Limitations of Angiotensin-Converting Enzyme Inhibitor in Restenosis of a Deep Arterial Injury Model

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Cilazapril (CLZ) has been reported to reduce intimal hyperplasia in a rat carotid model of restenosis. The purpose of this study was to determine whether CLZ inhibits restenosis after deep arterial injury in lathyrictic rats. The lathyrictic rat was used as a model of deep arterial injury; in this model the internal elastic lamina is easily broken by balloon injury because of the fragility of the connective tissue. Deep arterial injury is defined as rupture of the internal elastic lamina with damage to both the intima and the media. The rats were divided into 4 groups (n=40): mild injury (intimal damage with intact internal elastic lamina), mild injury+CLZ, deep injury, and deep injury+CLZ. In the CLZ-treated groups, the drug was administered orally (10 mg/day) from 7 days before balloon injury until the time of sacrifice 21 days after balloon injury. The intimal hyperplasia was determined histologically using a computerized morphometry program. At sacrifice, blood pressure was lower in the CLZ-treated groups than in the untreated (control) rats ($p<0.05$). In the mild injury model, CLZ decreased intimal hyperplasia markedly. In contrast, CLZ failed to reduce intimal hyperplasia in the rats with deep injury. CLZ markedly decreased neointimal hyperplasia in mild injury. In contrast, CLZ failed to reduce intimal area in deep injury. The type of arterial injury seems to determine the effectiveness of CLZ.

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Key Words: Intimal hyperplasia; Angiotensin-converting enzyme inhibitor; Restenosis; Deep injury

Coronary restenosis has been called the Achilles heel of balloon angioplasty not only by virtue of its frequency^{1,2} but also because of our inability to prevent it; it remains a major obstacle to an ideal lasting coronary angioplasty procedure^{3–6}. Moreover, factors that can prevent restenosis remain undefined, although many studies⁷ have examined the clinical, morphologic, and technical factors involved in restenosis. However, local renin-angiotensin system activity may play an important role in the pathophysiology of the response to injury⁸. This hypothesis was confirmed by the observation that cilazapril (CLZ), an angiotensin-converting enzyme (ACE) inhibitor, decreases neointimal formation in rat arteries after balloon injury⁹.

In contrast, ACE inhibitors do not prevent clinical restenosis or have any beneficial effect on overall

clinical outcome following percutaneous transluminal coronary angioplasty (PTCA)¹⁰. This failure may be attributed to the difference in doses, methods of measurement, and animal species used in clinical trials. We believe that the difference in the preventive effect of CLZ between rat models and humans could in part be caused by the type of arterial injury, ie in rat models arterial injury is limited to the internal elastic lamina^{9,11} whereas in humans the injury is more extensive and is inevitably accompanied by medial injury^{12,13}.

In a rat experimental model, deep injury could not be produced easily. Lathyrism was produced in weaning rats by administration of β -aminopropionitrile, a specific inhibitor of the enzyme lysyl oxidase¹⁴. These rats have fragile connective tissue^{15,16} resulting in easy disruption of the internal elastic lamina by balloon

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injury. The main goal of the present study was to determine whether CLZ inhibits the intimal proliferation that occurs after deep arterial injury in lathyrotic rats.

Methods

In this study, 3-week-old male Sprague-Dawley rats (Charles River, Kanagawa) were used. During the experiment, they were individually housed in metal cages and fed a pellet diet (Oriental Cobe MF, Tokyo) with tap water ad libitum for 4 weeks to acclimatize them [environmental conditions: temperature, $25 \pm 10^\circ\text{C}$; humidity, 45–60%; and lighting time, 12 h/day (08.00–20.00 h)]. All experiments and animal handling were conducted in such a manner as to minimize stress and discomfort to the animals and to conform with the guidelines of the Juntendo University Experimental Society. The protocol was approved by the Institutional Animal Care and Use Committee of Juntendo University, Tokyo.

Lathyrism was induced in 45 rats. Balloon injury was performed at 10 weeks of age and the rats were divided into 4 subsets: rats with and without CLZ and with and without deep injury. CLZ was administered 1 week before balloon injury and was continued until the rats were killed 3 weeks after balloon injury.

Induction of Lathyrism in Rats

Lathyrism was induced by feeding the rats special C-food including small doses of β -aminopropionitrile (0.2%), which is implicated in the formation of cross-links during the synthesis of elastic and collagen fibers.¹⁴ The rats were maintained on the β -aminopropionitrile-containing diet from weaning (3 weeks of age) until the age of 7 weeks. The administration of β -aminopropionitrile significantly reduced the occurrence of interluminal granular elastin.¹⁵ The rats had fragile connective tissue owing to a abnormality in the synthesis of collagen and elastic fibers.¹⁶ Thus, the internal elastic lamina in these lathyrotic rats could easily be broken by balloon injury.

Balloon Injury

Endothelial denudation and injury to the vascular wall were achieved in the left iliac and femoral artery in 10-week-old male normotensive rats (weight 350–400 g). After the rats were anesthetized with ethyl ether by inhalation, the abdominal aorta and the left common iliac artery were exposed. Immediately before ballooning, heparin was administered as a single bolus (100 units/kg). A 2-French arterial embolectomy catheter (Edwards Laboratories, Santa Ana, CA) was introduced through the abdominal aorta

and advanced into the left femoral artery. The balloon was filled with water to distend the femoral artery sufficiently and to resist withdrawal of the catheter. After removal of the catheter, the incision site on the abdominal aorta was sutured and repaired as blood flow was maintained as before operation. The right iliac artery was left untraumatized.

Deep injury was induced 3 times by withdrawal with the balloon inflated sufficiently (filling volume minimum to 0.25 ml) and also by finally pulling back the catheter with repeated inflation and deflation. A balloon inflation filling volume of up to 0.12 ml induced only mild injury. Deep injury¹⁷ was defined as rupture of the internal elastic lamina demonstrable in histologic sections. In contrast, endothelial denudation without a tear in the internal elastic lamina was considered to be mild injury.

The rats were randomly divided into 4 groups: mild injury (intimal damage with intact internal elastic lamina), mild injury+CLZ, deep injury (both intimal and medial damage), and deep injury+CLZ. In the CLZ-treated groups, the drug was administered orally (typically 10 mg/kg per day mixed with normal food) from 7 days before balloon injury until the time of killing 21 days after balloon injury. Systolic blood pressure was measured in conscious restrained animals by the tail-cuff plethysmograph method. Measurements were started 1 week before angioplasty and repeated twice weekly. The mean of 3 measurements in 1 session was used. In 5 of the CLZ-treated rats, the plasma concentration of cilazaprilate (activated metabolite of CLZ) was determined by the radioenzymatic method and measured at sacrifice.

After 21 days, the rats were anesthetized, killed with an overdose of an intravenous anesthetic agent and exsanguinated. The abdominal and iliac arteries were carefully isolated to minimize handling trauma. Immediately before removal, the abdominal aorta was cannulated and the distal aorta and iliac arteries were flushed by manual injection of physiologic saline to clear any residual blood and perfusion fixed under constant pressure (95–100 mmHg).

The iliac artery was divided into 5 segments and the middle segment was used for measuring the study parameters. Sections for histologic analysis were embedded in paraffin and thin-sectioned in the routine manner. Hematoxylin-eosin and elastica-van Gieson stains were applied in the usual way. The intimal area, the ratio of intimal to medial area, and the maximal intimal thickness were measured using a computerized image analyser (IBAS 2000, Kontron, Munich, Germany). The cross-sectional areas of the lumen, intima, and media were determined by tracing manually the

margin of the lumen, the internal elastic lamina, and the outer margin of the media. Using standard mathematical formulae¹⁸ for a circle, the lumen and outer margin values were used to calculate the intimal area and the ratio of intimal to medial area. At the sites of a defect in the internal elastic lamina, the natural curves connecting the interrupted ends were approximated. In all cases, a single section demonstrating the greatest extent of neointimal proliferation was selected for computerized image analysis.

For identification of smooth muscle cells (SMCs), antibody against rabbit α -smooth muscle (SM) actin (1A, Dako, Amsterdam, The Netherlands) was immunostained on paraffin-embedded sections. The number of α -actin-positive cells per high-power field ($\times 400$) was calculated using a superimposed grid to facilitate counting.

Histologic sections from the control and CLZ-treated animals were examined in random sequence by an independent investigator who was blinded to the type of treatment protocol.

Analysis of Data and Statistics

Data were recorded as the number of iliac arteries in each experimental group and are expressed as means $\pm$ SD. Histopathologic data were analyzed using the Student's *t* test. Paired and unpaired *t* tests were used as appropriate. A comparison of categorical data was made using the 2-tailed Fisher's exact test. The correlation between the intimal to media thickness ratio was determined by linear regression analysis. Statistical significance was set at a *p* value less than 0.05.

Results

Each group of animals was matched for age and weight at the start of the experiment. Weight gain and final weight did not differ significantly between groups. The dosage of CLZ used in this model was much higher than that used clinically in humans. The concentration of cilazaprilate was 147 ± 22 ng/L, which is 70-fold higher than the usual clinical concentration. CLZ was well absorbed by the rats. Before the study, mean tail arterial blood pressure was similar in the CLZ-treated and untreated rats. However, systolic blood pressure was significantly lower in the CLZ-treated group than in the untreated group after the administration of CLZ (Fig 1). Heart rate was similar in the CLZ-treated and the untreated rats before and at the end of the study. There was no significant difference between the deep injury and mild injury group with respect to body weight and arterial blood

Fig 1. Effect of cilazapril treatment on systolic arterial pressure at different time points. Cilazapril was administered for 4 weeks mixed with food at a daily dose of 10 mg, starting 7 days before ballooning. ○, cilazapril-treated rats; ●, untreated rats. Data are expressed as mean values and 95% confidence intervals.

Fig 2. Bar graph illustrating the effect of cilazapril on intimal lesion size after mild injury. Bar heights are group means $\pm$ SEM. The cilazapril-treated and untreated groups are compared with respect to intimal area (μm^2), the ratio of cross-sectional areas of the intima to media (%), and maximal intimal thickness (μm). **p* < 0.01, compared by Student's 2-tailed *t* test with the untreated groups.

pressure.

Morphologic Results

Forty-five rats underwent balloon intervention: 25 in the deep injury group and 20 in the mild injury group. Histologic analysis revealed that 80% (*n* = 20) of rats that received high-volume inflation sustained a deep injury, with the remaining 20% (*n* = 5) dying within a few days after balloon injury as a result of a rupture of the ballooning site. The use of low-volume inflation induced only the desired mild injury.

Three weeks after both the mild and the deep arte-

Fig 3. Photomicrographs showing intimal lesions 21 days after balloon catheter injury. Representative elastica-van Gieson-stained cross-sections of left iliac artery from cilazapril-treated rats (B and D) and untreated rats (A and C). A and B are in the mild injury group. Note that the structural integrity of the internal elastic lamina is intact. C and D are in the deep injury group. Note that the internal elastic lamina is disrupted. IP, intimal proliferation; IEL, internal elastic lamina; M, media. Bar = 100 μ m (original magnification $\times 40$).

Fig 4. Bar graph illustrating the effect of cilazapril on intimal lesion size after deep injury. Bar heights are group means $\pm$ SEM. The cilazapril-treated and untreated groups are compared with respect to intimal area (μm^2), the ratio of cross-sectional areas of the intima to media (%), and maximal intimal thickness (μm).

rial injury, neointimal thickening was observed at the site of arterial injury. The neointima was expanded by a combination of fibrocellular and extracellular matrix with endothelialization of the luminal surface. CLZ markedly prevented neointimal formation after superficial injury, as shown in Fig 2. The intimal area was significantly larger in the untreated group than in the CLZ-treated group (151 ± 25 vs $37 \pm 10 \times 10^3 \mu\text{m}^2$;

$p < 0.01$), as was the ratio of the intimal to medial area (116 ± 16 vs $27 \pm 6\%$; $p < 0.01$) and maximal intimal thickness (84 ± 7 vs $27 \pm 7 \mu\text{m}$; $p < 0.01$), which are shown in Fig 3. The neointima contained predominantly α -SM actin-positive cells (more than 95% of the total cell population). The difference in the number of α -SM actin-positive cells between the control group and the CLZ-treated group was statistically significant (321 ± 45 vs 57 ± 10 ; $p < 0.01$).

In contrast, CLZ failed to reduce intimal hyperplasia in rats with deep injury. Vessel wall damage after balloon injury was more extensive using high inflation pressure with disruption of the internal elastic lamina. There was no significant difference between the untreated and CLZ-treated groups with respect to intimal area (186 ± 25 vs $160 \pm 35 \mu\text{m}^2$), the ratio of intimal to medial area (116 ± 20 vs $105 \pm 8\%$), and maximal intimal thickness (103 ± 11 vs $97 \pm 4 \mu\text{m}$) (Fig 4). The difference in the number of α -SM actin-positive cells was not statistically significant (392 ± 51 vs 348 ± 64). The dominant cells, contributing at least 95% of the accumulation of neointima, were α -SM actin positive. The thickness of the neointima was more extensive in deep injury than in mild injury, and the differences were significant with respect to intimal area, the ratio of intimal to medial area, and the number of SMCs.

Discussion

Restenosis is a major clinical problem associated with PTCA.¹⁻⁶ Many drugs have been reported in experimental studies to be useful for preventing restenosis.⁹ Among these drugs, CLZ is considered to be the most effective,⁹ inhibiting intimal hyperplasia by up to 80%. The present results showed that CLZ prevents neointima formation after mild vascular injury, confirming the finding of previous reports.⁹ However, CLZ was ineffective in a model of deep arterial injury with a ruptured internal elastic lamina despite adequate drug absorption. The presence or absence of rupture in the internal elastic lamina and the severity of injury may determine the extent to which CLZ inhibits intimal hyperplasia after balloon injury.

This study is unique and different in several aspects from previous studies. First, the use of lathyrictic rats as a model of deep arterial injury is a novel and intriguing idea. The principal advantages of the rat model are that the animals are readily available, easy to handle, and inexpensive to purchase and maintain. For these reasons, rats have been widely used to study intimal hyperplasia. However, in the rat model, the Fogarty catheter is of less robust construction than the catheters used for PTCA, which means that damage to the intima only is a prominent feature. This is a disadvantage when the rat model is used as a model for humans. To solve this problem, various species, such as pigs²⁰ or monkeys,²¹ have been introduced as potential deep injury models. However, the number of institutes where such experiments can be carried out is limited because these models are expensive and large-scale equipment is needed. Taking this into consideration, introduction of the rat deep injury model is a novel idea as the lathyrictic rat is an excellent model with good reproducibility of destruction of the internal elastic lamina after balloon injury. Additionally, the response of neointimal formation in the mild injury using lathyrictic rats was compared with previous studies using Sprague-Dawley rats.^{9,22-24} The degree of neointimal formation and the histologic features in lathyrictic rats were similar to those seen in Sprague-Dawley rats. The neointima was composed predominantly of SMCs in both rats. Thus, lathyrictic rats might be useful as a model of restenosis as deep injury is easily induced in lathyrictic rats.

Second, in conventional experimental models, the artery into which the balloon catheter was inserted is ligated after ballooning. This may cause thrombus formation extending to the injured artery, thus accelerating SMC proliferation. Moreover, it is known

that intimal hyperplasia is augmented proximal and distal to arterial ligations.^{25,26} In this study, however, the incision site of catheter insertion was sutured, not ligated, so that blood flow could be maintained as it was before operation and, although it is true that vessel suture requires some technical skills, it provides an excellent model in which the effects of thrombus formation could be minimized because postoperative blood flow through the abdominal aorta is not interrupted.

In this study, we focused on the severity of injuries because there are differences in the pathologic findings between human PTCA sites and rat injury models.⁹⁻¹³ At the angioplasty site in humans, medial and intimal disruption and fragmentation of the internal elastic lamina in the arterial wall occur as a consequence of this procedure, which is deep injury or type III injury according to the classification of Ip et al.²⁷ However, injuries in rat models are mainly mild injury or type II, in which the internal elastic lamina is not destroyed.

The question arises as to why CLZ does not prevent restenosis after balloon injury in the deep injury model. It is evident that the ineffectiveness of CLZ in deep injury is not due to inadequate drug absorption compared with mild injury, because there were no significant differences in serum cilazaprilate or in the decrease in blood pressure between the deep injury and the mild injury groups.

Firstly, deep arterial wall injury with exposure of the media appears to be a potent thrombogenic stimulus.^{28,29} Mural thrombus is a major contributor to the intimal proliferation that leads to restenosis;³⁰ if injuries are limited to the intima, it is generally considered that mural thrombus has not formed.³⁰ Thus, the degree of injury is said to be an important determinant of thrombus formation.³¹ In fact, in our preliminary study, mural thrombus overlying the exposed area of the media was seen in the presence of deep injury in 100% of arteries (5 of 5 deep injury arteries at 3 days after ballooning). In contrast, in the absence of fragmentation of the internal elastic lamina, complete endothelial cell loss but no mural thrombus was observed (5 of 5 mild injury arteries at 3 days after ballooning). In the presence of deep injury created by the balloon technique, which is an important stimulus for thrombus formation, the beneficial effects of CLZ may be limited if its antiproliferative effect is linked to inhibition not of thrombus formation but of angiotensin II production, as proposed previously.^{19,32} Furthermore, platelet adhesion in deep injury is the result of exposure of medial collagen types I and III, which are potent in promoting platelet aggregation;³³ in contrast, mild injury results in exposure of basement

membrane collagen type IV and elastin, which are not potent stimulators of platelet aggregation.^{34,35} Thus, following mild injury, platelets are found in very small numbers and tend to be deposited in a monolayer deposition.³⁶ In contrast, deep injury promotes both adhesion and aggregation of platelets to the deeply injured areas.³¹ The degree of platelet accumulation has been related to the extent of vessel wall damage.³⁷ In the case of deep injury, continuous platelet aggregation leads to continuous stimulation of SMC proliferation. Therefore, it is clear that CLZ does not have a preventive effect in deep injury.

Secondly, the elastic lamina performs a barrier role, protecting the SM in the blood vessels from migratory factors;³⁸ the internal elastic lamina also has an important defense function, which is compromised if the lamina is ruptured. Deep injury weakens the defense barrier and migration continues unchecked and proliferation is not terminated as long as inhibition by the autocrine system continues.

Thirdly, focal lethal damage to the endothelium can lead to increased local mitogenic activity, which can attract and stimulate neighboring SMCs.^{27,39} In contrast, endothelial cells with intact function may exert an inhibitory effect on neighboring SMCs.^{27,39} In other words, intact endothelium is a potent inhibitor of the growth of SMCs;⁴⁰ their proliferation ceases when a mechanically denuded area is re-endothelialized. In the case of deep or wide endothelial injuries, recovery of the endothelial cells takes a long time⁴¹ and some areas are never re-endothelialized.⁴⁰ As stimulation of mitogenic activity in denudated areas is thought to be sustained over a long period in deep injury, the inhibitory effects of CLZ may be incomplete.

Finally, a local angiotensin system plays an important role in the regulation of the vascular SMC response to arterial wall.^{8,42,43} Moreover, inhibition of tissue ACE is a key determinant of the effect of the drug on neointima formation. Interestingly, an increase in neointimal size is associated with a proportional increase in the residual tissue ACE activity.^{42,43} Thus, in this study, tissue ACE activity may be greater in rats with deep injury than with mild injury. The dose of CLZ used in this study may have been too low to suppress the ACE level in deep injury. Severe arterial damage requires more antiproliferative drug to be administered to prevent intimal hyperplasia than does mild injury. However, significantly higher doses than those used in our study would most probably not be tolerated because of clinical side-effects. The doses administered in this study were the same as published previously,⁹ which already achieved 70 concentration compared with human concentration. Our data show

that response to a drug with known antiproliferative activity differs markedly depending on the type of arterial injury.

The limitations of this model include the use of elastic iliac arteries as opposed to muscular human coronary arteries. Moreover, in the rat model there is no complicating pre-existing factors such as human atheromatous plaque. The process of human restenosis is complex,⁴³ involving many factors, including remodeling, thrombus formation, and SMC proliferation; in contrast, the rat model is primarily a SMC proliferation model. Thus, rat models do not reproduce the clinical situation exactly. Despite these limitations, this model may be an important first step in evaluating drugs that appear to be valuable in the prevention of restenosis as deep injury models provide a great deal of information about the drug response to injury.

In conclusion, in our mild injury model, CLZ decreased neointimal hyperplasia markedly. In contrast, CLZ failed to reduce intimal hyperplasia in deep injury. The type of arterial injury influences the effectiveness of CLZ in intimal hyperplasia after balloon injury.

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