Smooth Muscle Cell Migration and Matrix Metalloproteinase Expression After Arterial Injury in the Rat

Michelle P. Bendek, Nobuya Zempo, Alexander W. Clowes, Richard E. Galardy, Michael A. Reidy

Abstract We have characterized matrix metalloproteinase expression in the rat carotid artery after two forms of arterial injury, balloon catheter denudation and nylon filament denudation. Gelatinolytic enzymes with molecular masses of 70 and 62 kD were produced constitutively in the rat carotid. Production of an 88-kD gelatinase was induced after balloon catheter injury, and proteinase production continued during the period of migration of smooth muscle cells from the media to the intima, from 6 hours to 6 days after balloon catheter injury. In addition, a marked increase in 62-kD gelatinolytic activity was observed between 4 and 14 days after arterial injury. Gelatibase activities (88 and 62 kD) were also increased after nylon filament denudation but were markedly less after this injury than after balloon catheter injury. These results suggested a correlation between gelatibase activity and smooth muscle cell migration after arterial injury. Administration of a metalloproteinase inhibitor after balloon catheter injury resulted in a 97% reduction in the number of smooth muscle cells migrating into the intima. Therefore, we hypothesize that gelatibase expression directly facilitates smooth muscle cell migration within the media and into the intima. These results suggest that gelatinases are involved in the vascular smooth muscle cell activation and neointimal formation that characterize arterial tissue remodeling after injury. (Circ Res. 1994;75:539-545.)

Key Words • matrix metalloproteinases • artery • injury

The proliferation of medial smooth muscle cells, migration of cells to the intima, and proliferation of intimal cells to form a thickened neointima are sequential events that characterize tissue remodeling in the arterial wall after balloon catheter injury. Medial smooth muscle cell proliferation begins immediately after injury, and the proliferation rate reaches a maximum of 46% at 2 days after injury. The first smooth muscle cells appear in the intima 4 days after injury, and they proliferate with a maximum rate of 73% at 7 days. Collectively, the increase in smooth muscle cell number and the newly synthesized extracellular matrix lead to a significantly thickened intima. Maximum intimal thickness is achieved by 3 to 4 weeks after injury. Recent evidence suggests that the migration and proliferation of smooth muscle cells are controlled by distinct mechanisms. Basic fibroblast growth factor is clearly important in controlling the replication of medial smooth muscle cells, whereas platelet-derived growth factor (PDGF) regulates cell migration with only minimal effects on replication, and recent experiments have shown that cell migration is essential to the development of neointimal thickening. When migration is inhibited by administration of antiplatelet or anti-PDGF antibodies, neointimal thickening is dramatically reduced. Conversely, when PDGF is infused after injury, neointimal thickening is significantly enhanced.

Replication and migration of smooth muscle cells presumably require degradation of basement membrane and extracellular matrix surrounding the cells, but little is known about the proteolytic properties of these cells. We have shown that smooth muscle cells express mRNA for urokinase plasminogen activator (UPA) and tissue-type plasminogen activator (TPA) after balloon catheter injury, and TPA activity is increased at 4 days after arterial injury. This is the time when smooth muscle cells are known to migrate to the intima. Further, the addition of tranexamic acid, a plasminogen activator inhibitor, inhibits smooth muscle cell migration. Plasminogen activators catalyze the conversion of plasminogen to plasmin, which in turn can degrade several matrix molecules and can activate matrix metalloproteinases (MMPs), a family of molecules involved in degradation and remodeling of connective tissues. Activation of such a proteolytic cascade would provide the potential to degrade virtually all vascular extracellular matrix components; the matrix in the blood vessel wall includes elastin, type I and type III collagen, and proteoglycans, and the basement membrane underlying endothelial cells and surrounding smooth muscle cells contains type IV collagen, laminin, and fibronectin. Proteinase activity may be an important mechanism for matrix degradation during smooth muscle cell replication and migration. In fact, coordinate expression of proteinase activity has been shown to mediate cell migration and tissue remodeling during angiogenesis, tumor cell invasion and metastasis, rheumatoid arthritis, wound healing, and blastocyst implantation and placentation.

In the present study, we have characterized MMP mRNA expression and proteolytic activity in the arterial wall.
wall after two forms of arterial injury, balloon catheter denudation and nylon filament denudation. Inhibition of MMP activity results in a nearly complete inhibition of smooth muscle cell migration into the intima. These studies suggest that MMPs may be important mediators of tissue remodeling after arterial injury.

Materials and Methods

Surgery

Male Sprague-Dawley rats (3 to 4 months old) from Bantin and Kingman Laboratories, Edmonds, Wash, were used in all experiments. Rats were anesthetized by intraperitoneal injection of xylazine (Anased, 4.6 mg/kg body wt, Lloyd Laboratories) and ketamine (Ketaset, 70 mg/kg body wt, Aveco Co Inc). A midline incision in the neck was made to expose the left external carotid artery. A 2F balloon catheter (Baxter Healthcare Co) was introduced through the left external carotid artery and passed into the common carotid artery. The balloon was distended with saline until a slight resistance was felt and then was rotated while pulling it back through the common carotid to denude the vessel of endothelium. This procedure was repeated two more times, the catheter was removed, the external carotid was ligated, and the wound was closed. Some left carotids were injured by gentle filament denudation. 17 A loop of 5-0 nylon monofilament suture (Dermalon, Davis and Geck) was held open by inserting its ends into polyethylene tubing (PE-10, Clay Adams), and the loop was introduced into the left external carotid artery via a trocar made of polyethylene tubing (PE-60). The loop was pushed through the common carotid to the aortic arch; the filament was long enough (3 cm) to reach the arch while the supporting PE-10 tubing remained inside the trocar not touching the common carotid. The filament was pulled back along the carotid with constant rotation; a total of three passes were made to ensure complete denudation. Contralateral right carotid arteries and carotid arteries from unmanipulated rats served as controls. Rats were killed at various times after injury by intravenous injection of sodium pentobarbital (Aniphen, Lacticated Ringer's Injection U.S.P. [Baxter]) was infused at a pressure of 120 mm Hg retrogradely via a catheter placed in the abdominal aorta. The left and right common carotid arteries were excised, adhering connective tissue was removed, and the vessels were snap-frozen in liquid nitrogen and then stored at −80°C.

Zymograms

Tissues were processed as we have described in a recent publication.28 Briefly, the vessels were pulverized under liquid nitrogen with a mortar and pestle, and the samples were vortexed in lysis buffer containing 1% sodium dodecyl sulfate (SDS), 1 mmol/L phenylmethylsulfonyl fluoride, and 10 mg/mL leupeptin in 50 mmol/L Tris buffer, pH 7.6. Insoluble matter was removed by centrifugation at 13,500g. A small sample of the supernatant was reserved for protein determination; the rest was diluted 1:1 with electrophoresis sample buffer (0.5 mmol/L Tris, pH 6.8, 2% SDS, 20% glycerol, and 0.002% bromphenol blue). The protein concentration of each sample was determined by biocinchnonic acid assay (Pierce Chemical Co) with bovine serum albumin (BSA) used as a standard. Arterial extracts were subject to zymography according to the procedures of Herron et al.14 Type I gelatin (DIFFCO Laboratories) or α-casein (Sigma Chemical Co) was added to a standard 8% polyacrylamide gel mix for a final concentration of 0.1% substrate in the gel. Gelatin and casein were used as substrates because they are readily cleaved by connective tissue–degrading proteinases and are easily incorporated into polyacrylamide gels. Equal amounts of total protein from each arterial extract were loaded onto the gel and electrophoresed at a constant voltage of 200 V. After electrophoresis, the gels were soaked in 2.5% Triton X-100 (Sigma), with gentle shaking for 30 minutes at room temperature with one change of detergent solution. The gels were rinsed and incubated overnight at 37°C in incubation buffer (0.05 mol/L Tris, pH 8.0, 2.5 mmol/L CaCl2, and 0.02% sodium azide). Some gels were incubated in the presence of 20 mmol/L EDTA (Sigma) or 20 mmol/L 1,10-phenanthroline (Sigma) to inhibit metalloproteinase activity. After incubation, the gels were rinsed in 10% trichloroacetic acid (Baker) and then stained for 30 minutes in rapid Coomassie stain (Diversified Biotech), followed by destaining in 30% methanol and 10% acetic acid in water. The gels were then photographed and dried for permanent records.

RNA Isolation and Northern Blot Analysis

Frozen arterial tissue was ground to a fine powder under liquid nitrogen, and total cellular RNA was prepared by acid phenol extraction.20 The pulverized tissue was homogenized in 4 mol/L guanidine isothiocyanate and 0.5% sarkosyl in 25 mmol/L sodium citrate with a Polytron homogenizer (Brinkman). This was followed by two phenol/chloroform extractions and ethanol precipitation. Equal amounts of total cellular RNA, as determined by absorption of the extracts at 260 nm, were loaded on a 1.2% agarose gel. Formaldehyde–phosphate gel electrophoresis and RNA transfer to nylon membranes (Zeta-Probe, Bio-Rad) were carried out as previously described.24 After transfer, RNA was cross-linked to the membrane by baking at 80°C for 2 hours. The blot was photographed under UV light to verify equal loading in each lane. Blots were hybridized by using cDNA probes labeled with [32P]dCTP by random primer extension (Multi-Prime, Amersham). After overnight hybridization with probes, the blots were washed at 65°C in two changes of 0.045 mol/L NaCl, 0.0045 mol/L sodium citrate (pH 7.0), and 0.1% SDS for 15 minutes each and then exposed to Hyperfilm-MP (Amersham) at −70°C. Northern blots were probed with the following cDNAs: rat MMP-9 (800-bp probe; gift from Dr Birkdal-Hansen, University of Alabama, Birmingham), human MMP-2 (2.73-kb probe; gift from Dr Goldberg, Washington University School of Medicine, St Louis, Mo),23 rat MMP-1 (2.9-kb probe; gift from Dr Jeffries, Albany [NY] Medical Center),24 and rat MMP-3 (1.6-kb probe; gift from Dr Matrisian, Vanderbilt University, Nashville, Tenn.).25

In Situ Hybridization

A technique was used that allowed us to carry out in situ hybridization on the luminal surface of arteries.26 The surface smooth muscle cells of the artery were hybridized with labeled riboprobes and then separated from the artery as a single monolayer (Hautchen procedure). For these en face preparations, rat common carotid arteries were perfusion-fixed at 7 and 14 days after injury as described below; then the middle 1 cm of the artery was cut open longitudinally and pinned in a Teflon card.18 UTP–labeled riboprobes were synthesized from rat MMP-9 cDNA by using a kit from Promega: T7 and SP6 polymerases were used to generate sense and antisense strands, respectively. The Hautchen procedure for en face preparations was carried out after probe hybridization.27 Slides were coated with autoradiographic emulsion (Kodak, NTB2), exposed for 3 weeks, and developed. En face preparations were studied and photographed under the light microscope by using dark-field illumination.

Metalloproteinase Inhibitor

A peptide hydroxamic acid (GM 6001) supplied by Glycomed Inc was used to inhibit MMP activity in injured rat carotid arteries.28 The ability of this inhibitor to effectively inhibit rat arterial MMPs was tested by incubating a zymogram gel containing rat arterial extracts with incubation buffer containing 0.5 mmol/L GM 6001. GM 6001 completely inhibited all gelatinolytic activity on the zymogram (results not shown). Immediately before balloon catheter injury, rats were injected intraperitoneally with 100 mg/kg GM 6001 dissolved in 4% carboxymethylcellulose (CMC). Control rats were injected with 4% CMC vehicle. The rats were injected daily with...
either GM 6001 or CMC until they were killed, 4 days after carotid injury. To label all cells entering S phase during the last 24 hours before death, three injections of 5-bromo-2'-deoxyuridine (BrdU, Boehringer Mannheim Corp) were given subcutaneously to all rats (25 mg/kg body wt) at 17, 9, and 1 hours before they were killed. The carotid arteries were perfusion-fixed with 0.1 mol/L phosphate-buffered 4% paraformaldehyde at 110 mm Hg. Vessels were excised and immersed in 4% paraformaldehyde for 1 hour and then transferred to Ringer’s solution. A 1-cm length was excised from the middle of the common carotid artery and used for the smooth muscle cell migration assay described below. Adjacent sections 5 mm in length were cut and embedded in paraffin. Cross sections were cut and immunostained for BrdU as previously described.29

Smooth Muscle Cell Migration Assay

We developed a technique to quantify smooth muscle cell migration into the intima. Previous work in this laboratory has shown that smooth muscle cells first appear in the intima 3 to 4 days after balloon injury.8 Smooth muscle cell migration was measured by staining the intimal cell nuclei with an antibody against histone H1 and counting the number of cells in the intima at 4 days after injury. This technique takes advantage of the fact that the antibody does not permeate the internal elastic lamina and thus stains only intimal cell nuclei. The middle 1 cm of the denuded common carotid artery was cut lengthwise and pinned intimal side up onto a Teflon card. The carotid arteries were rinsed in phosphate-buffered saline (PBS) and then placed in 0.3% hydrogen peroxide in cold methanol to block endogenous peroxidase. Nonspecific protein binding was blocked by incubating for 30 minutes in 2% normal horse serum diluted in PBS containing 1% BSA. After incubation with the histone monoclonal antibody (MAB 1276, mouse anti-human nuclei and chromosomes, Chemicon International Inc) diluted 1:100 in PBS/BSA for 30 minutes, the sections were incubated with 1% biotinylated horse antimouse IgG in PBS/BSA for 30 minutes. Peroxidase labeling was carried out by using an avidin-biotin complex (Elite ABC, Vector Laboratories) and visualized by using 0.05% 3,3'-diaminobenzidine (DAB) in 0.05 mol/L Tris-HCl (pH 7.6) with 50 μL of 30% hydrogen peroxide. Tissues were exposed to the DAB solution for 5 minutes. All steps were followed by three rinses in PBS. After staining, the tissues were placed intimal side up on glass slides, and coverslips were applied using a 50:50 mixture of PBS-glycerol. For a negative control, matching tissues were stained with a matching concentration of normal mouse IgG substituted for the anti-histone antibody. There were no positive-staining cells in these control tissues. Another series of tissues was counterstained with hematoxylin to ensure that all surface nuclei were stained with the anti-histone antibody. The intimal surface of the vessel was visualized by light microscopy, and the number of intimal cell nuclei per square millimeter of surface area was determined under a magnification of x100 with an eyepiece graticule to measure surface area.

Results

Gelatinolytic Activity

Several bands of lytic activity were observed when arterial extracts from both normal and balloon catheter-injured arteries were subject to gelatin zymography. In normal uninjured rat carotid arteries, two prominent bands of activity were present, with molecular masses of ~70 and 62 kD. Two additional bands with molecular masses of ~238 and 88 kD were induced in the artery after balloon catheter injury (Fig 1). We noted that the activity of the 238- and 88-kD bands was increased between 1 and 4 days after injury but had decreased significantly by 6 days after injury. The activity of the 70-kD gelatinase did not change at any time after injury, but an increase in the 62-kD band was noted between 4 and 14 days after injury. The time course of increased 62-kD activity did vary after injury (eg, see Fig 5), where 62-kD gelatinase activity at 8 days after injury was not different from that observed in uninjured control arteries. All lytic activity was abolished on incubation of the gel with 20 mmol/L EDTA or 2 mmol/L 1,10-phenanthroline, suggesting that the gelatinases were metalloproteinases. On the basis of similarity of the molecular masses on nonreducing zymogram gels, we believe that the gelatinolytic activities in arteries correspond to gelatinases previously identified in other tissues. SDS present in the zymogram gel denatures the enzyme, exposing the active site so that latent and active forms of the enzyme are evident on the gel. We believe that the 70- and 62-kD lytic bands correspond to latent and active forms of 72-kD type IV collagenase/gelatinase (MMP-2),30 whereas the 88-kD band probably corresponds to an active form of rat 95-kD type IV collagenase/gelatinase (MMP-9).32 The 238-kD lytic band may represent a novel proteinase unique to rat arterial tissue or may be a multimer of smaller gelatinolytic molecules.31 There are several reports of larger gelatinases in other tissues,22,32,34; however, none is exactly the same size as the band we observed in the rat carotid artery.

Metalloproteinase mRNA Expression

Northern blots containing total arterial RNA were probed with MMP-9 cDNA derived from rat mammary carcinoma cells.27 Expression was induced as early as 6 hours after injury and peaked at 3 days after injury (Fig 2). No MMP-9 mRNA was detected 2 weeks after injury. This change in mRNA levels for MMP-9 paralleled the change in enzyme activity of the 88-kD gelatinase observed by gelatin zymography. The presence of two bands on the Northern blot probably represents the presence of two mRNA transcripts. Similarly sized MMP-9 mRNA species have been reported on Northern blots of RNA from mice.35 By contrast, there was constitutive expression of MMP-2 mRNA in control animals, and expression actually decreased during the first week after injury (Fig 3). Thus, there was no correlation between MMP-2 activity (the 70-kD gelatinase measured on gelatin zymograms) and MMP-2 mRNA levels present in the vessel wall. Northern blots were probed with cDNAs for stromelysin (MMP-3)25 and interstitial collagennase (MMP-1),24 but no mRNA species were detected in arterial extracts, although MMP-3–transfected Chinese hamster ovary cells were positive (results not shown). We were also
unable to detect lytic activity due to MMP-3 on casein substrate zymograms (data not shown).

In Situ Hybridization for MMP-9

We used an en face Hautchen preparation in conjunction with in situ hybridization to investigate MMP-9 mRNA expression by migrating intimal smooth muscle cells after arterial injury. Smooth muscle cells expressed MMP-9 mRNA at 7 days after balloon catheter injury (Fig 4A). Sections hybridized with a control sense riboprobe showed negligible levels of hybridization (Fig 4B). At 14 days after injury, no intimal smooth muscle cells expressed MMP-9 mRNA (not shown). All cells present in the middle 1 cm of the common carotid artery showed positive immunostaining with an anti-smooth muscle α-actin antibody (Boehringer Mannheim) (not shown). Previous studies of immunostained vessel wall cross sections have shown that only 1% of the cells in the vessel wall are leucocytes and 0.7% are monocytes. More recently, we have immunostained similar en face Hautchen preparations with the anti-macrophage antibody, ED-1, and we detected no macrophages present at these times (personal communication, Dr Volkhard Lindner, Department of Pathology, University of Washington).

Modulation of Gelatinase Activity After Injury

In previous studies, we have shown that the extent of neointimal development is dependent on the type of arterial injury. Injury with a balloon catheter stimulates a more vigorous medial cell proliferation and migration in the artery compared with injury with a nylon filament. The gelatinase activities in these two sets of injured arteries were examined, and activities in the 238-, 88-, and 62-kD bands were significantly increased at 2, 4, and 6 days after balloon injury compared with gentle denudation injury (Fig 5). No change in 70-kD gelatinase activity was observed in these vessels.

Inhibition of MMPs

An MMP inhibitor, GM 6001, was administered to rats to assess the contribution of MMPs to smooth
muscle cell migration after balloon injury. As shown in Fig 6, administration of the MMP inhibitor almost completely inhibited the migration of smooth muscle cells to the intima at 4 days after balloon injury. The number of intimal smooth muscle cells decreased from 99.7±30.8 cells per square millimeter in control animals to 2.84±0.79 cells per square millimeter in GM 6001-treated animals. The medial smooth muscle cell replication rate was not significantly affected by GM 6001 treatment (Fig 7), confirming that the MMP inhibitor directly inhibited migration and did not reduce the number of cells available to migrate from the media to the intima.

**Discussion**

There are no smooth muscle cells in the intima of a normal rat carotid artery,27,28 and so after balloon catheter injury, medial smooth muscle cells must migrate to the intima to develop an intimal lesion.1 In the present study, we have shown induction of an 88-kD gelatinase in the rat carotid artery after balloon catheter injury. The early induction of this enzymatic activity (by 1 day after injury) preceded the appearance of smooth muscle cells in the intima (4 days after injury) and thus may have served to facilitate migration of the cells within the media. Our studies showed that 88-kD gelatinase activity was present between 1 and 6 days after injury but was not present in the vessel at 2 weeks. In situ hybridization studies confirmed that intimal smooth muscle cells express MMP-9 mRNA at 7 days after injury, but expression of this mRNA stops by 14 days. We believe that this time course of activity is significant, because 14 days after injury, intimal smooth muscle cells continue to replicate. Thus, there is no correlation between 88-kD gelatinase activity and intimal cell replication. We propose that expression of 88-kD gelatinase is associated with migration of cells from media to intima but not with intimal cell replication.

We obtained further evidence in support of this hypothesis when we compared gelatinolytic activities in balloon- and filament-injured arteries. The replication rate of intimal smooth muscle cells is very similar after both injuries, but the neointima is significantly smaller after filament injury, indicating that smooth muscle cell migration is greatly reduced in this model.17 The gelatinolytic activity (62- and 88-kD enzymes) of arteries after gentle denudation was considerably less than that observed after balloon injury. Taken together, the results of the present study suggest a strong association between 88- and 62-kD gelatinase activity and smooth muscle cell migration into the intima.

Administration of an MMP inhibitor, the peptide hydroxamic acid GM 6001,28 dramatically inhibited the migration of medial smooth muscle cells into the intima. We believe that GM 6001 inhibited migration directly, since there was no change in the smooth muscle cell replication rate. Thus, the number of cells in the media available to migrate was not decreased. These data support the hypothesis that MMP expression and activity are necessary to enable smooth muscle cells to migrate through the vessel media into the intima. GM 6001 inhibits all MMPs by binding to the active site with K_i values ranging from 0.1 to 27 nmol/L (R.E. Galardy, unpublished data). Thus, both 62- and 88-kD gelatinases were inactivated by GM 6001 treatment.

We believe that the 88-kD gelatinase activity that we have observed on zymogram gels corresponds to an activated form of rat MMP-9 (the latent form has a molecular mass of 95 kD22). The 70- and 62-kD gelatinase activities probably represent latent and active MMP-2. The present study provides evidence that MMP-9 and MMP-2 gelatinase activities are regulated at different levels. MMP-9 was present mainly in an active 88-kD form, and the pattern of mRNA expression and activity on zymograms was similar after injury, suggesting transcriptional regulation of this proteinase. By contrast, the majority of MMP-2 activity on gelatin zymograms was present in the proenzyme form (70-kD gelatinase), and the amount of proenzyme did not change after injury. Further, mRNA levels for MMP-2 actually decreased during the first week after injury. However, we did notice an increase in the amount of an activated MMP-2 species (molecular mass, 62 kD) between 4 and 14 days after balloon injury. Thus, it seems that MMP-2 may be regulated at least in part at the level of proenzyme activation. At present, we know little about mechanisms of MMP activation in the arterial...
wall; however, the tissue inhibitors of metalloproteinases (TIMPs) may serve to limit and control proteolytic degradation of matrix. TIMP-2 binds to latent and active MMP-2.39 Preliminary experiments show that TIMP-2 is an abundant inhibitor in the vessel wall, and TIMP-2 activity and mRNA levels increase within 24 hours after balloon catheter injury. Interestingly, the increase in TIMP-2 at 7 days after injury was coincident with the increase in gelatinase activity (personal communication, Reza Forough, Department of Surgery, University of Washington, Seattle). This increase in TIMP activity may represent preparation of a defense mechanism to limit matrix degradation.

Potentially, there are other proteinases produced by smooth muscle cells, although we did not find any evidence of synthesis of stromelysin (MMP-3) or interstitial collagenase (MMP-1) by rat vascular smooth muscle cells in vivo. When casein, a preferred substrate for MMP-3 in zymogram gels, was incorporated into the gels, no MMP-3 lysis bands were detected, despite prolonged incubation of the gels. Similarly, Northern blots of arterial tissue probed with MMP-3 or MMP-1 cDNA contained no complementary mRNA species. Previous studies have shown that cultured rabbit vascular smooth muscle cells produce MMP-2 and MMP-9 but do not produce MMP-3 or MMP-1.40,41 Similarly, tissues obtained from human atherosclerotic aortas did not contain MMP-1 activity,42 but Yanagi et al43 have reported that human vascular smooth muscle cells in vitro synthesize MMP-1. Also Au et al44 have demonstrated MMP-1 production by baboon smooth muscle cells in vitro. Thus, smooth muscle cell proteinase expression appears to vary in vivo and in vitro or according to species.

In summary, we have shown induction of an active 88-kD gelatinase and increased activity of a 62-kD gelatinase after balloon catheter injury in the rat carotid artery. The time course of expression of these gelatibaseases correlated with smooth muscle cell migration, and their activities were decreased in a model in which migration was reduced. Furthermore, inhibition of MMP activity with a peptide hydroxamic acid resulted in inhibition of smooth muscle cell migration into the intima. These results suggest that gelatinases are involved in tissue remodeling and neointimal formation after arterial injury.

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