Bradykinin $B_1$ Receptor Mediates Inhibition of Neointima Formation in Rat Artery After Balloon Angioplasty

Jun Agata, Robert Q. Miao, Katsutoshi Yayama, Lee Chao, Julie Chao

Abstract—We evaluated the effects of the kallikrein-kinin system on the proliferation and migration of primary cultured vascular smooth muscle cells (VSMCs) in vitro and neointima formation in balloon-injured rat carotid arteries in vivo. In cultured rat VSMCs, tissue kallikrein inhibited cell proliferation, and this inhibitory effect was blocked by Sar-Tyr-Aca($\epsilon$)-Lys [d-$\beta$Nal$^7$,Ile$^8$]-des-Arg$^9$-bradykinin, a bradykinin $B_1$ receptor antagonist, and by icatibant, a bradykinin $B_2$ receptor antagonist. Platelet-derived growth factor significantly increased the expression of the $B_1$ receptor but not the $B_2$ receptor in VSMCs. Platelet-derived growth factor–induced cell migration was significantly attenuated by des-Arg$^9$-bradykinin and to a lesser degree by bradykinin. Endogenous $B_1$ receptor mRNA increased in rat carotid arteries after balloon angioplasty. After local delivery of adenosine carrying the human tissue kallikrein gene into the rat carotid artery, we observed a 54% reduction in the intima/media ratio at the injured site compared with the control ratio (n=7, P<0.01). Administration of the $B_1$ receptor antagonist via minipumps blocked the protective effect of kallikrein and partially reversed the intima/media ratio toward the control ratio. Kallikrein gene delivery results in the regeneration of endothelium compared with the control groups, and the $B_1$ receptor antagonist abolished this effect. Nitrite/nitrate, cGMP, and cAMP levels in balloon-injured arteries significantly increased after kallikrein gene delivery, whereas the $B_2$ receptor antagonist abolished these increases (n=4 or 5, P<0.05). These results indicate that the $B_1$ receptor contributes to the reduction of neointima formation via the promotion of reendothelialization and inhibition of VSMC proliferation and migration through NO-cGMP and cAMP signaling pathways. This study provides significant implications in treating restenosis after revascularization. (Hypertension. 2000;36:364-370.)

Key Words: bradykinin $\bullet$ kallikrein-kinin system $\bullet$ gene delivery $\bullet$ cell migration $\bullet$ neointima formation

Balloon catheter injury to the rat carotid artery triggers a sequence of events, including early platelet accumulation, proliferation of medial smooth muscle cells, migration into the intima, and proliferation of intimal cells to form neointima hyperplasia. It was reported that the angiotensin-converting enzyme (ACE) inhibitor has been shown to suppress neointima formation after endothelial injury in the rat carotid artery and abdominal aorta.1 Because angiotensin II exhibits mitogenic activity in vascular smooth muscle cell (VSMC) growth and proliferation,2 it was thought that the inhibition of angiotensin II is related to the suppression of neointima formation by the administration of ACE inhibitors. Inhibition of ACE activity not only prevents the formation of angiotensin II but also increases kinin levels by preventing kinin degradation. Previous studies have shown that icatibant, a bradykinin (BK) $B_2$ receptor antagonist, can partially block the inhibitory effect of ACE inhibitors on neointima formation.3,4 Furthermore, we have recently reported that a continuous supply of tissue kallikrein by gene delivery suppressed neointima formation in the balloon-injured artery and that this effect was inhibited by icatibant.5 These combined results indicate a protective role of kinins in neointima formation after balloon angioplasty.

There are 2 BK receptor subtypes, $B_2$ and $B_1$ receptors. Kinin and its kininase I metabolite, des-Arg$^9$-BK or des-Arg$^9$-Lys-BK, activate $B_2$ and $B_1$ receptors, respectively. The $B_2$ receptor is not expressed or is expressed at low levels in tissues under normal conditions, but it is induced under pathological conditions. It has already been reported that the $B_1$ receptor is induced by cytokines such as interleukin-1$\beta$, bacterial lipopolysaccharides, or vascular injury.6–9 Induction of $B_1$ receptor–dependent vasoconstriction has also been shown in the rabbit carotid artery after balloon injury.10 In addition, a previous study has shown that the $B_1$ receptor agonist inhibits platelet-derived growth factor (PDGF)-stimulated proliferation in rat mesenteric smooth muscle cells in vitro.11 However, whether $B_1$ receptors affect VSMC migration and/or neointima formation in injured blood vessels after balloon angioplasty has not been investigated. In the present study, we evaluated the potential role of $B_1$ receptors in the migration and proliferation of VSMCs in vitro and neointima formation in the rat artery after balloon angioplasty in vivo by both pharmacological and gene delivery approaches. The present study provides new insights into the role of BK $B_1$ receptors in VSMC growth, migration, and reendothelialization after vascular injury.
Methods

Preparation of Adenovirus Carrying the Human Tissue Kallikrein Gene

Adenovirus containing the human tissue kallikrein gene under the control of the cytomegalovirus promoter (Ad.CMV-cHK) was generated as previously described. Large quantities of high-titered adenoviruses, Ad.CMV-cHK and control virus containing the luciferase gene (Ad.CMV-Luc), were prepared and purified for gene delivery.  

Primary Cultured Aortic Smooth Muscle Cell Proliferation Assay

Rat VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats (180 to 200 g, Harlan Sprague Dawley, Inc, Indianapolis, Ind) by the combined collagenase and elastase digestion method. The cells exhibited a “hill-and-valley” growth pattern and were characterized by positive immunostaining with monoclonal antibodies against smooth muscle α-actin. Cells were serially passaged and used between passages 3 and 10. Quiescent VSMCs in 24-well plates were treated with 0.1 μmol/L of rat tissue kallikrein and Sar-Tyr-Aca(ε)-Lys-[D-β-Val(ε),Ile(ε)]-des-Arg9-BK, a BK B1 agonist provided by Dr D. Regoli, Institut de Pharmacologie de Sherbrooke, Canada, or icatibant, a BK B2 agonist kindly provided by Hoechst Roussel Pharmaceuticals Inc, Germany, at 1 μmol/L in 0.1% FBS DMEM for 18 hours and then pulse-labeled with 1.0 μCi/mL [3H]thymidine for another 6 hours. At the end of the incubation, cells were washed 3 times with PBS, precipitated with 10% trichloroacetic acid for 30 minutes at 4°C, washed 2 times with 95% ethanol, and then solubilized with 0.25 mol/L NaOH plus 0.1% SDS. After neutralization with 1 mol/L of acetic acid, the radioactivity was determined by a liquid scintillation counter (Packard). Each experiment was performed in triplicate.

Cell Migration Assay

VSMC cell migration was assessed by using modified Boyden chambers (Corning Inc). The transwell inserts were coated with a solution of 10 μg/mL fibronectin and 50 μg/mL type I collagen (Sigma Chemical Co) and then air-dried. VSMCs (2×10⁴ cells) suspended in a 200 μL aliquot of DMEM containing 0.1% BSA were added to the upper chamber and incubated in DMEM containing 0.1% BSA for 1 hour. Transwell apparatuses were then incubated with testing samples for 4 hours at 37°C. Rat PDGF-BB (Sigma) at 15 ng/mL or des-Arg9-BK or BK (Sigma) at 1 mol/L dissolved in DMEM containing 0.1% BSA was used as the chemoattractant and was placed in the bottom chamber of Boyden apparatus. At the end of incubation period, cells on both sides of the membrane were fixed and stained with modified Wright-Giemsa stain (Sigma). Cells on the upper surface of the membrane were then mechanically removed, and the average number of cells from 5 randomly chosen high-power (×400) fields on the lower side of the membrane was counted. Each experiment was performed in triplicate.

Animal Treatment

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, Md). Male Sprague-Dawley rats (weight 350 to 400 g) were anesthetized with ketamine (90 mg/kg IP) and xylazine (10 mg/kg IP), and a 2F embolectomy balloon catheter (weight 350 to 400 g) were anesthetized with ketamine (90 mg/kg IP) and xylazine (10 mg/kg IP), and a 2F embolectomy balloon catheter (Baxter Health Care Corp) was introduced into the left common carotid artery by way of the external artery. The balloon was inflated with sufficient saline to distend the common carotid artery and was then withdrawn to the external artery. This procedure was repeated 3 times, and the catheter was then removed. After balloon injury of the left common carotid artery, the injured distal segment was isolated by temporary ligature. The adenoviral particle of Ad.CMV-cHK or Ad.CMV-Luc (4×10⁹ plaque-forming units in 20 μL) was infused into the distal injured segment and incubated for 15 minutes at room temperature. After incubation, the cannula was removed, and blood flow to the common carotid artery was restored. To investigate the potential kinin-mediated effect after kallikrein gene delivery, Sar-Tyr-Aca(ε)-Lys-[D-β-Val(ε),Ile(ε)]-des-Arg9-BK (B, antagonist) was infused intraperitoneally at a rate of 70 μg/kg per day after balloon angioplasty and Ad.CMV-cHK infusion. At 7 and 14 days after gene delivery, rats were anesthetized and perfused with saline through the ascending aorta. Both carotid arteries were isolated for nitrite/nitrate (NOx), cGMP, and cAMP assays or morphometric analysis.

RT-PCR and Southern Blot Analysis

Total RNA was extracted from Trizol reagent according to the protocol recommended by the manufacturer (BRL). Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) and Southern blot analysis were used to determine the abundance of B1 receptors and B2 receptor mRNA in nontreated, sham-operated, and injured carotid arteries at 7 and 14 days after angioplasty in vivo and in quiescent cultured VSMCs treated with or without PDGF at 15 ng/mL for 30 minutes in vitro. Specific oligonucleotide probes for the B1 receptor (5' primer, 5'-AAGACACGATCCACCATC-3'; 3' primer, 5'-GACAACCTAAGGCCAACCGTG-3'; and internal probe, 5'-CCGTCTGACCTCCTTGAAC-3') and the B2 receptor (5' primer, 5'-GGATGGGTTTGCCTGCACG-3'; 3' primer, 5'-CGTGCCTCCATGGCTGTCAACACC-3'; internal probe, 5'-GTAGTCCTTATGGCTGTCAACACC-3') were used. The quality of RNA was evaluated by RT-PCR and Southern blot of rat cytoplasmic β-actin by using specific primers and internal probe (5' primer, 5'-GAACCTTAAAGGCAACCGTG-3'; 3' primer, 5'-TGCGATAGAGGTCCTTACG-3'; and internal probe, 5'-CCGACAGTTCCCTCCTGCACG-3').

Assays for NOx, cGMP, and cAMP levels

At 7 days after angioplasty and gene delivery, the rats were anesthetized, and the carotid artery was dissected and homogenized (Polytron, Brinkmann Instruments) in 400 μL of 0.1% HCl at 4°C and centrifuged at 15 000g for 30 minutes. cGMP and cAMP levels were measured by radioimmunoassay, and NOx was measured by a fluorometric assay as previously published. Protein concentrations were determined by Lowry’s method as previously described.

Morphometric Analysis

Two weeks after gene delivery, the rats were anesthetized, and the left and right carotid arteries were removed and embedded in paraffin. Each artery was divided into 3 segments, which were separately embedded in paraffin. Cross-sectional rings (4 μm) were cut from each segment and stained with hematoxylin and eosin. The slides were photographed with a microscope at a magnification of ×100. The luminal, neointimal, and medial areas were measured by use of the NIH Image 1.60 software package. For evaluating recovery of the endothelium, antibody against von Willebrand factor was used to identify endothelial cells (Dako). The standard immunoperoxidase procedure using the avidin-biotin-peroxidase complex (Vectastain ABC kits, Vector Laboratory) was performed according to the manufacturer’s instructions. The sections were then developed with 0.02% H₂O₂ and 0.1% diaminobenzidine tetrahydrochloride.

Statistical Analysis

Data are expressed as mean±SEM. Data were compared between experimental groups by 1-way ANOVA. Differences between kallikrein and control groups were further evaluated by the Fisher protected least squares differences. Differences were considered significant at a value of P<0.05.
Results

Effects of Tissue Kallikrein and B1 and B2 Receptor Antagonists on the Proliferation of Primary Cultured VSMCs

Figure 1 shows that tissue kallikrein (0.1 μmol/L) significantly inhibited the proliferation of VSMCs compared with control proliferation as measured by [3H]thymidine incorporation (67.8±3.6% of control, n=4, P<0.01). The inhibitory effect of tissue kallikrein was abolished by 1 μmol/L of Sar-Tyr-Aca(ε)-Lys [β-βNalβ,Ille]-des-Argβ-BK, a B1 receptor antagonist (87.0±7.8% of control, n=4, P<0.05), or icatibant, a B2 receptor antagonist (89.0±6.3% of control, n=4, P<0.05).

Effects of B1 and B2 Receptor Agonists on the Migration of Primary Cultured VSMCs

Figure 2 shows that B1 and B2 agonists have no effect on VSMC migration in the absence of PDGF. PDGF increased the migration of VSMCs by 3-fold compared with the control migration of VSMCs by 3-fold compared with the control (92±5 cells per field, n=3). The B1 agonist, des-Argβ-BK, reduced 44% of PDGF-stimulated cell migration (59±1 cells per field, n=3, P<0.01), whereas the B2 agonist, BK, had a minor effect, with only a 13% reduction in cell migration (92±5 cells per field, n=3, P<0.01) (Figure 2).

Differential Expression of B1 and B2 Receptors After Administration of PDGF In Vitro or Balloon Angioplasty In Vivo

The expressions of endogenous B1 and B2 receptors were analyzed by RT-PCR followed by Southern blot analysis with the use of 3 gene-specific oligonucleotides for each transcript. Figure 3A shows the transcripts of B1 and B2 receptors in primary cultured VSMCs with or without PDGF. Expression of the B1 receptor was significantly increased by PDGF, whereas no significant change was observed in the expression of the B2 receptor. Figure 3B shows the transcripts of B1 and B2 receptors in rat carotid arteries after balloon angioplasty. Expression of the B1 receptor was significantly increased at 7 days in rats after balloon angioplasty compared with control or sham-operated rats, whereas the expression of the B2 receptor was unaltered in the injured carotid artery. Identical levels of β-actin were detected among these samples in both experiments, indicating that the RNA quality of these samples is internally consistent.

Effects of Local Kallikrein Gene Delivery and B1 Receptor Antagonists on Neointima Formation in Rat Artery After Balloon Injury In Vivo

Figure 4 shows typical morphology of the rat carotid artery 14 days after angioplasty and tissue kallikrein gene delivery. The artery of a sham-operated rat showed normal morphology (Figure 4A), whereas angioplasty caused neointima formation in the artery (Figure 4B). Kallikrein gene delivery significantly reduced thickening of the arterial wall (Figure 4C), whereas B1 antagonist treatment abolished the protective effect of kallikrein (Figure 4D). Figure 5 shows morphometric analysis of the intimal area and the intima/media ratio in the carotid artery after balloon angioplasty. The intimal area in rats receiving adenovirus-mediated kallikrein gene delivery significantly decreased compared with that in control rats (cross-sectional area, 62.0±6.0 versus 119.0±6.7 μm² [mean±SEM], respectively; n=7, P<0.01). A 54% reduction in the intima/media ratio was found in rats receiving kallikrein gene delivery compared with control rats (0.47±0.04 versus 1.03±0.09 [mean±SEM], respectively; n=7; P<0.01). No significant difference in intimal area or the
intima/media ratio was detected between the angioplasty groups with or without the control virus containing the luciferase gene. Suppression of the intimal area and reduction of the intima/media ratio after kallikrein gene delivery were partially blocked by the B₁ antagonist (62.0±6.0 μm² and 0.47±0.04 versus 94.8±6.2 μm² and 0.82±0.06 [mean±SEM], respectively; n=7; P<0.01). There were still significant differences in intimal area and the intima/media ratio between the group receiving Ad.CMV-cHK with the B₁ antagonist and the control group (P<0.05). No significant difference in the medial area was found among these groups.

Effects of Local Kallikrein Gene Delivery and B₁ Receptor Antagonists on Reendothelialization in Rat Artery After Balloon Injury In Vivo

Figure 6 shows reendothelialization 14 days after balloon angioplasty among the different groups. The index of endothelialization was 37.7±13.7% and 35.6±6.9%, respectively, in balloon-injured arteries of rats with and without Ad.CMV-Luc (n=7 in each group). A prominent recovery of endothelium was observed in the balloon-injured arteries of rats receiving kallikrein gene delivery compared with control group arteries (88.6±5.8% versus 37.7±13.7% and 35.6±6.9%, n=7, P<0.01). Administration of the B₁ antagonist abolished the kallikrein-mediated promotion of reendothelialization (57.7±10.9% versus 88.6±5.8%, n=7, P<0.05).

Effects of Kallikrein Gene Delivery and B₁ Receptor Antagonists on NOx, cGMP, and cAMP Levels in the Carotid Artery After Balloon Angioplasty

Figure 7 shows the effect of kallikrein gene delivery and B₁ receptor antagonist on NOx, cGMP, and cAMP levels in the carotid artery after balloon angioplasty. NOx content in the artery increased significantly in the group receiving the adenovirus containing the tissue kallikrein gene versus the angioplasty group and the group that received Ad.CMV-Luc (225.4±32.8 versus 94.8±27.0 and 68.7±37.4 pmol/mg protein, n=4 or 5,
Administration of the B₁ antagonist inhibited the increase of NOx content (80.4±28.2 pmol/mg protein) (Figure 7A). cGMP levels in the carotid artery increased significantly in the group receiving the adenovirus containing the kallikrein gene compared with the angioplasty groups with and without Ad.CMV-Luc (32.9±2.3 versus 18.9±2.0 and 20.9±4.9 pmol/mg protein, n=4 or 5, P<0.05). Administration of the B₁ antagonist inhibited the increase of cGMP (19.5±4.6 pmol/mg protein) (Figure 7B). Similarly, cAMP levels in the artery increased significantly in the group receiving the adenovirus containing the kallikrein gene compared with the angioplasty groups with and without Ad.CMV-Luc (581.9±67.2 versus 376.6±51.6 and 339.4±79.4 pmol/mg protein, n=4 or 5, P<0.05). Administration of the B₁ antagonist inhibited the increase of cAMP (303.4±43.4 pmol/mg protein) (Figure 7C).

**Discussion**

This is the first study to demonstrate that the BK B₁ receptor plays an important role in the reduction of neointima formation after balloon angioplasty. Local delivery of the tissue kallikrein gene at the injured site results in significant reduction of the intima/media ratio and neointima formation as well as prominent recoveries of endothelium and increases of NOx, cGMP, and cAMP levels at the injured site. The B₁ receptor antagonist abolished the protective effects of kallikrein on arterial thickening and the increases in vascular NOx, cGMP, and cAMP levels. In primary cultured VSMCs, the B₁ antagonist abolished the inhibitory effect of kallikrein on cell proliferation, and the B₁ receptor agonist inhibited PDGF-stimulated VSMC migration. These results indicate that the BK B₁ receptor attenuates neointima formation in response to balloon angioplasty.26 It has been well established that the expression of endogenous kininogen and tissue kallikrein is a significant component of neointima formation after balloon angioplasty.25 On the other hand, VSMC migration is another important component of neointima formation after balloon angioplasty.26 It has been well established that the expression of PDGF increased markedly in injured tissues and that this growth factor plays an important role in VSMC migration and growth after balloon injury.27–29 In the present study, we showed that the B₁ receptor plays a major role in the inhibition of PDGF-stimulated cell migration but that the B₂ receptor has only a minor effect. The B₁ agonist reduced 44% of PDGF-stimulated cell migration, whereas the B₂ agonist reduced only 13%, and the BK B₁ or B₂ agonist alone did not have any effect on VSMC migration in the absence of PDGF (Figure 2). These results suggest a synergistic action of the B₁ agonist and PDGF. Another potential mechanism for the inhibition of neointima formation is via reendothelialization, which leads to recovery of beneficial functions of the endothelium.22 The key role of the endothelium in regulating underlying intimal growth is well known, and endothelium...
has a major influence on the degree of intimal hyperplasia.\textsuperscript{30,31} We showed that kallikrein gene delivery accelerated the growth of endothelium in the injured artery and that the B\textsubscript{2} antagonist abolished this effect (Figure 6). This finding is consistent with previous reports that the B\textsubscript{2} receptor can induce endothelial cell proliferation and angiogenesis.\textsuperscript{32,33} Taken together, our results indicate that activation of the B\textsubscript{1} receptor has multiple effects on neointima formation, including inhibitory effects on both migration and proliferation of VSMCs as well as the promotion of reendothelialization, whereas the B\textsubscript{2} receptor mainly inhibits VSMC proliferation. However, we found that the inhibitory effect of the B\textsubscript{1} receptor antagonist against kallikrein gene delivery appears to be stronger than that of the B\textsubscript{2} receptor antagonist,\textsuperscript{5} because it must be noted that the B\textsubscript{1} receptor is induced after vascular injury, whereas the B\textsubscript{2} receptor is constitutively expressed. Moreover, the effect of kallikrein gene delivery is completely blocked by the NO synthase (NOS) inhibitor, inasmuch as both effects of B\textsubscript{1} and B\textsubscript{2} receptors are mainly mediated by NO.\textsuperscript{34}

It has been reported that the overexpression of human endothelial NOS mediates the decrease of neointima formation after balloon injury\textsuperscript{35} and that NO inhibits smooth muscle cell growth through the cGMP signaling pathway.\textsuperscript{36} Our results have shown that local kallikrein gene delivery into the carotid artery after angioplasty results in increased NOx and cGMP levels at the injured vessel and that the B\textsubscript{1} antagonist suppresses these elevations. These findings suggest that the inhibitory effect of neointima formation after kallikrein gene delivery is in part mediated by B\textsubscript{1} receptor–NO–cGMP signaling pathways. Previous reports have shown that the NOS inhibitor N\textsuperscript{\textendash}nitro-L-arginine methyl ester could block the beneficial effect of ACE inhibition\textsuperscript{4} and kallikrein gene delivery\textsuperscript{34} on neointima formation in balloon-injured rat carotid arteries. Also, BK significantly increased nitrite release from isolated canine coronary microvessels, and the increased release of nitrite was dramatically reduced with NOS inhibition.\textsuperscript{37} In the present study, we showed that the B\textsubscript{1} antagonist abolished the elevation of NOx and cGMP levels in the carotid artery after kallikrein gene delivery. These findings indicate that the metabolites of kinin may stimulate NO and cGMP production through the activation of NOS via the BK B\textsubscript{1} receptor. One potential source of NO may be inducible NOS from VSMCs. This notion is supported by a report that arterial smooth muscle cells express inducible NOS in response to endothelial injury.\textsuperscript{38} Elevated cGMP formation regulates the cell proliferation and migration through cGMP-mediated activation of tyrosine phosphatases,\textsuperscript{39} cGMP-dependent protein kinase,\textsuperscript{40–43} or cross-activation of cAMP-dependent protein kinase.\textsuperscript{44}

Prostacyclin activates adenylate cyclase, leading to increased cAMP levels. Elevation of cAMP production attenuates mitogen-activated protein kinase signaling induced by PDGF in VSMCs and thus inhibits cell proliferation.\textsuperscript{45} Furthermore, elevated cAMP production also suppresses VSMC migration via protein kinase A.\textsuperscript{41–43} Interestingly, we found that vascular injury increased cAMP production in the carotid artery (Figure 7C). We speculate that inflammation may cause cAMP elevation by means of stimulation of prostaglandin production,\textsuperscript{46} and elevated levels of cAMP could contribute, in part, to self-protection for reduced neointima formation. Our results also showed that kallikrein gene delivery significantly increased cAMP levels in the carotid artery and that the B\textsubscript{1} antagonist blocked this elevation. These results suggest that the inhibitory effect of neointima formation after kallikrein gene delivery is, in part, mediated by B\textsubscript{1} receptor–cAMP signaling pathways, because binding of kinin metabolites to the B\textsubscript{1} receptor stimulates the production of prostacyclin.\textsuperscript{47} Detailed mechanisms by which the B\textsubscript{1} receptor contributes to the inhibition of VSMC proliferation and migration remain to be further elucidated.

In conclusion, using pharmacological and gene delivery approaches, we showed that the BK B\textsubscript{1} receptor contributes to the attenuation of neointima formation after balloon injury through the promotion of reendothelialization and the inhibition of VSMC proliferation and migration via NO-cGMP and cAMP signaling pathways. These results provide new insight into the role of the vascular BK B\textsubscript{1} receptor and have significant implications for gene therapy in the treatment of restenosis and arteriosclerosis.

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References
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