. 1). TBC-1900, which differs from bimosiamose in only one bond located within the 6-carbon chain joining the two biphenyl moieties, was used as an inactive control compound. Bimosiamose and TBC-1900 were kindly donated by Texas Biotechnology (Houston, TX).

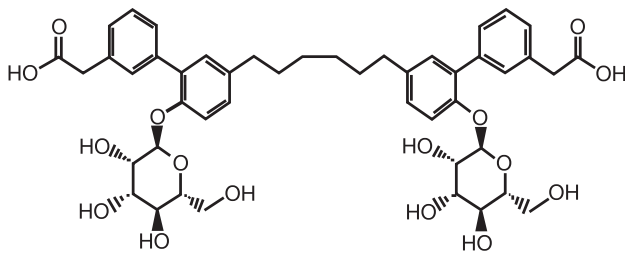

Fig. 1. Structure of bimosiamose (TBC 1269). Bimosiamose is a non-oligosaccharide glycomimetic and characterized as a small molecule (molecular weight 862.94).

2.3. Treatment protocol

The animals were assigned randomly into one of three treatment groups ($n = 7$ each), as follows: (a) intravenous bimosiamose (25 mg/kg) at reperfusion; (b) intravenous TBC1900 (25 mg/kg) at reperfusion; (c) sham-operated animals (thoracotomy with left anterior descending artery isolation but without ligation).

2.4. Measurement of area at risk and infarct sizes

At the end of 24 h of reperfusion, the anesthetized rats were intubated and thracotomy was repeated. The left anterior descending artery was religated tightly and Evans blue dye (2 ml of 1.0% solution) was infused via an inferior vena cava to determine the nonischemic zone (area not at risk). Hearts were then sliced transversely into four slices, and incubated in 2.0% triphenyl tetrazolium chloride (TTC) (Sigma, Tokyo, Japan) for 15 min at 37 °C to verify the viable and necrotic area in the ischemic myocardium (area at risk) as described earlier (Vivaldi et al., 1985). Each slice was weighed and photographed, and the areas of infarction, risk, and left ventricle were evaluated using computer-assisted planimetry (Scion Image β4.0.2) by observers blinded to the treatment protocol. The volumes of each area were determined by the following process: volume of area= $(A_1 \times Wt_1)+(A_2 \times Wt_2)+(A_3 \times Wt_3)+(A_4 \times Wt_4)$ where A is the percentage of each area by planimetry from subscripted numbers 1–4 indicating sections and Wt is the weight of the same numbered sections.

2.5. Measurement of rat heart cardiac function

Rats were anesthetized mildly with sodium pentobarbital. Transthoracic echocardiography was performed with a commercially available ultrasound machine (*Nemio*, Toshiba, Tokyo, Japan) before the operation, immediately after reperfusion and 24 h after reperfusion. A 7-MHz annular array transducer was used. Hearts were imaged in the two-dimensional mode in short-axis views at the level of papillary muscle. M-mode views were used to measure the left ventricular (LV) dimensions. LV end-diastolic dimension (LVDd) and end-systolic dimension (LVDs), and fractional shortening (FS%=[(LVDd − LVDs)/LVDd] × 100) were calculated from the M-mode recordings. Each dimension was presented as the average of measurements of three selected consecutive beats.

2.6. Assessment of myocardial neutrophil infiltration

To determine the extent of the polymorphonuclear neutrophil infiltration, histological staining was performed on the midventricular cardiac sections. After the myocar-

dial ischemia/reperfusion protocol, hearts were cut into sections and immediately fixed and stored in a 10% neutral buffered formalin solution (Wako, Tokyo, Japan). The tissue slices were then embedded in paraffin and cut into sections. The tissue sections were stained with Gill no. 3 hematoxylin and eosin. The number of polymorphonuclear neutrophils in area at risk was counted using microscopy. Five hearts from each group were examined, and the numbers of polymorphonuclear neutrophils per high power field were counted in five fields using the random observations and the counts were averaged.

2.7. Measurement of myeloperoxidase activity in cardiac tissue

Myeloperoxidase activity was determined in the ischemic cardiac tissue as described previously (Mullane et al., 1985). Briefly, myocardial tissue samples were weighed and homogenized in a potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The homogenates were centrifuged and then the supernatants were reacted with a 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine hydrochloride (Sigma) and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured using the microplate reader (*Ultramark*, BIO-RAD), and the results were expressed as units (U) of myeloperoxidase/100 mg tissue. One unit of myeloperoxidase was defined as that degrading 1 μmol peroxide/min at 25 °C.

2.8. Cell culture

Human umbilical vein endothelial cells were isolated from the normal-term umbilical veins and cultured on 0.1% gelatin-coated tissue culture dishes as described previously (Yoshida et al., 1998) in RPMI-1640 with 20% fetal calf serum (Life Technologies Oriental), endothelial growth factor (25 μg/ml, Funakoshi, Tokyo, Japan), and porcine intestinal heparin (50 μg/ml, Sigma), along with penicillin and streptomycin as antibiotics. HL-60, a maturation-arrested promyelocytic cell line, which has a SLe^x-bearing structure on the surface, was used in this study. The cells have been utilized for the analysis of selectin-dependent leukocyte adhesion in several studies (Ostrovsky et al., 2000; Jeong et al., 2001). HL-60 cells

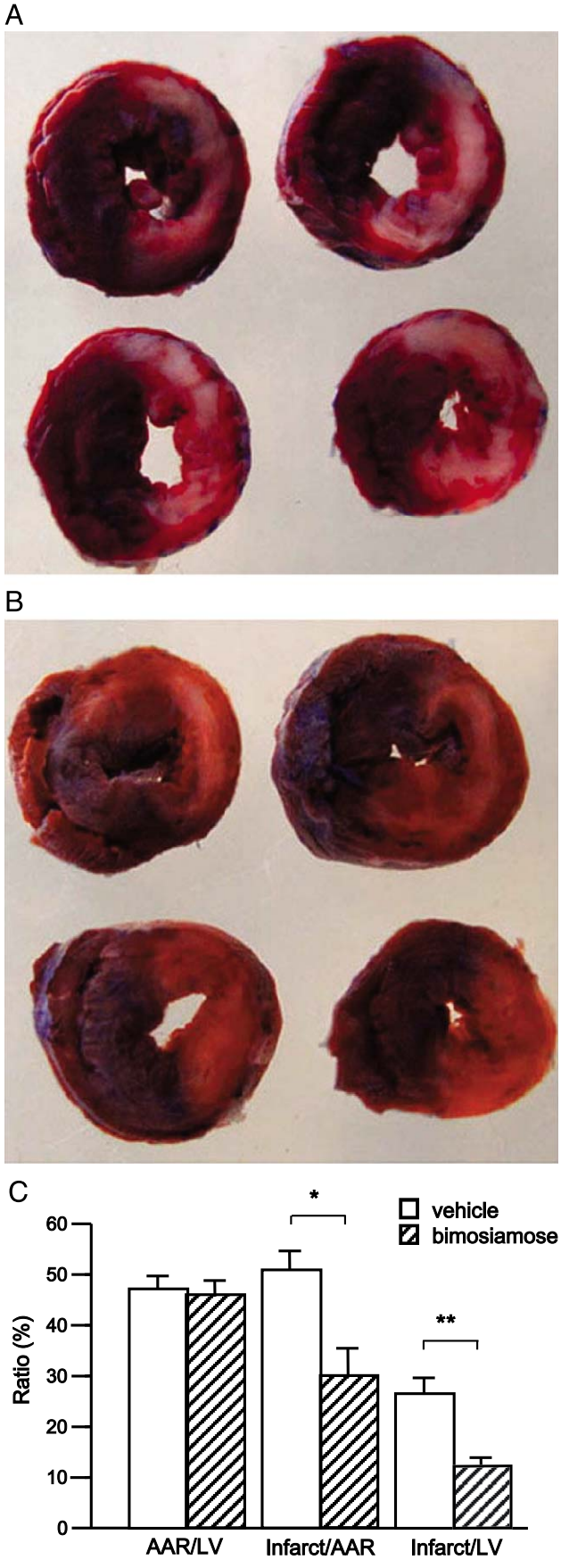

Fig. 2. (A and B) Representative photographs of transverse ventricular sections of the vehicle- and the bimosiamose-treated hearts. Hearts were stained by Evans Blue and triphenyl tetrazolium chloride to confirm the infarct area (white color), the area at risk (AAR) (brick-red color) and the area not at risk (ANAR) (blue color) after 30 min of ischemia and 24 h of reperfusion. (A) Photographs of the vehicle-treated heart. (B) Photographs of the bimosiamose-treated heart. (C) Statistical analyses of infarct size and AAR in hearts from the vehicle- and bimosiamose-treated rats. $*P = 0.01$, $**P < 0.01$ compared with rat given vehicle.

were cultured in RPMI-1640 containing 10% fetal calf serum with penicillin and streptomycin. For use in the flow-chamber experiment, human umbilical vein endothelial cell (passages 2 and 3) were plated onto 22-mm fibronectin-coated glass coverslips as previously described (Gerszten et al., 1999).

2.9. Adhesion assay under laminar flow

The parallel-plate flow chamber used in the present study was described previously (Yoshida et al., 2001). Briefly, the monolayer of human umbilical vein endothelial cells was stimulated with 20 U/ml of interleukin-1β (Genzyme) for 4 h and incubated with bimosiamose (10 or 100 μM) or vehicle (10 or 100 μM) for 30 min at 37 °C. In vitro dosage of bimosiamose was referred to the previous study (Abraham et al., 1999). After the incubation period, the monolayer of human umbilical vein endothelial cells was then positioned in a flow chamber mounted on an inverted microscope (IX70, Olympus, Tokyo, Japan). The monolayer of human umbilical vein endothelial cells was perfused for 5 min with a perfusion medium (Dulbecco's phosphate-buffered saline containing 0.2% human serum albumin) and then HL-60 cells (1×10^6/ml) were drawn through the chamber at controlled flow rates to generate calculated wall shear stress of 1.0 dyn/cm^2 for 10 min. The entire period of perfusion was recorded on the videotapes, and then transferred to a PC computer (Sony, Tokyo, Japan) for image analysis to determine the number of rolling and adherent HL-60 cells in 10 randomly selected × 100 microscopic fields. Cells were considered to be adherent after 10 s of stable contact with the monolayer. Rolling cells were easily recognized, because their velocity was much slower (up to 80 μm/s) than that of free-flowing cells.

2.10. Statistical analysis

Results are presented as mean ± S.D. All data were analyzed using analysis of variance (ANOVA), with a value of $P < 0.05$ considered significant.

3. Results

3.1. Bimosiamose reduced I/R-induced myocardial infarction

Representative photographs of transverse ventricular sections of bimosiamose- and vehicle-treated rats are shown in Fig. 2A and B. Area at risk/LV ratios were not significantly different between bimosiamose- and vehicle-

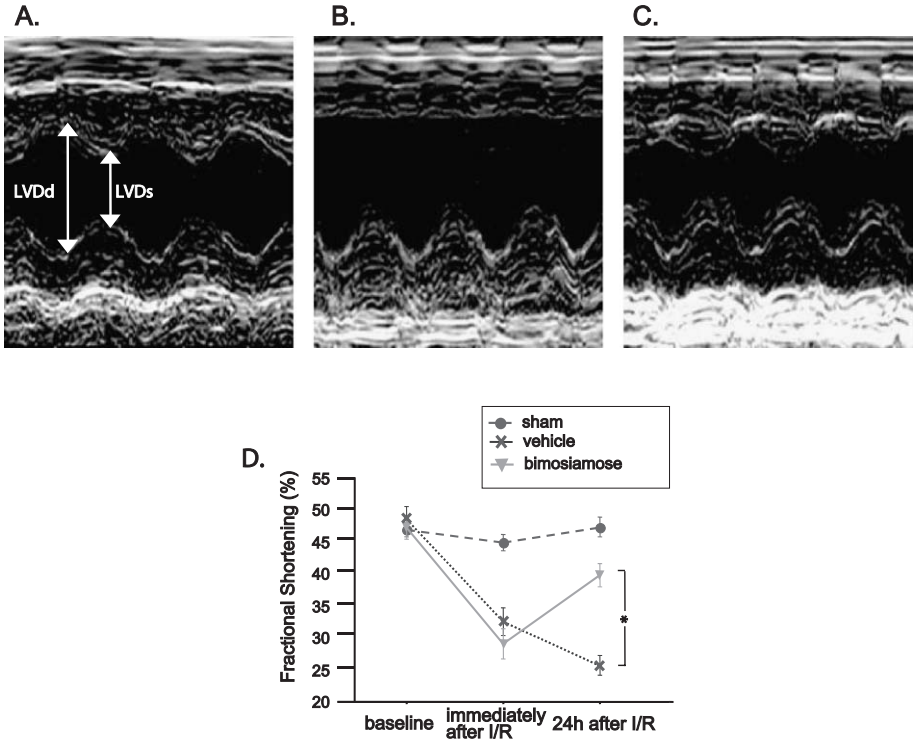

Fig. 3. M-mode echocardiogram was obtained from left ventricular short-axis view at the timing of before operation, immediately after reperfusion and after 24 h of reperfusion. (A) Normal M-mode echocardiogram as a baseline. (B) Representative M-mode echocardiogram of a vehicle-treated rat after 24 h of reperfusion. (C) Representative M-mode echocardiogram of a bimosiamose-treated rat after 24 h of reperfusion. (D) Serial change of FS% in each group. *$P < 0.001$ compared with rat given vehicle.

treated groups (bimosiamose 45.7 ± 2.9%; vehicle 46.7 ± 3.0%). In contrast, infarct/area at risk was significantly different between bimosiamose- and vehicle-treated groups after 24 h of reperfusion (bimosiamose 29.8 ± 5.7%; vehicle 50.7 ± 4.2%; $P = 0.01$). Bimosiamose also significantly decreased Infarct/LV compared to the vehicle-treated group (bimosiamose 11.8 ± 2.0%; vehicle 26.1 ± 3.5%; $P < 0.01$) (Fig. 2C).

3.2. Bimosiamose prevents myocardial dysfunction after infarction

As a baseline, normal M-mode echocardiograms were obtained from age- and weight-matched rats and their average baseline of %FS was 47.4 ± 1.9% (Fig. 3A). Fig. 3B is a representative M-mode echocardiogram of the vehicle-treated rat after 24 h of reperfusion showing that the wall motion in antero-lateral region of left ventricle is decreased remarkably. However, bimosiamose improved the regional wall motion in rat after 24 h of reperfusion (Fig. 3C). Serial change of %FS is presented in Fig. 3D. There was no difference in %FS at baseline and at immediately after ischemia/reperfusion between the bimosiamose- and the vehicle-treated groups. However, after 24 h of reperfusion, %FS of the bimosiamose-treated group was improved significantly compared to the vehicle-treated group (bimosiamose 39.9 ± 1.8%; vehicle 25.7 ± 1.6%; $P < 0.001$). Sham-operated group showed no significant change in cardiac function though serial echocardiographic measurements.

3.3. Bimosiamose reduces myocardial polymorphonuclear neutrophils accumulation

The number of polymorphonuclear neutrophils infiltrating into the area at risk of the reperfused myocardium was reduced obviously in the bimosiamose-treated rats. Significantly fewer polymorphonuclear neutrophils were found in the bimosiamose-treated hearts compared to the vehicle-treated hearts (bimosiamose 11.0 ± 2.6 vs. vehicle 23.2 ± 1.6/high-powered field; $P < 0.005$) (Fig. 4A). Myeloperoxidase activity was very low in the area not at risk myocardium of each group, however, was increased remarkably in the area at risk myocardium of the vehicle-treated hearts. Ischemia/reperfusion-induced myeloperoxidase activity was reduced significantly in the heart tissue of area at risk by treatment with bimosiamose as shown in Fig. 4B. Reduction rate was 78% compared to the vehicle-treated group ($P < 0.0001$).

3.4. Bimosiamose inhibits neutrophil rolling and adhesion under physiological flow conditions

Very few rolling or adherent HL-60 cells were observed when unactivated human umbilical vein endothelial cell (without Interleukin-1β stimulation) were used, and

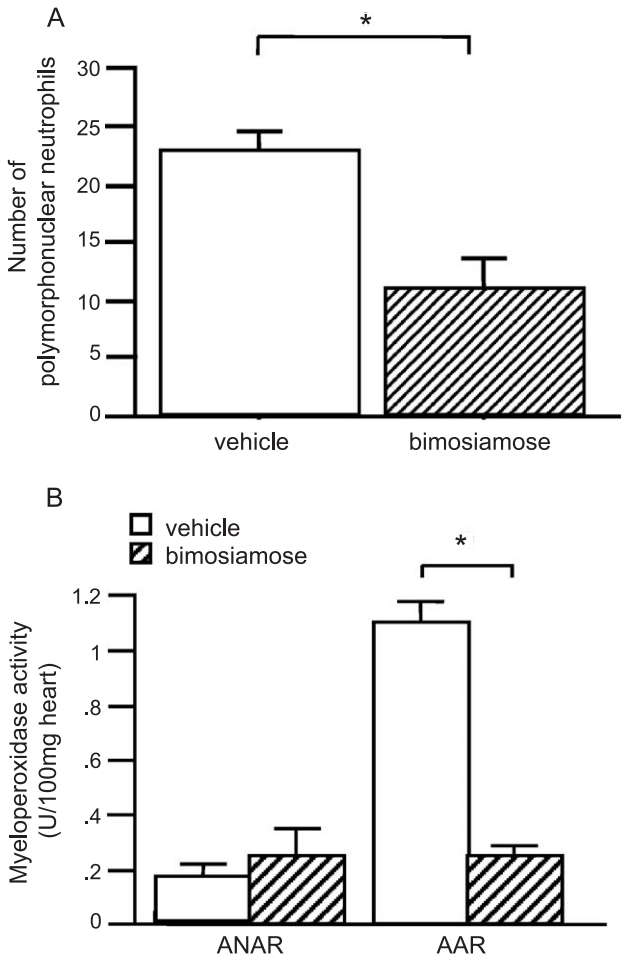

Fig. 4. (A) Average numbers of infiltrating polymorphonuclear neutrophils per × 400 field in the myocardium collected from bimosiamose-treated rats ($n = 5$) and vehicle-treated rats ($n = 5$). Polymorphonuclear neutrophils were counted in five fields of each heart and averaged. *$P < 0.005$ compared with rat given vehicle. (B) Myocardial myeloperoxidase activity in area at risk (AAR) and area not at risk (ANAR) cardiac tissue samples obtained from vehicle- and bimosiamose-treated rats. **$P < 0.0001$ compared with rat given vehicle.

similar results were obtained when unactivated human umbilical vein endothelial cells were incubated with bimosiamose (100 μM, 30 min) or vehicle (100 μM, 30 min). However, the number of HL-60 cells that showed rolling and adhesion to human umbilical vein endothelial cells was increased significantly after cytokine-activation (Interleukin-1β, 20 U/ml, 4 h), and similar degree of increase in HL-60 cells interactions was observed when activated human umbilical vein endothelial cells were pretreated with vehicle (Fig. 5A). In contrast, pretreatment with bimosiamose reduced remarkably the number of HL-60 cells that interacted with activated human umbilical vein endothelial cells (Fig. 5B). Reduction rate was significant in both rolling (62%, $P < 0.001$) and adhesion (38%, $P < 0.05$), when 100 μM of bimosiamose was used, compared with the vehicle-pretreatment cells as shown in Fig. 5C and D. The

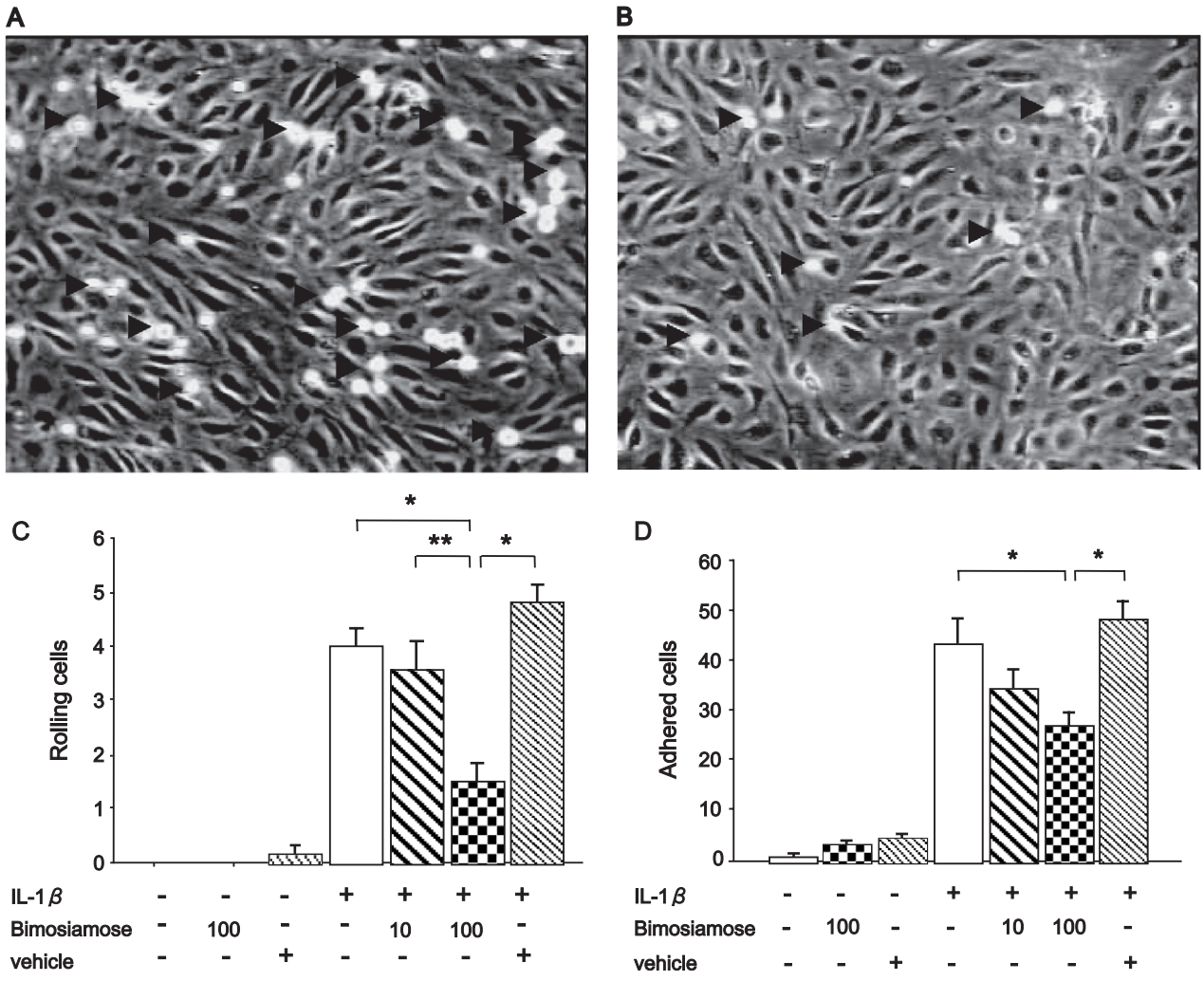

Fig. 5. Efficacy of bimosiamose in neutrophil rolling and adhesion under physiological flow condition. (A and B) Representative photographs of flow plate in condition of HL-60 cells and activated human umbilical vein endothelial cells (Interleukin-1β, 20 U/ml, 4 h) with vehicle pretreatment (100 μM) (A), and with bimosiamose pretreatment (10 or 100 μM) (B). Arrow heads are indicating HL-60 cells adhered to the monolayer of human umbilical vein endothelial cell. (C) Counts of rolling HL-60 cells per high power field under physiological flow condition. *$P < 0.001$ compared with non- or vehicle-pretreated human umbilical vein endothelial cells (Interleukin-1β+). **$P < 0.02$ compared with bimosiamose (10 μM)-pretreated human umbilical vein endothelial cells (Interleukin-1β+). (D) Counts of adhered HL-60 cells per high power field under physiological flow condition. *$P < 0.05$ compared with non- and vehicle-pretreated human umbilical vein endothelial cells (Interleukin-1β+).

effect of bimosiamose in inhibition of cell interaction was observed in a dose-dependent manner.

4. Discussion

Early reperfusion of the ischemic myocardium is the most important strategy for treatment of acute myocardial infarction to salvage the jeopardized myocardium. However, recent studies have revealed that reperfusion evokes a new myocardial damage which is related closely to accumulation of the activated inflammatory cells, especially polymorphonuclear neutrophils (Engler et al., 1983; Neumann et al., 1997; Dreyer et al., 1991). Several adhesion molecules are intervening in attachment of the circulating polymorphonuclear neutrophils to the myocardial vascular endothelium. The most important class of adhesion molecules related to leukocyte rolling, a precursor to firm adhesion and transmigration, is the selectin family. Previous studies have reported that correlation between soluble P- and E-selectin level and the incidence (Shimomura et al., 1998; Suefuji et al., 2000) and the severity (Li et al., 1997) of acute myocardial infarction. Moreover, several studies using animal models have demonstrated that expression of P-selectin (Seko et al., 1996a) and SLe^x (Seko et al., 1996b) is induced by ischemia/reperfusion in myocardium. Furthermore, administration of monoclonal antibody of L-selectin (Ma et al., 1993), P-selectin (Weyrich et al., 1993), E-selectin (Altavilla et al., 1994) and SLe^x (Seko et al., 1996b) and genetic deficiency of P-selectin and E-selectin in mice (Jones et al., 2000) attenuates the myocardial reperfusion injury in vivo.

In this study, we used a small molecule selectin antagonist bimosiamose to inhibit the binding of E-, P- and L-selectin to their counter ligands (e.g., SLe^x) via competitive binding of the glycomimetic. Although the beneficial effects of neutralizing antibodies of selectins for reperfusion injury have been demonstrated, their antigenicity hampers clinical utility (Friedman et al., 1996). In contrast, bimosiamose has several advantages, e.g., low molecular weight, simplicity and stability of the chemical structure, low probability of antigenicity, and a good safety profile observed in clinical testing including phase II clinical trials (Aydt and Wolff, 2002–2003). Furthermore, bimosiamose is more potent in blocking the binding of each selectin than their natural ligand SLe^x (Kogan et al., 1998; Abraham et al., 1999). The beneficial effects of bimosiamose on acute renal failure (Nemoto et al., 2001), liver damage due to hemorrhage shock (Ramos-Kelly et al., 2000) and ischemic liver damage (Palma-Vargas et al., 1997) have been reported in animal models. Although efficacies of bimosiamose for treatment of diseases associated with inflammatory reactions have been presented, the effectiveness of bimosiamose on myocardial reperfusion injury in vivo has not been reported.

As we demonstrated, TTC staining revealed that remarkable reduction of necrotic area in the bimosiamose-treated myocardium compared to the control vehicle-treated myocardium. Moreover, bimosiamose was effective not only in a reduction of myocardial necrosis but also in a preservation of physiological cardiac function assessed by echocardiogram. We also found treatment with bimosiamose resulted in a reduction of polymorphonuclear neutrophil infiltration into the post-ischemic myocardium. Furthermore, treatment with bimosiamose tended to reduce the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of cardiac small venules at the area at risk of postischemic myocardium (data not shown). Apparently, in the reperfused myocardium of the vehicle-treated rats, many polymorphonuclear neutrophils were observed with marked increase of myeloperoxidase activities in contrast to the non-ischemic myocardium, indicating that reperfusion after short-period ischemia induced the accumulation of polymorphonuclear neutrophils in the ischemic area. Activated polymorphonuclear neutrophils release a variety of cytotoxic substances (Neumann et al., 1997), which directly induce vascular endothelial dysfunction and myocardial inflammation. Hence, cardioprotective effect of bimosiamose in vivo could be based on the suppression of baleful polymorphonuclear neutrophils accumulation in the reperfused myocardium resulted in the inhibition of inflammation.

Neutrophil adherence to the vascular endothelium is a first step and essential event of polymorphonuclear neutrophil accumulation, which occurs after myocardial ischemia/reperfusion. Various adhesion molecules mediate this phenomenon. The selectin family is regarded as one of the most important class of adhesion molecules responsible for leukocyte rolling, a process important for subsequent firm adhesion and transmigration. We tested the in vitro effect of bimosiamose on the leukocyte attachment using HL-60, which is a maturation-arrested promyelocytic cell line and has a SLe^x-bearing structure on the surface, in the parallel-plate flow chamber. Treatment with bimosiamose showed a significant reduction in leukocyte rolling on and adhesion to Interleukin-1β stimulated human umbilical vein endothelial cells in a dose-dependent manner. As a general inhibitor of selectins, bimosiamose reduced strongly the leukocyte–endothelium interaction not only in rolling attachment but also in adhesion, a step following rolling interaction. Previous studies reported that decrease of rolling attachment in P-selectin knock-out mice (Mayadas et al., 1993). Further reduction of rolling attachment has been shown in E- and P-selectin double-knock-out mice (Frenette et al., 1996). However, no modification in rolling attachment has been observed in E-selectin knock-out mice (Labow et al., 1994). In addition, delay of late phase rolling has been observed in L-selectin knock-out mice (Arbones et al., 1994). These data suggested that coordination of E-, P- and L-selectin is necessary for proper localization of polymorphonuclear neutrophils to the inflammation site and leukocyte–leukocyte tethers (Alon et al., 1996). Because the function of each selectin differs from each other, it would be effective to inhibit all three selectins simultaneously to block their coordination. Therefore, general inhibition of each selectin is indispensable for definite anti-inflammatory effect.

Although several studies using anti-selectin agents in animal models of myocardial reperfusion injury showed significant effect, there still remains no clinical evidence of anti-leukocyte therapy. Several reasons for this discrepancy might be based on the study limitations. It is possible that the myocardial reperfusion is not completely equal between human and animal models in the several conditions such as the duration of myocardial ischemia, the existence of preconditioning, the degree of coronary atherosclerosis, and the difference of cardiomyocyte aging and collateral circulation. Since myocardial reperfusion injury is induced by various factors including calcium overload, oxygen-derived free radicals, altered metabolism, edema and inflammatory cells, it might be insufficient to target only one factor to improve clinical outcome of the patients who suffered from the reperfusion injury. Additional investigations about anti-leukocyte therapy are required in order to clarify the difference between experimental and clinical results.

In conclusion, the results of the present study demonstrate that robust cardioprotective effect against myocardial reperfusion injury by treatment with a novel small molecule selectin antagonist. This cardioprotective effect is based on the suppression of neutrophil rolling attachment to inhibit following polymorphonuclear neutrophil accumulation into the myocardial tissue. It makes great sense that pan-selectin blockade results in reduction of necrotic area as well as in preservation of cardiac physiological function. Although further investigation will be required to

evaluate the anti-leukocyte therapy, bimosiamose could potentially become a part of strategy to prevent the awkward reperfusion injury.

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