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Activation of two angiotensin-generating systems in the balloon-injured artery

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Participation of angiotensin II in the myointimal proliferation following a vascular injury was postulated. This study assessed the potential involvement of the local angiotensin II-forming enzymes in injured arteries of dogs. The potential angiotensin II-forming enzymes are angiotensin-converting enzyme (ACE) and chymostatin-sensitive angiotensin II-generating enzyme (CAGE) which is highly homologous to or could be identical to the mast cell chymase. Both ACE and CAGE catalyze the conversion of angiotensin I to angiotensin II. We found that the enzymatic activities of ACE and CAGE, and the mRNA levels of ACE and chymase were increased in the injury-induced hypertrophied vessels. The results suggest that ACE and CAGE participate in the hypertrophy through the production of angiotensin II which is a growth promoter for vascular smooth muscle cells.

Angiotensin converting enzyme; Mast cell chymase; Chymostatin; Angiotensin II; Neointima; Wound healing

1. INTRODUCTION

An injury to vascular intima promotes a series of events which are characterized by the migration of the smooth muscle cells (SMC) to the intima and proliferation of SMC in the intima [1]. These events imply the wound healing responses to injury [1], and eventually lead to the restenosis after the percutaneous transluminal coronary angioplasty (PTCA) in humans and the experimentally induced balloon injury in laboratory animals. Although the cause of the restenosis has not been clearly identified, the involvement of various growth factors and cytokines which are released from the migrated macrophages and damaged endothelial and medial cells has been suggested [1]. With the help of these substances, angiotensin (ANG) II promotes migration [2] and proliferation [3] of SMCs, although ANG II is not mitogenic by itself but is a comitogen for SMC [3]. ANG II is also a known stimulator for synthesis of extracellular matrix by SMCs [4].

It is clear that ANG II participates in the myointimal proliferation. However, it is not clear which are the primary responsible cells and enzymes that generate ANG II in the injured vascular tissues. It is generally believed that angiotensin-converting enzyme (ACE; EC 3.4.15.1) is responsible for the local conversion of ANG I to ANG II in the vascular wall. Participation of ACE in the proliferative responses is suspected since ACE was increased in the hypertrophic vascular tissues in response to a balloon injury [5], and since ACE inhibitors [6] as well as an ANG II antagonist [7] were effective in preventing the neointimal hypertrophy in the balloon-injured rats.

However, the effect of ACE inhibitors differed among the species. The agents failed to exert the preventive effect in humans [8], non-human primates [9], and pigs [10]. The difference in the effect among the species strongly suggested the presence of a system which produces ANG II other than the action of ACE. We have pursued the possibility and have shown the presence of an ANG II-forming enzyme other than ACE in primate and dog vessels [11–13]. This enzyme was a serine-class neutral protease and named chymostatin-sensitive angiotensin II-generating enzyme (CAGE), the enzymatic activity of which was inhibited by chymostatin. After partial purification and characterization, CAGE was found to have strikingly similar properties to mast cell chymase such as its capability to generate ANG II [14], molecular size (ca. 30 kDa), pH profile, inhibitor susceptibilities, and localization to the vascular adventitia [12]. Another ANG II-generating enzyme which is highly specific to ANG I was isolated to homogeneity from human heart [15], and its genomic DNA was sequenced [16]. This enzyme, named human heart chy-

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Abbreviations: ACE, angiotensin converting enzyme; ANG, angiotensin; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; PCR, polymerase chain reaction; SMC, smooth muscle cell.

mase, was found to be identical to human mast cell chymase [17] based on their genomic DNA sequences. This evidence suggests that the mast cell chymase is involved in the alternative ANG II-generating system in the local vascular sites.

In the present study, we have examined the potential involvement of the two types of ANG II-forming enzymes, ACE and CAGE, in the myointimal hypertrophy which was induced by a balloon injury to dog vessels.

2. MATERIALS AND METHODS

2.1. *Animal treatments*

Six beagles weighing 9–13 kg (Nihon Nosan Kogyo K.K., Yokohama, Japan) were used. After the dogs were anesthetized with sodium pentobarbital (50 mg/kg i.v.), both sides of the common carotid arteries were exposed. The left common carotid arteries were remained intact and used as controls. A balloon catheter (3F, Arterial Embolectomy Catheter, VERMED, Neuilly en Thelle, France) was inserted into the right common carotid artery through a proximal incision of the arterial wall. The balloon was filled with water to distend the common carotid artery and was pulled back. After this procedure was repeated three times, the catheter was removed and the incision was sutured. Thirty days after the procedure, the animals were killed by bleeding under pentobarbital anesthesia. Both sides of their common carotid arteries were dissected. The tissues were cut into small blocks; one was processed for histological examination while the others were immediately frozen in liquid nitrogen and stored at −80°C. The experimental procedures and treatment of the animals were in accordance with our institutional guidelines.

2.2. *Measurement of activities for ACE and CAGE*

A synthetic substrate, Bz-Gly-His-Leu (Peptide Institute, Inc., Minoh, Japan) was used for determining ACE activity [18]. Angiotensin I was used as a substrate for determining CAGE activity [12]. The protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL, USA) in which bovine serum albumin was used as a standard.

2.3. *Polymerase chain reaction*

Total RNA was isolated from the common carotid arteries by the procedure described previously [19]. A single-stranded cDNA was synthesized from 0.5 μg of the total RNA with cDNA synthesis kit (Super Script Preamplification System, Gibco BRL, Gaithersburg, MD, USA). Two oligonucleotide primers (5′-GAAGCTGAGGA-GATCATCGG-3′ and 5′-CAGCTTCACCTCTTGCAGAG-3′) were synthesized to amplify the 449-bp fragment of the mast cell chymase mRNA which corresponded to its nucleotides 61–510. The oligonucleotides were synthesized on a DNA synthesizer (Model 391, Applied Biosystems, Osaka, Japan) and were purified by high-performance liquid chromatography. Two additional oligonucleotide primers (5′-GCCTCCCCAACAAGACTGCCA-3′ and 5′-CCACATGTCTC-CCAGCAGATG-3′) were designed based on the human ACE cDNA sequence, and synthesized to amplify the 388-bp fragment of ACE mRNA between nucleotides 492 and 880.

Four microliters of the reverse transcription reaction mixture were added to 96 μl of the polymerase chain reaction (PCR) buffer containing 25 pmol each of the upstream and downstream primers and 1 U of Ampli Taq Polymerase (Takara, Osaka, Japan). The PCR reaction was carried out on a DNA thermal cycler (Zymoreactor AB-1800, Atto, Tokyo, Japan). The amplification conditions for the mast cell chymase were 94°C for 1 min, 62°C for 1 min and 72°C for 2 min over 35 cycles. The conditions for ACE were 94°C for 1 min, 62°C for 1 min and 72°C for 2 min over 30 cycles. The PCR fragments were subcloned into the TA cloning vector (Invitrogen, San Diego, CA, USA) and their nucleotide sequences were determined by the dideoxynucleotide chain termination method. The PCR fragments were also subjected to electrophoresis (1.5% agarose gel) to quantitate the induced ACE mRNA and mast cell chymase mRNA levels. The gel was stained with ethidium bromide, and the stained images were scanned on a densitometer (Model CS-9000, Shimadzu, Kyoto, Japan). The mRNA levels were expressed as the ratio of densitometrically integrated areas for the injured group to those for the control group. The PCR procedure was also performed with serial dilutions of the total RNA (2.0, 1.0, 0.5, 0.25 and 0.125 μg) in order to verify the linearity of the PCR procedure.

2.4. *Statistical analysis*

All numerical data shown in the text are expressed as mean ± S.E.M. Significant differences in the means between the control and injured vessels were evaluated by the Student's *t*-test for unpaired data.

3. RESULTS AND DISCUSSION

The vascular injury induced by the balloon catheterization elicited remarkable neointimal formation in the dog carotid arteries (Fig. 1). The mast cell chymase mRNA levels were 3.4 times and CAGE activities were 22 times higher in the injured arteries than in the non-injured arteries (Figs. 2 and 3). The ACE mRNA levels and ACE activities were 4.8 and 4.6 times higher, respectively, in the injured as compared to the non-injured arteries (Figs. 2 and 3). These results indicate that two distinct ANG II-generating systems, the CAGE and ACE systems, are present in the vascular wall and that they are activated concomitantly when the vessels are injured and thereby hypertrophied.

There is some evidence to suggest a strong possibility that the mast cell chymase is identical to CAGE. These two enzymes share similar properties such as their molecular sizes, pH profiles, susceptibilities to a panel of inhibitors, and unique localization to the adventitial tissues. The dog mast cell chymase exhibited an extremely high sequence homology and similar enzymological characteristics to human mast cell chymase [20,21] which is known to convert ANG I to ANG II [14,22]. Mast cells are known for their localization to the adventitial and perivascular tissues [23]. In addition, the perivascular localization of human heart chymase in the human heart has also been reported (H. Urata et al., presented at the Meeting of the 14th International Society of Hypertension, Madrid, 1992). The findings mentioned above strongly suggest that the chymase, presumably identical to CAGE, in the vascular wall is capable of generating ANG II and may also play a role in raising the ANG II concentration at the injured vascular wall.

CAGE appears to contribute to the ANG II formation in the vascular wall independently from the ACE action. We have observed that both human and dog arteries possess the CAGE-dependent ANG II-forming pathway. As much as 70% of the total ANG II formation depended on the CAGE activity in human arteries (Okunishi et al., submitted for publication), and 30–

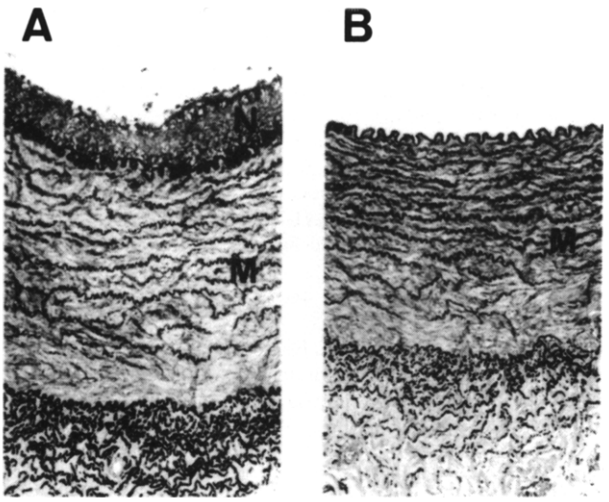

Fig. 1. Histological cross sections of the injured common carotid artery (panel A), and non-injured contralateral common carotid artery (panel B). M, media; N, neointima. Van Gieson staining. Original magnification, × 40.

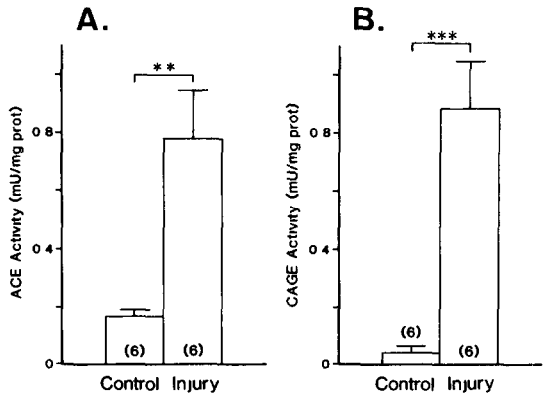

Fig. 3. ACE activity (panel A) and CAGE activity (panel B) from the injured common carotid arteries (Injury), and non-injured contralateral common carotid arteries (Control). Number of dogs is shown in parentheses. The vertical bars represent S.E.M. $^{**}P < 0.01$ and $^{***}P < 0.001$; significantly different from respective controls.

40% of the total ANG II formation took place via the CAGE-dependent pathway in dog vessels [11].

CAGE activity was detected in dog, monkey and human arteries [11,12], but not in rodent arteries (Okunishi et al., submitted for publication). This may be due to the differences in the properties of the mast cell chymases among the animal species. For example, the human mast cell chymase is highly efficient to convert ANG I to ANG II by cleaving the Phe^8–His^9 bond of ANG I. This enzyme is 9 times more efficient than cathepsin G [14], and 4 times more efficient than human lung ACE [22]. On the other hand, rat mast cell chymase is efficient in degrading ANG II by cleaving the Tyr^4–Ile^5 bond of ANG II [24]. The different properties of the mast cell chymase among the animal species may explain why ACE inhibitors are effective in rodent [6], but not in pig [10] or primates [8,9].

As mentioned above, about one-third of the total ANG II formation depended on CAGE activity in non-injured vessels from dogs. Interestingly, the percentage of CAGE-dependent ANG II formation was increased to 70–80% of the total in the injury-induced hypertrophied vessels (Okunishi et al., to be published). The tremendous increase in CAGE activity after balloon injury, the 22-fold increase as shown in the present study, indicates significant involvement of CAGE in the formation of ANG II, particularly in pathological states.

After balloon injury, the ACE activity and ACE mRNA levels were increased to a similar extent while CAGE activity and mast cell chymase mRNA levels were increased in a significantly different manner. Thus,

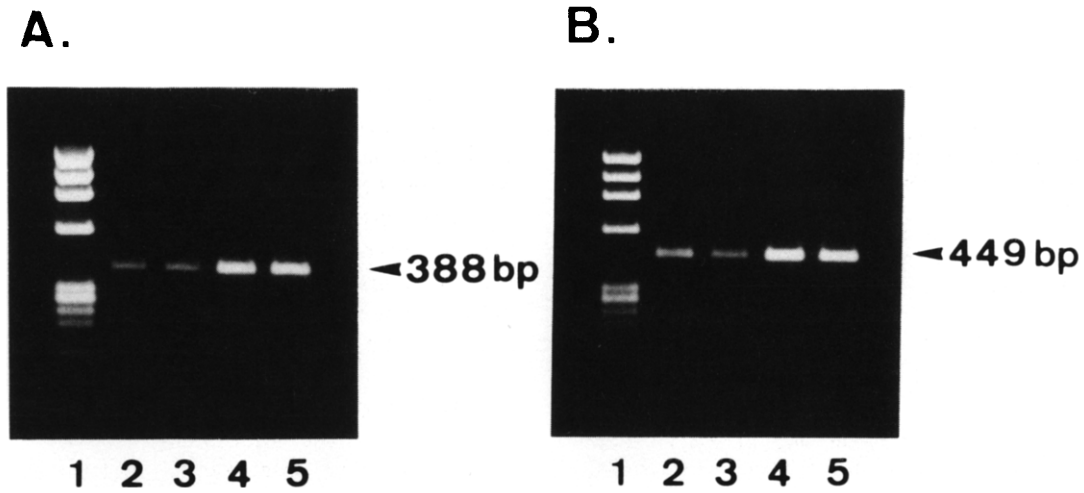

Fig. 2. Representative ethidium bromide-stained agarose gels (1.5%) of PCR products with specific primers for ACE (panel A) and mast cell chymase (panel B). Lane 1 contains ϕx174-*Hae*III digested DNA marker. Lanes 2 and 3 contain PCR products using RNA samples isolated from non-injured common carotid arteries. Lanes 4 and 5 contain PCR products from injured common carotid arteries. The arrowheads at 388 bp and 449 bp indicate the positions of PCR products for the ACE and mast cell chymase, respectively.

the increase in CAGE activity cannot be explained solely by the increase in mast cell chymase levels, and the results suggest that other chymase-like enzymes such as cathepsin G, a neutrophil protease which is also found in mast cells [25], may also participate in the generation of ANG II. The detailed mechanism for the induction of ACE or mast cell chymase in the injured vessels remains to be determined. A histological study reported that the increase in ACE immunostaining was observed in neointima and medial lesion after vascular injury [5]. A similar histological work on CAGE and/or mast cell chymase would provide additional information on their role in vascular injury.

This study has shown that both ACE and CAGE which is identical to or closely homologous to mast cell chymase are activated in the injured vascular wall. This indicates that these local ANG II-forming systems may play a major role in vascular restenosis which is an exaggerated wound healing response to injury [1] and is a serious problem to the current therapeutic regimen. The development of ANG II antagonists and/or CAGE inhibitors may benefit individuals with hypertension or hypertrophic vascular diseases, particularly when ACE inhibitors are not effective.

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