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GR144053, a fibrinogen receptor antagonist, enhances the suppression of neointima formation by losartan, an angiotensin II receptor antagonist, in the injured carotid artery of hamster

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1 The present study investigated the inhibitory effect of losartan, a type 1 angiotensin II receptor (AT1) antagonist, and of combined treatment with losartan and GR144053, a fibrinogen receptor (GPIIb/IIIa) antagonist, on neointima formation subsequent to vascular injury in the hamster carotid artery. Vascular injury was achieved by a roughened-tip 2F catheter and the neointimal area was measured up to 2 weeeks inducing the injury.

2 Compared to non-treated hamsters (intimal area (IA)/internal elastic laminal area (IELA) ratio = $60.3 \pm 5.9\%$, $n=12$), losartan dissolved in drinking water (1, 3 and 10 mg kg^{-1} per day, $n=8$ each) reduced neointimal area dose-dependently, a significant decrease (IA/IELA = $39.7 \pm 5.6\%$) being attained with the highest dose when it was administered from 1 day before injury. However, neointima formation was not prevented even with the highest dose of losartan when the administration was started after injury.

3 When the administration of GR144053 (1.0 mg kg^{-1} per hour) via an implanted osmotic pump was started 30 min before the injury and continued for the next 2 weeks, no suppression of neointima formation was observed, although platelet aggregation evoked *ex vivo* by adenosine diphosphate (ADP) at the end of treatment period was efficiently inhibited.

4 In separate experiments in which 5-bromo-2-deoxy-Uridine (BrdU) was used to test smooth muscle cell (SMC) proliferation 1 and 7 days after injury, the ratio of SMC proliferation in the injured area was only slightly decreased by losartan when its administration was started after the injury, despite the marked reduction of SMC proliferation when treatment was started before the injury. Treatment with GR144053 as indicated above also significantly decreased the SMC proliferating index 1 day after the injury.

5 To examine the potential benefit of the coadministration of the GPIIb/IIIa antagonist with the AT1 receptor antagonist, GR144053 (1.0 mg kg^{-1} per hour) was combined with post-injury treatment with losartan (10 mg kg^{-1} per day). This markedly reduced the proliferation of SMCs and significantly decreased the neointimal area (IA/IELA = $31.2 \pm 4.6\%$) measured 2 weeks following the catheterization.

6 According to the results of a time-dependent study in which GR144053 was given in combination with post injury treatment with losartan for 1, 3, 7 or 14 days, neointima formation could be reduced by treatment with GR144053 for just 7 days.

7 In conclusion, GR144053, a fibrinogen receptor antagonist, enhanced the inhibitory effect of losartan, an AT1 receptor antagonist, on neointima formation in the damaged carotid artery of hamsters.

Keywords: AT1 antagonist; combined therapy; fibrinogen receptor antagonist; neointima formation; SMC proliferation

Introduction

Despite the high primary success rate of percutaneous transluminal coronary angioplasty (Detre *et al*., 1988), the late restenosis rate still limits the long-term benefit of this procedure (Serruys *et al*., 1988). Recently, successful experimental approaches to prevent neointima formation have been reported using platelet-derived growth factor (PDGF) antagonists (Liu *et al*., 1990; Ferns *et al*., 1991), a β3 integrin antagonist (Matsuno *et al*., 1994), angiotensin converting enzyme (ACE) inhibitors (Clozel *et al*., 1993; Rakugi *et al*., 1994), an angiotensin II (ATII) receptor antagonist (Azuma *et al*., 1992), and antisense c-*myb* oligonucleotides (Simons *et al*., 1992). These reports indicate that the development of neointima formation is complicated. It has been suggested that vascular angiotensin is an important contributing factor in the development of neointima lesions (Gibbons *et al*., 1992). However, ACE inhibition begun after angioplasty in patients failed to prevent restenosis in clinical trials (Itoh *et al*., 1993; David *et al*., 1995). While Linz *et al*. (1995) reported that it was necessary to start the administration of ACE inhibitors before vascular injury in order to prevent neointima formation. Indeed, it has been shown in experimental studies on animals that ACE inhibits and an ATII receptor antagonist reduce neointima formation following vascular injury when the compounds were administered from before the injury (Azuma *et al*., 1992; Clozel *et al*., 1993; Rakugi *et al*., 1994).

On the other hand, platelets also contribute the development of restenosis since platelets provide several kinds of stimulation factors, such as PDGFs (Ferns *et al*., 1991) and β-transforming growth fact (Assoian *et al*., 1984), leading to SMC migration and proliferation. Recently, potent antiplatelet agents, such as inhibitors of the platelet fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa) have become available and are highly effective both *in vitro* and *in vivo* in animal models of angioplasty (Coller *et al*., 1986; Kohmura *et al*., 1993; Kiss *et al*., 1994). Indeed a GPIIb/IIIa antagonist was shown to improve vascular potency after percutaneous

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transluminal coronary angioplasty (Konstantopoulos *et al*., 1995).

Recently, we have developed a fast and reproducible restenosis model in hamsters (Matsuno *et al*., 1995) and have investigated the inhibitory effect of antiplatelet agents (Matsuno *et al*., 1994; Kaida *et al*., 1997). These observations indicated that vascular smooth muscle cells (SMCs) were stimulated immediately after vascular injury and the proliferation rate of SMC in the media gradually decreased. In contrast, in the newly formed intima, activated SMCs reached a maximum 5 or 7 days after injury. Therefore, the number of proliferating SMCs in the injured area showed two peaks, at 1 and at 7 days after injury, i.e. the first peak in the injured media and the second in the newly formed intima. For this reason we hypothesized that it is necessary to continuously prevent SMC activation in order to prevent neointima formation.

In view of the postulated roles of ATII and platelets in the newly forming intima, we have now investigated in our hamster model (Matsuno *et al*., 1995) whether losartan, an angiotensin II, AT1 receptor antagonist (Azuma *et al*., 1992), and GR144053, a fibrinogen receptor antagonist, prevent restenosis or not, and whether the combination of losartan and GR144053 can enhance the inhibition effect on neointima formation.

Methods

Male hamsters (Gold, SLC, Japan) weighing 100–120 g were fed a standard chow (RC4, Oryental Yeast Co., Ltd, Japan). All experiments were performed in accordance with institutional guidelines.

Reagents

GR144053, 4-[4-{4-(Aminoiminomethyl)phenyl}-1-piperazinyl}-1-piperidineacetic acid hydrochloride trihydrate, was synthesized in Glaxo Research & Development Limited. GR144053 and losartan were kind gifts from Glaxo Wellcome U.K. and Banyu, Co. Ltd, Tokyo, Japan, respectively. ADP was obtained from Sigma (St Louis, MO).

Production of arterial injury

The experimental procedure to induce arterial injury in the carotid artery has been described in detail previously (Matsuno *et al*., 1995). In brief, the animal was anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) the distal right common carotid artery and the region of bifurcation were exposed. A 2FG catheter (Portex) with roughened tip was inserted through the external carotid artery and advanced into the thoratic aorta. The catheter was left in position for 30 s and rotated completely three times. The external carotid artery was ligated after the catheter was slowly and carefully withdrawn. According to our previous immunohistochemical observations using vWF antibody (Matsuno *et al*., 1995), endothelial cells in the injured area were completely stripped as the staining area of vWF had disappeared after injury. This procedure achieved constant neointima formation over 2 weeks following arterial injury, the percentage of narrowing area being 60% on an average. After recovery from anaesthesia the animals were kept in individual cages and fed standard chow (RC4, Oryental Yeast Co., Ltd, Japan).

Quantitation of neointima formation

At the end of observation period, the hamster was anaesthetized by sodium pentobarbitone (50 mg kg^{-1}, i.p.) and the common carotid artery was excised, rinsed with saline and frozen. After removal of the artery, the animal was killed by intraperitorial injection of an overdose of sodium pentobarbitone. The frozen sections were cut transversely into several sections, followed by staining with hematoxylin and eosin (Siguma Chemical Co., St Louis, CA) after a reflux fixation. The total areas within the internal elastic lamina (IELA) and lumen (LA) were measured using computerized image graphic analysis system. For this analysis, three consecutive carotid artery cross-section (4 μm thick) were taken at 100 μm intervals in the most stenotic area and the intima area (IA = IELA-LA) was then expressed proportional to IELA by averaging the three measurements performed for each of the three cross-sections.

Experimental protocol

Experimental procedure was shown in Figure 1. GR144053 (0.1, 0.3 or 1.0 mg kg^{-1} per hour, $n=8$ each) was infused intravenously through the left jugular vein via a cannula (ID = 0.5 mm, OD = 0.8 mm, polyethylene sp3, Natsume Co. Ltd, Japan) connected to an implanted osmotic pump (2ML1, Alzet, Palo Alto, CA). The osmotic pump was incubated for 30 min in saline (37°C) before GR144053 was injected by a exclusive syringe and then it was subcutaneously implanted on the back of the hamster. The infusion was begun 30 min before the vascular injury and was continued for the next 14 days. Losartan was dissolved in the drinking water from 1 day before vascular injury and the end of the observation period (14 days): the consumption of drinking water in each group was measured during the observation period. Animals were killed by an overdose of sodium pentobarbitone at the end of experiment.

Dose-dependent study

Hamsters were divided into eight groups, control groups for losartan ($n=8$), and for GR144053 ($n=8$) which were treated with vehicle, groups treated with losartan at doses of 1.0, 3.0, 10.0 mg kg^{-1} per day ($n=9$ each) from day −1 (1 day before injury) or at 10.0 mg kg^{-1} per day from day 0 (the day of injury) and groups treated with GR144053 from 30 min before vascular injury and the end of the observation period (day 14) at doses of 0.1, 0.3 or 1.0 mg kg^{-1} per hour ($n = 9$ each). Blood pressure was monitored for 10 min on day 1 and day 14. For this purpose, a cannula connected to a pressure transducer (AP601G Nihon Koden, Tokyo, Japan) was inserted into the right carotid artery on day 1. It was left in position for 10 min, whilst the animal was anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Then it was removed and replaced with the modified catheter that was used to induce arterial injury (see above). The same proce-

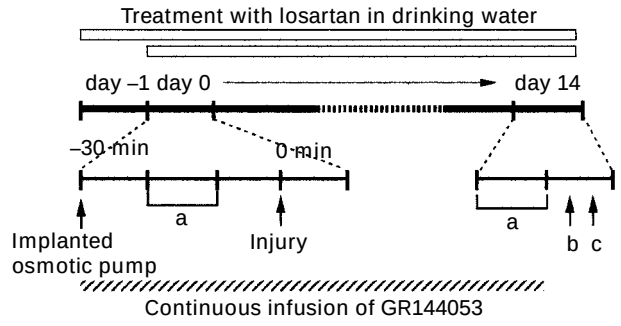

Figure 1 Catheter induced carotid artery stenosis model in hamster was used. When GR144053 (dashed column) was infused, the osmotic pump was implanted 30 min before the initiation of injury. Losartan (open column) in drinking water was administered from either 1 day before injury or after recovery from surgery under anaesthesia. The drugs were continuously treated until the end of experiments (14 days after injury). (a, b, c) Measurement of arterial blood pressure for 10 min, taking a blood sample for platelet aggregation and the removal of injured artery, respectively.

dure was used for measurement of blood pressure from the right femoral artery on day 14. After monitoring blood pressure for 10 min the cannula was removed, the artery ligated and the animal allowed to recover from anaesthesia. Platelet aggregation was assessed at the end of each experiment. Blood (4 ml) was collected by cardiac puncture in tubes containing sodium citrate (3.15%) whilst the animal was anaesthetized with ether, this being administered when the animal had fully recovered from pentobarbitone anaesthesia: in our experience ether interferes less with measurement of aggregation than pentobarbitone. The blood was centrifuged for 10 min at 155 g to obtain platelet-rich plasma. Platelet aggregation was induced by adding 2.5 μM ADP (Sigma Chemical Co., St Louis, CA) and followed in an aggregometer (Aggrecorder II, DA-3220, KYOTO DAIICHI-Chemical, Japan) at 37°C and at 800 g. Aggregation was expressed as percentage of maximum light transmission.

Proliferation of SMC

In separate experiments, proliferating SMCs were indentified by the thymidine analogue 5-bromo-2-deoxy-Uridine (BrdU) labeling technique (Lindner *et al*., 1992). BrdU tests were performed both 1 day and 7 days after injury. In each experiment, hamsters were divided into four groups (n = 6 each). The first group was the control group, the second and third groups were treated with losartan (10 mg kg^{-1} per day) from either before, or immediately after the vascular injury, and the fourth group was treated with a continuous infusion of GR144053 (1.0 mg kg^{-1} per hour) for 30 min before, until 14 days after injury. BrdU (50 mg kg^{-1}) was injected subcutaneously 1, 8, 16 and 24 h prior to removal of the carotid arteries. Following removal of the artery, at 1 and 7 days after injury, frozen cross-sections were prepared from these arteries. BrdU positive cells were stained with a murine monoclonal antibody (SIGMA, St Louis, MO), followed by goat anti-mouse Ig-antibodies conjugated to peroxidase and detection with diaminobenzidine (DAB). Sections were also stained for background with hematoxylin. The number of positive and negative nuclei were counted in the media and newly formed intima. The BrdU labelling index was calculated using the following formula: (the number of positive nuclei stained with DAB) / (the number of total nuclei stained with haematoxylin) $\times$100.

Combined treatment

Hamsters were divided into two groups (n = 10 each). GR144053 (1.0 mg kg^{-1} per hour) was given by implanted osmotic pump as mentioned above (the paragraph of dose-dependent study). In addition, losartan (10.0 mg kg^{-1} per day) was given via the drinking water from either 1 day before the vascular injury or from after recovery from anaesthesia, until the end of observation period (day 14). In the groups in which losartan was given after injury, we used some hamsters (two groups, n=4 each) for BrdU test on days 1 and 7.

Time-dependent effect of GR144053 with losartan

Hamsters were divided into four groups for a study on the time-dependent effect of GR144053 on neointima formation. GR144053 (1.0 mg kg^{-1} per hour) was infused from 30 min before vascular injury and continued for 1, 3, 7 or 14 days (n=8 each) after vascular injury by using the implanted osmotic pump, this being combined with post-injury administration of losartan (10.0 mg kg^{-1} per day).

Statistics

All data are represented as mean$\pm$s.e.mean. The effect of the drugs versus the control was identified by ANOVA followed by the Student–Newman–Keuls test.

Results

Effect of losartan or GR144053 alone on neointima formation

Figures 2a and 3a show the neointima formation in the hamster carotid artery subsequent to vascular injury, expressed as the ratio 'intimal area/internal-elastic lamina area' (IA/IELA) $\times$ 100 at 2 weeks after the catheterization. The ratio was zero without injury, whereas it was as high as 60.3$\pm$3.1% (Figure 1a and 59.5$\pm$5.5% (Figure 3a) at 2 weeks after injury in the control (non treated) groups. The neointimal area was reduced in a dose-dependent manner by losartan treatment and that with 10 mg kg^{-1} per day losartan from day -1 until day 14 resulted in a significant reduction of IA/IELA ratio to 39.0$\pm$5.6%, corresponding to 35.8$\pm$6.8% inhibition of restenosis. However, when administration of losartan was started after vascular injury, neointima formation was not prevented even when the highest dose of losartan was used (Figure 1a, open circle). The average intake of losartan was 1.13$\pm$0.02, 3.04$\pm$0.33, 9.89$\pm$0.88 and 10.02$\pm$1.01 mg kg^{-1} per day for the four treatment groups that received 1.0, 3.0, 10.0 mg kg^{-1} per day from day 1, or at 10.0 mg kg^{-1} per day from day 0, respectively. When infusion of GR144053 was started 30 min before vascular injury and continued for the next 2 weeks, neointima formation was not significantly reduced (Figure 3a). Losartan did not inhibit *ex vivo* platelet aggregation (Figure 2b) whereas the continuous infusion of GR144053 suppressed platelet aggregation

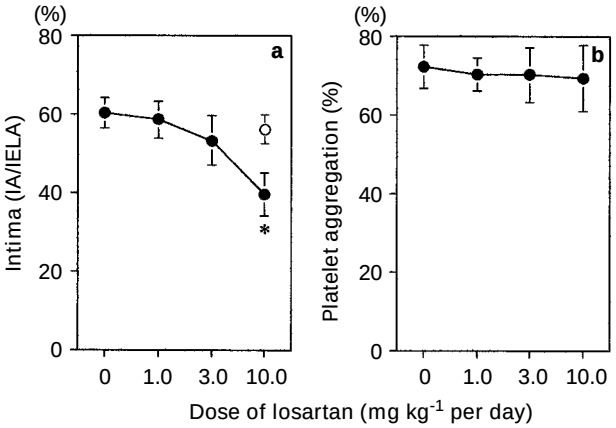

Figure 2 Dose-dependent inhibition of neointima formation (a) and *ex vivo* ADP (2.5 μM) platelet aggregation (b) in hamsters treated with losartan. Administration of losartan was started 1 day before (●) or after recovery from surgery in which injury was indicated (○; n=12) and was maintained for 14 days. Neointimal area was analysed on day 14 (control: n=8, losartan 1.0, 3.0, 10.0 mg kg^{-1} per day: n=9 each). *P<0.01, compared to the non-treated group.

Table 1 Mean arterial blood pressure

	Dose	*Day 0*	*Day 14*
GR144053	0	106$\pm$6	107$\pm$5
(mg kg^{-1}	0.1	102$\pm$4	108$\pm$5
per hour)	0.3	104$\pm$5	107$\pm$9
	1.0	107$\pm$3	110$\pm$9
Lostartan	0	106$\pm$4	102$\pm$5
(mg kg^{-1}	1.0	104$\pm$12	104$\pm$11
per day)	3.0	100$\pm$12	105$\pm$9
	10.0	96$\pm$11	93$\pm$14
	10.0*	105$\pm$5	94$\pm$8

Day 0: blood pressure was measured before injury. Day 14: blood pressure was measured 14 days after injury. *Losartan in drinking water was administered after injury.

in a dose dependent manner (Figure 3b). Mean arterial blood pressures in each group were shown in Table 1.

Proliferation index of SMC with losartan or GR144053 alone

Figure 4 shows the percentage of proliferating SMC in media on day 1 and in newly formed intima on day 7 after vascular injury. Pretreatment with losartan (10 mg kg^{-1} per day) caused a significant reduction of SMC proliferation in the media and newly formed intima. By contrast, GR144053 efficiently inhibited the early proliferation of SMCs, measured on day 1 as a $51.3 \pm 12.1\%$ drop in proliferation index, while post-treatment with losartan (10 mg kg^{-1} per day) reduced the percentage of proliferating SMC in newly formed intima on day 7, but not on day 1. The average intake of losartan in this experiment was not significantly different from that measured in the dose-dependent study.

Effect of combination therapy

It was as a results of the above findings that experiments were conducted in which GR144053 (1.0 mg kg^{-1} per hour) was coadministered with losartan (10 mg kg^{-1} per day) begun either before or after arterial injury. In both cases, combined treatment with the drugs reduced neointima formation significantly (Figure 5, hatched bars) as compared to treatment with losartan alone (Figure 5, open bars). In the group given combined treatment with losartan (10 mg kg^{-1} per day) after injury and a continuous infusion of GR144053 (1.0 mg kg^{-1} per hour), the index of SMC proliferation was significantly reduced in the injured media (day 1) and newly formed intima (day 7) as compare to non-treated control group: the data were 10.5 ± 0.7 and $11.8 \pm 1.3\%$, respectively ($n = 4$ each). Typical microphotographs of neointima formation after vascular injury on day 14 in each group are shown in Figure 6.

Time-dependent inhibition of neointima formation by combination therapy

Figure 7 shows that the neointimal area was significantly decreased by continuous infusion of GR144053 (1.0 mg kg^{-1} per hour) for 7 and 14 days when combined with post-injury with losartan (10 mg kg^{-1} per day). However, when GR144053 was continuously infused in combination with losartan for only 1 or 3 days after injury, the neointimal area was not significantly decreased.

Discussion

In the present study, we have demonstrated that losartan, an AT1 receptor antagonist, has an inhibitory action on neointima formation in the injured hamster carotid artery and, in addition, that this inhibitory action was enhanced by coadministration of GR144053, a fibrinogen receptor (GPIIb/IIIa) antagonist.

The angiotensin II receptor subtype AT1 was reported to be a trigger of SMC proliferation as blockade of the AT1 receptor was shown to markedly reduce neointima formation (Prescott *et al*., 1991). In our hamster stenosis model, the suppression of neointimal area by losartan was confirmed when it was administered from before vascular injury. However, post-treatment with losartan did not reduce neointimal area. This phenomenon was clearly supported by the results on SMC proliferation index using BrdU. Thus, when treatment with losartan was started before the injury, the proportion of pro-

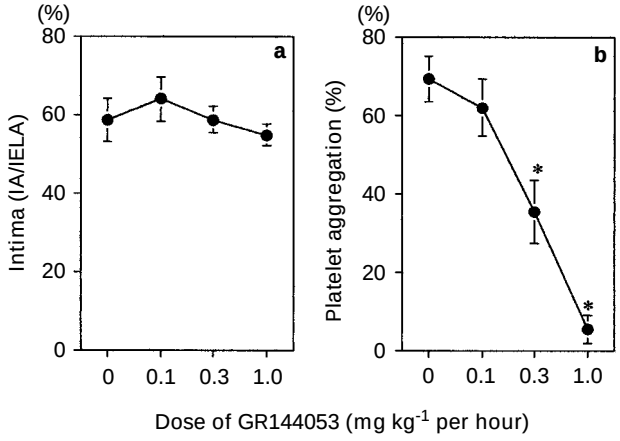

Figure 3 Dose-dependent inhibition of neointima formation (a) and *ex vivo* ADP (2.5 μM) platelet aggregation (b) in hamsters treated with GR144053. GR144053 was continuously infused i.v. from 30 min before, until 14 days after carotid artery injury. Control ($n = 8$) and treated animals (0.1, 0.3 and 1.0 mg kg^{-1} per hour: $n = 8$ each) were analysed on day 14. $^*P < 0.01$ vs control.

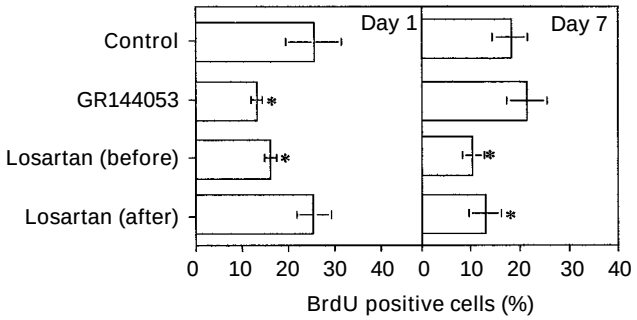

Figure 4 Percentage of proliferating SMC measured as BrdU index in neointima formation on days 1 and 7 following vascular injury in controls, treated with GR144053 (1.0 mg kg^{-1} per hour), or with losartan (10.0 mg kg^{-1} per day) from either before or after injury. $^*P < 0.01$ vs control.

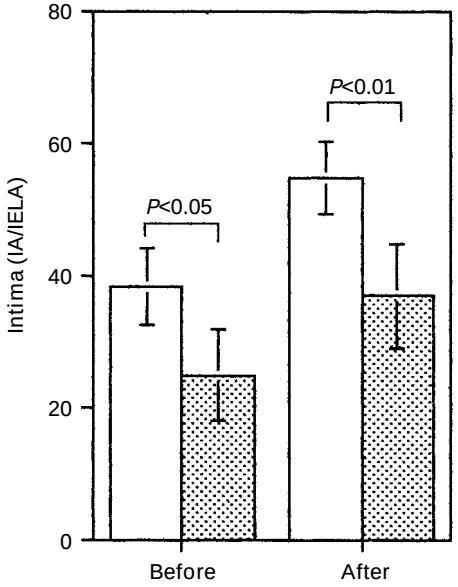

Figure 5 Reduction of neointima formation by combined therapy. Administration of losartan (10.0 mg kg^{-1} per day) was started on 1 day before or after the vascular injury and was maintained for 14 days. GR144053 (1.0 mg kg^{-1} per hour) was continuously infused i.v. from 30 min before until 14 days after injury. Open bars and hatched bars show treatment with losartan alone and combined treatment with both drugs, respectively.

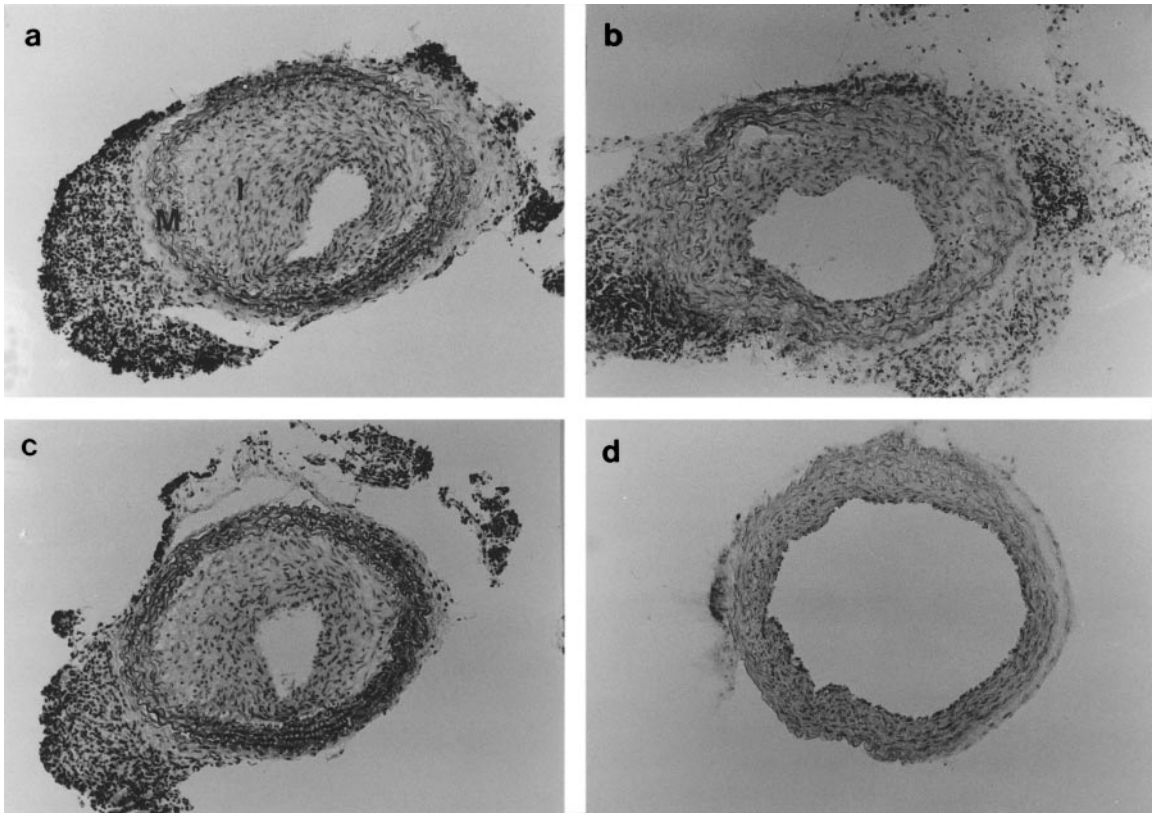

Figure 6 Light microphages ($\times 20$) of newly formed neointima in damaged carotid arteries, control (a), the start of treatment with losartan alone (10 mg kg^{-1} per day) either before (b) or after (c) the catheterization and the combination therapy both GR144053 (1.0 mg kg^{-1} per hour) and losartan (10 mg kg^{-1} per day) started to administer before injury (d).

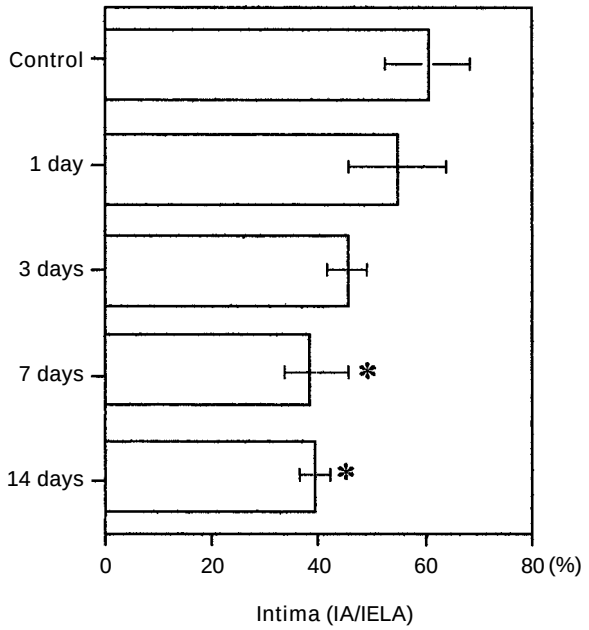

Figure 7 Time-dependent inhibition of neointima formation in hamsters treated with both GR144053 (1.0 mg kg^{-1} per hour) and losartan (10 mg kg^{-1} per day). Treatment with losartan was started just after injury and continued for 14 days. Treatment with GR144053 was started 30 min before the injury and continued for 0 (control, $n=8$), 1 ($n=6$), 3 ($n=6$), 7 ($n=8$) and 14 days ($n=8$). *$P<0.01$ vs control.

liferating SMCs in the injured area was significantly reduced on days 1 and 7. However, BrdU index was decreased only on day 7 when treatment with losartan was started post-injury. These results that prompted us to investigate whether neointima formation could be inhibited even by post-injury administration of losartan if it were combined with other drug, i.e. able to suppress the early activation of SMC in the injured area.

We have previously shown that a non-selective platelet αIIb/β3 (GPIIb/IIIa) and smooth muscle cell αv/β3 receptor antagonist prevents restenosis (Matsuno *et al*., 1994). Moreover the final pathway to platelet aggregation is the fibrinogen receptor and blockade of these receptors markedly prevented platelet aggregation *in vitro* (Peerlinck *et al*., 1993) and reduced thrombus formation *in vivo* (Coller *et al*., 1986). Since GR144053 is a selective GPIIb/IIIa antagonist (Nicholas & Lumley, 1995; Kawasaki *et al*., 1996), we therefore investigated whether this compound would reduce neointima formation or not, as the continuous supply of factors that stimulate SMC by activated platelets, such as PDGF, should be interrupted by antagonism of GPIIb/IIIa receptor. In fact, in the present study the continuous infusion of hamster with GR144053 during the observation period did result in a marked suppression of platelet aggregation in response to ADP *ex vivo*; there was no significant inhibition of neointima formation, but the BrdU index was significantly decreased on day 1. These findings therefore suggested that treatment of hamster with GR144053 alone had an inhibitory effect on the early stimulation of SMC proliferation (day 1).

Thus, we investigated whether we could attain a more potent inhibition of neointima formation due to SMC proliferation by post-injury administration of losartan if we coadministered it with GR144053. As expected, the BrdU index of SMC proliferation was significantly decreased on days 1 and 7. This effect may be due to continuous inhibition of the supply of a stimulating substance, for instance PDGF, in the early phase of development of neointima formation because platelet activation was prevented by the GPIIb/IIIa antagonist.

Further, this inhibition may also decrease the migration of SMCs to newly formed intima from the injured media. Indeed, the neointimal area was significantly reduced after combined treatment with both drugs even though treatment with either losartan or GR144053 alone did not prevent neointima formation when administered of each of these drugs was started after the injury.

Finally, we also investigated the period of treatment with GR144053 that was required to significantly reduce neointima formation when administration of losartan was started after injury. According to the results obtained, a full 14 days treatment with GR144053 was not necessary. These findings suggested that the activated platelets are mainly involved in the early phase of the neointima formation and raise the possibility that inhibition of neointima formation by an angiotensin II antagonist could be improved in the clinical situation by the coadministration of a powerful antiplatelet agent such as a GPIIb/IIIa receptor antagonist for a short time period after vascular injury.

In conclusion, the present study indicated that the combination of an AT1 receptor antagonist with a fibrinogen receptor antagonist can efficiently improve vascular patency after endothelial damage even if administration of both drugs begins after injury. This observation suggested that such a combination therapy may efficiently prevent restenosis after thrombolysis or angioplastic interventions.

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