Basic Fibroblast Growth Factor Mediates Carotid Plaque Instability Through Metalloproteinase-2 and -9 Expression

P. Sapienza,* **L. di Marzo, V. Borrelli, A. V. Sterpetti, A. Mingoli,**
R. Piagnerelli and A. Cavallaro

Department of Surgery 'Pietro Valdoni', University of Rome 'La Sapienza', Rome, Italy

Objective(s). *We hypothesized that basic fibroblast growth factor (bFGF) may exert a role in carotid plaque instability by regulating the expression of matrix metalloproteinases (MMP).*

Methods. *Plaques obtained from 40 consecutive patients undergoing carotid endarterectomy were preoperatively classified as soft or hard. Serum bFGF was pre- and postoperatively measured. The release of MMP-2 and MMP-9 in the blood serum, and the activity, production and expression in the carotid specimens was analyzed. Specific anti-bFGF inhibition tests were performed* in vitro *on human umbilical artery smooth muscle cells (HUASMC) to evaluate the role of bFGF in the activity, production and expression of MMP-2 and -9.*

Results. *Twenty-one (53%) patients had a soft carotid plaque and 19 (48%) a hard plaque. Preoperative bFGF serum levels were higher in patients with soft plaques [soft = 34 (28−39) pg/mL and hard = 20 (17−22) pg/mL—p < 0.001] and postoperatively returned to normal values (when compared to 10 healthy volunteers). The serum levels of MMP-2 in patients' with soft plaques were higher than those in patients' with hard plaques [soft = 1222 (1190−1252) ng/mL and hard = 748 (656−793) ng/mL—p < 0.0001]. MMP-9 serum values were 26 (22−29) ng/mL for soft plaques and 18 (15−21) ng/mL for hard plaques (p < 0.0001). We found increased activity, production and expression of MMP-2 and -9 in soft plaques compared to hard plaques (p < 0.001). In* vitro *inhibition tests on HUASMC showed the direct influence of bFGF on the activity, production and expression of MMP-2 and -9 (p < 0.001).*

Conclusions. *bFGF seems to exert a key role in carotid plaque instability regulating the activity, production and expression of MMP thus altering the physiologic homeostasis of the carotid plaque.*

Key Words: bFGF; Metalloproteinases; Carotid endarterectomy; Atherosclerosis; Unstable carotid plaques; Stable carotid plaques.

Introduction

Atherosclerosis leads to myocardial infarction and stroke and is the principal cause of death in Western countries.[1] Advanced atherosclerotic plaques are characterized by a lipid core covered by a fibrous cap composed of smooth muscle cells (SMC) and extracellular matrix. Lesions with a thin fibrous cap overlying a large lipid-rich core are more vulnerable to plaque rupture.[2] The fibrous cap is composed of extracellular matrix that includes collagens, elastins and proteoglycans.[3] A number of proteolytic enzymes, particularly matrix metalloproteinases (MMP), are present in atherosclerotic plaques.[4] Degradation of extracellular matrix by MMP, causing intraplaque hemorrhage, cap rupture, and cap ulceration, may promote plaque instability and prelude to the onset of cerebral ischemic events.[2,4] Recent studies suggest that MMP are important in atherosclerosis development and complications. These enzymes are the physiological regulators of the extracellular matrix[5] and are secreted as proenzymes requiring extracellular activation after which they are capable of degrading virtually all components of the extracellular matrix.[6]

MMP-2 (72 kDa gelatinase/type IV collagenase, gelatinase A) cleaves a number of substrates including gelatins, collagen types IV, V, VII, X and XI, fibronectin, elastin and proteoglycan. MMP-2 has been identified in a number of tissues, cells and plasma/serum and it is secreted as a precursor form.[7,8] MMP-9 (92 kDa gelatinase/type IV collagenase, gelatinase B) has a broad range of substrate specificity for native collagens including types I, III, IV and V as well as gelatin, proteoglycans and elastin, it is secreted as a precursor form.[9] Since MMP-2 and -9 efficiently degrade type IV collagen, the major structural component of basement

*Corresponding author. Dr P. Sapienza, MD, PhD, Department of Surgery 'Pietro Valdoni', University of Rome 'La Sapienza', Policlinico Umberto I°, Viale del Policlinico, 155, 00161 Rome, Italy.

membrane, they may have a predominant role in carotid plaque instability.

Cytokines and growth factors secreted by SMC, endothelial cells (EC) and macrophages might also induce the release of matrix-degrading proteases. Basic fibroblast growth factor (bFGF), a growth factor member of the heparin-binding growth factors,[10] ubiquitously distributed and with mitogenic activity both for EC and SMC,[11] may theoretically have a role in extracellular matrix remodelling regulating the expression of MMP.

We investigated the role of bFGF in plaque instability, in particular whether it regulated the expression of MMP.

Materials and Methods

Forty consecutive patients (26 men and 14 women; mean age 71 ± 7 years; median 72 years; range min. 60-max. 87 years) presenting at our institution with internal carotid artery stenosis greater than 70% were entered into this study after informed consent was obtained. All patients were studied preoperatively with color Doppler, magnetic resonance angiography to confirm the degree of stenosis and cerebral magnetic resonance.

The carotid plaque was echographically (Acuson 128 XP/4, Computed Sonography, USA) characterized as uniformly anechogenic, predominantly hypoechogenic, predominantly echogenic, uniformly echogenic. The luminal surface of the plaque was also defined as regular, irregular (with recess between 0.4 and 2 mm in depth and width) and irregular (with recess more than 2 mm in depth and width). The presence of disruption or marked irregularities of the plaque surface, on two-dimensional cross-sectional and longitudinal views of carotid artery, defined unstable plaque (soft carotid plaque).

All operations were performed in a standard fashion under general anesthesia and EEG monitoring with minimal manipulation of the carotid plaque specimen. The plaques were obtained and processed immediately after endarterectomy.

All tissues were stored at $-70\,°C$ under liquid nitrogen after the plaque was divided longitudinally into four pieces for further analyses. Two blood samples were also drawn from each patients 24 h before and after surgery to determine bFGF and MMP plasma levels. Ten healthy volunteers were enrolled to measure their bFGF circulating levels. The release, expression and activity of MMP-2 and MMP-9 were then evaluated in the carotid plaque specimens. Thereafter, similar experiments were conducted on

human umbilical artery SMC (HUASMC) culture and inhibition tests with anti-bFGF antibody were performed to assess the role of this growth factor in regulating the expression of MMP.

In vivo *experiments*

Enzyme linked immunosorbant assay (ELISA)
Plasma was obtained from EDTA-anticoagulated peripheral blood samples centrifuged at $4000g$ for 10 min. Plasma levels of 72 kDa MMP-2 and 92 kDa MMP-9 were determined with ELISA technique (Quantikine humanMMP-2 and -9, R&D Systems Europe Ltd, UK). Aliquots of diluted plasma (1:50 and 1:20, respectively) from all patients were analyzed. Similarly, bFGF plasma levels were determined with ELISA technique (Quantikine humanFGF basic, R&D).

Zymography
Zymography, able to differentiate between active and latent enzyme forms, quantified MMP activities. The carotid plaque specimens were homogenized (Ultra-Turrax T 25, IKA-Labortechnik), lysed (0.3 g/tissue/mL) with an ice-cold lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.2% sodium azide, 200 μM PMSF, 1 μM Leupeptina, 1 μM Pepstatin A), clarified ($16,000g$ for 30 min), and the protein concentration determined using Bradford protein assay (Sigma Chemical Co., St Louis, MO, USA). Zymography was performed with 1 μg total protein. The presence of MMP-2 and MMP-9 was demonstrated using 10% SDS-polyacrylamide gels containing 1 mg/mL gelatin (Sigma) under non reducing conditions at constant voltage (120 V) for approximately 1.5 h. Gels were washed twice (30 min) with 2.5% Triton X-100 (Sigma) on a rotary shaker and incubated (24 h at 37 °C) in zymographic buffer (0.5 M Tris/HCl pH 7.5, 50 mM CaCl$_2$, 2 M NaCl, 0.2% Brij 35). Gels were stained using 0.5% Coomassie brilliant blue R-250. HT-1080 fibrosarcoma cells were used as positive control. Clear areas on the Coomassie-stained gel which indicated the presence of proteolitic activity, were quantified by densitometric scanning. Densitometry of destained areas was quantified with the use of an Imaging Fluor-S (Bio-Rad, Hercules, CA, USA) densitometer connected to a personal computer. All results were normalized to the positive control. The identities of the lytic zones on the zymograms were confirmed as MMP-2 and MMP-9 (72 kDa for MMP-2 and 92 kDa for MMP-9) by immunoblotting.

Western blot analysis

The specimens were homogenized (Ultra-Turrax T 25), lysed with a ice-cold lysis buffer, clarified, and the proteins concentration was determined. Forty microgram of total protein of tissue extracts were suspended in reducing buffer. The samples were then separated by 8% SDS-PAGE for 1.5 h at 120 V and blotted into nitrocellulose for 1 h at 100 V. The membranes were incubated in blocking buffer (5% nonfat dry milk, 0.1% Tween 20, PBS) and probed overnight with the mouse monoclonal anti-human MMP-2 and -9 (R&D) (1 μg/mL) IgG antibody. The membranes were incubated with specific secondary horseradish peroxidase-conjugated anti-mouse antibody (1:2000 dilution) (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and developed with the use of chemiluminescence ECL Kit (Amersham), and exposed to Kodak XAR X-ray film for 1, 5 and 10 min. Human recombinant MMP-2 and -9 (150 ng) were used as positive controls. Densitometry of the bands was performed described above.

Reverse transcription polymerase chain reaction (RT-PCR)

The total cellular RNA by the RNAfast method (Molecular system, San Diego, CA, USA) from the resulting fine tissue powder was extracted. Reverse transcription was performed with 1 μg of total RNA by using, in each sample, 150 pmol of random hexamers (Boehringer Mannheim, GmbH, Germany), with 50 units per sample of M-MuLV Reverse Transcriptase (HT Biotechnology Ltd, Cambridge, UK) at 42 °C for 1 h, in the manufacturer's assay buffer (20 μL final volume) plus 20 units of human placental ribonuclease inhibitor (HT Biotechnology), and 200 μM dNTPs, followed by heat inactivation at 94 °C for 5 min. Subsequently, PCR reactions were performed with specific primers for MMP-2 (accession number NM_004530; sense: GGATGATGCCTTTGCT-CGTGC, antisense: CATCGTAGTTGGCTGTGGTCG; annealing temperature 60 °C; product 729 bp), MMP-9 (accession number AF538844; sense: GTGGCAGA-GATGCGTGGAGAGTCG, antisense: TTGCCCAGG-GACCACAACTCGTC; annealing temperature 64 °C; product 472 bp) and β-actin (accession number NM001101; sense: GCGAGAAGATGACCCAGAT-CATGTT, antisense: GCTTCTCCTTAATGTCACG-CACGAT; annealing temperature 60 °C; product 300 bp) primers as an internal control. In all reactions 10 pmol of each primer and one unit of Super Taq DNA polymerase (Thermus thermophilus DNA polymerase) (HT Biotechnology) were used. PCR was performed in a final volume of 50 μL in the manufacturer's assay buffer plus 200 μM dNTPs. After an initial denaturation step of 5 min at 94 °C, the PCR

procedure consisted of scalar cycle number (from 30 to 20) of 1 min. at 94 °C, 1 min at specific annealing temperature for each primers, 1, 5 min at 72 °C, followed by 10 min at 72 °C, on a Perkin–Elmer Cetus Thermal Cycler model 480. Electrophoresis of 15 μL of the PCR reaction was performed under standard conditions. All gels were photographed with a Polaroid DS-34 camera using Polaroid 667 films and scanned by a Kodak camera Megaplus model 1.4. The images were acquired by Imaging Fluor-S (Bio-Rad) densitometer. The MMP-2, MMP-9 and β-actin expressions were assessed starting, for each experimental condition, from aliquots of the same cDNA preparation. The linearity range of the assay was checked by the method of scalar cycle number. The results were expressed as the ratio MMP-2/β-actin and MMP-9/β-actin.

Immunohistochemical analysis

Specimens from carotid plaques were fixed in 10% neutral buffered formalin and subsequently decalcified in formic acid. The plaques were dehydrated in graded ethanol and embedded in paraffin. Serial sections (3 μm thick) were obtained every millimeters throughout the length of the plaque. The resulting sections were mounted on APES (DAKO, Glostrup, Denmark) coated slides. Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol and treated with methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity. Slides, in citrate buffer, were placed in microwave oven for 10 min at 65 °C and then incubated in 10% normal horse serum (NHS) (Vector Laboratories Ltd, Peterborough, England) for 30 min at room temperature. NHS was removed and slides were incubated overnight at 4 °C with mouse anti-human MMP-2 and -9 (1:100) (R&D). Slides were then washed twice in PBS and horseradish peroxidase-conjugated anti-mouse secondary antibody (1:200 dilution) were used. Subsequently avidin-biotin complex (Vector) was added and the peroxidase reaction was performed using 0.06% diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. After a final rinse, sections were dehydrated in ethanol, cleared in xylene and mounted in Permount.

In vitro *experiments*

Stimulation of MMP-2 and -9 release, activity and expression by bFGF

To study the effect of bFGF on MMP-2 and MMP-9 release, expression and activity, HUASMC (Clonetics, Cambrex BioScience Rockland, Inc., USA) were cultured in SmBM (smooth muscle cell basal medium)

(Clonetics-Cambrex) supplemented with 20% Fetal Calf serum (FCS). They were grown in an environment of 5% CO_2 at 37 °C. Culture medium was changed every two days. For stimulation experiments cells at confluence were stimulated after 24 h of cultivation in a serum-free medium with 20, 30 and 100 ng/mL of bFGF (Peprotech EC Ltd, UK). The conditioned medium was then removed, centrifuged for 5 min at 16,000g and stored at −80 °C. The presence of MMP-2 and -9 was assayed with ELISA technique (Quantikine humanMMP-2 and -9, R&D).

To determine whether bFGF affected the levels of proteolytic enzymes, the conditioned medium from HUASMC exposed to bFGF, was examined for its gelatinolytic activity by zymography. MMP-2 and -9 protein levels were tested by Western blotting in the same conditioned medium. To further determine whether the regulation occurs at the mRNA level, RT-PCR was performed on mRNA, isolated with the RNA fast method, from HUASMC, stimulated for 24 h at confluence, with bFGF (20, 30 and 100 ng/mL) in a serum free medium.

Inhibition of MMP-2 and -9 release, activity and expression by anti-bFGF antibodies
To assess the role of bFGF on the release, activity and expression of MMP-2 and -9, HUASMC were stimulated with bFGF (20, 30 and 100 ng/mL) and an excess of monoclonal antibody anti-bFGF (5 μg/mL) (Peprotech) was added for 72 h for ELISA, zymography and Western blot analysis and for 24 h for RT-PCR analysis.

To exclude nonspecific effect of antibody for bFGF on MMP-2 and -9, HUASMC were cultured in SmBM supplemented with 20% FCS. They were grown in an environment of 5% CO_2 at 37 °C. Cells at confluence were stimulated after 24 h of cultivation in a serum-free medium with 20, 30 and 100 ng/mL of bFGF (Peprotech) and an excess both of monoclonal antibody anti-bFGF (5 μg/mL) (mouse IgG isotype) (Peprotech) and monoclonal antibody anti-PDGF (5 μg/mL) (mouse IgG isotype) (Peprotech) added for 72 h for ELISA, zymography and Western blot analysis and for 24 h for RT-PCR analysis.

Statistical analysis
Data were analyzed with a computer software program (SPSS 12.0 for Windows, Basic and Advanced Statistics, 1989–2003; SPSS Inc., Chicago, IL, USA). All results are expressed as median and range (between brackets) of at least 6–8 different experiments. Mann–Whitney U test and Kruskal–Wallis one-way analysis of variance were used to compare means. A p-value less than 0.05 was considered significant.

Results

Nineteen (48%) carotid plaques were echographically defined as hard and 21 (53%) as soft.

In vivo *experiments*

ELISA
The circulating MMP-2 and -9 levels were higher in the plasma of patients affected with soft plaques when compared to those with hard plaques [MMP-2, soft = 1222 (1190–1252) ng/mL and hard = 748 (656–793) ng/mL—$p < 0.0001$; MMP-9, soft = 26 (22–29) ng/mL and hard = 18 (15–21) ng/mL—$p < 0.0001$].

Healthy volunteers had a significantly lower bFGF plasma levels when compared to patients with unstable carotid plaques ($p < 0.001$) and stable plaques ($p < 0.01$) (Table 1). Preoperatively, patients affected with unstable carotid plaques had higher bFGF plasma levels when compared to patients with stable plaques ($p < 0.001$) (Table 1). Twenty-four hours after endarterectomy, bFGF levels were similar to healthy volunteers in both groups (Table 1).

Zymography
Four zones of lysis at 62, 72 (active/latent MMP-2), 83 and 92 kDa (active/latent MMP-9) were identified on zymography of the carotid specimens. The amount of active MMP-2 and -9 was significantly higher in patients affected with unstable plaques when compared to stable plaques [MMP-2, soft = 1.3% (1.2–1.5%) and hard = 1.2% (1.1–1.4%)—$p < 0.0001$; MMP-9, soft = 11% (10–13%) and hard 1.4% (1.2–1.6%)—$p < 0.0001$]. The amount of latent MMP-2 and -9 was significantly higher in the soft plaques when compared to hard plaques [MMP-2, soft = 60% (53–68%) and hard = 29% (24–33%)—$p < 0.0001$; MMP-9, soft = 33% (29–36%) and hard = 16% (10–24%)—$p < 0.0001$].

Table 1. bFGF plasma levels

Healthy volunteers ($n = 10$)	
bFGF	pg/mL
	16, (12–19)
Soft carotid plaques ($n = 21$)	
bFGF	pg/mL
Preoperative	34, (28–39)
Postoperative	18, (16–21)
Hard carotid plaques ($n = 19$)	
bFGF	pg/mL
Preoperative	20, (17–22)
Postoperative	15, (12–18)

Values are expressed as median and range (between brackets).

Western blot

Immunoblotting confirmed the identities of the lytic zones on the zymograms as MMP-2 and MMP-9 (72 and 92 kDa, respectively). Protein levels of MMP-2 and -9 were more abundant in the soft than in the hard plaques [MMP-2, soft = 79% (72–84%) and hard = 50% (46–54%)—$p < 0.0001$; MMP-9, soft = 69% (64–72%) and hard = 48% (44–53%)—$p < 0.0001$].

RT-PCR

The densitometric analysis of the amplified products corresponding to the MMP-2 and -9 mRNA (729 bp and 472 bp, respectively) showed that mRNA expression was significantly higher in the soft plaques when compared to the hard plaques [MMP-2, soft = 1.4 (0.9–2.1) and hard = 0.7 (0.3–1.1)—$p < 0.0001$; MMP-9, soft = 0.9 (0.4–1.3) and hard = 0.3 (0.1–0.6)—$p < 0.0001$].

Immunohistochemistry

Immunostaining for MMP-2 and -9 showed the presence of MMP within the plaque, revealing intense staining around the plaque core, especially in the plaque shoulder and cap of the soft plaques when compared to hard plaques.

In vitro *experiments*

ELISA

The increase of bFGF concentration from 20 to 100 ng/mL augmented, in a dose dependent manner, the release of MMP-2 ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) and -9 ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) when compared to controls (Table 2). The addition of monoclonal anti-bFGF antibody significantly reduced the release of MMP-2 ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) and -9 ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) when compared to controls (Table 2).

Zymography

Two zones of lysis at 72 (latent MMP-2) and 92 kDa (latent MMP-9) were identified. The amount of latent MMP-2 and MMP-9 significantly augmented in a dose dependent manner with the increase of bFGF concentration from 20 to 100 ng/mL when compared to controls [MMP-2: 60% (55–68%) $p < 0.001$, 67% (59–71%) $p < 0.001$ and 74% (68–79%) $p < 0.001$, respectively; MMP-9: 30% (26–38%) $p < 0.05$, 40% (35–48%) $p < 0.001$ and 45% (37–51%) $p < 0.001$, respectively] (Fig. 1). The addition of monoclonal anti-bFGF antibody significantly decreased the proteolytic activity of MMP-2 and MMP-9 at all concentrations when compared to controls [MMP-2: 42% (35–48%) $p < 0.001$, 50% (42–59%) $p < 0.001$ and 46% (41–52%) $p < 0.001$, respectively; MMP-9: 20% (13–26%) $p < 0.001$, 35% (31–38%) $p < 0.001$ and 38% (33–44%) $p < 0.001$, respectively] (Fig. 1).

Western blot

Protein levels of MMP-2 and MMP-9 significantly augmented in a dose dependent manner with the increase of bFGF concentration from 20 to 100 ng/mL when compared to controls [MMP-2: 49% (44–53%) $p < 0.001$, 53% (47–61%) $p < 0.001$ and 60% (52–67%) $p < 0.001$, respectively; MMP-9: 52% (47–60%) $p < 0.001$, 66% (59–73%) $p < 0.001$ and 71% (59–80%) $p < 0.001$, respectively] (Fig. 2). The addition of monoclonal anti-bFGF antibody significantly decreased the protein levels of MMP-2 and MMP-9 when compared to controls (MMP-2: 36% (31–44%) $p < 0.001$, 39% (33–46%) $p < 0.001$ and 60% (55–69%) $p < 0.001$, respectively; MMP-9: 42% (32–51%) $p < 0.001$, 31% (23–38%) $p < 0.001$ and 19% (15–27%) $p < 0.001$, respectively] (Fig. 2).

RT-PCR

The densitometric analysis of the amplified products corresponding to the MMP-2 and -9 mRNA (729 bp and 472 bp, respectively) showed that mRNA expression significantly increased in a dose dependent

Table 2. Release of MMP-2 and -9 after bFGF stimulation and inhibition

MMP-2			
Control	25, (18–28)		
Stimulation	20 ng/mL	30 ng/mL	100 ng/mL
	40, (36–42)	71, (64–73)	243, (237–258)
Inhibition	20 ng/mL + 5 μg/mLαbFGF	30 ng/mL + 5 μg/mLαbFGF	100 ng/mL + 5 μg/mLαbFGF
	20, (17–22)	54, (51–57)	160, (155–167)
MMP-9			
Control	5.5, (4.9-6.1)		
Stimulation	20 ng/mL	30 ng/mL	100 ng/mL
	16, (13–19)	18, (14–21)	20, (16–27)
Inhibition	20 ng/mL + 5 μg/mLαbFGF	30 ng/mL + 5 μg/mLαbFGF	100 ng/mL + 5 μg/mLαbFGF
	8, (7–11)	7, (4–9)	8, (5–11)

Values are expressed as median and range (between brackets).
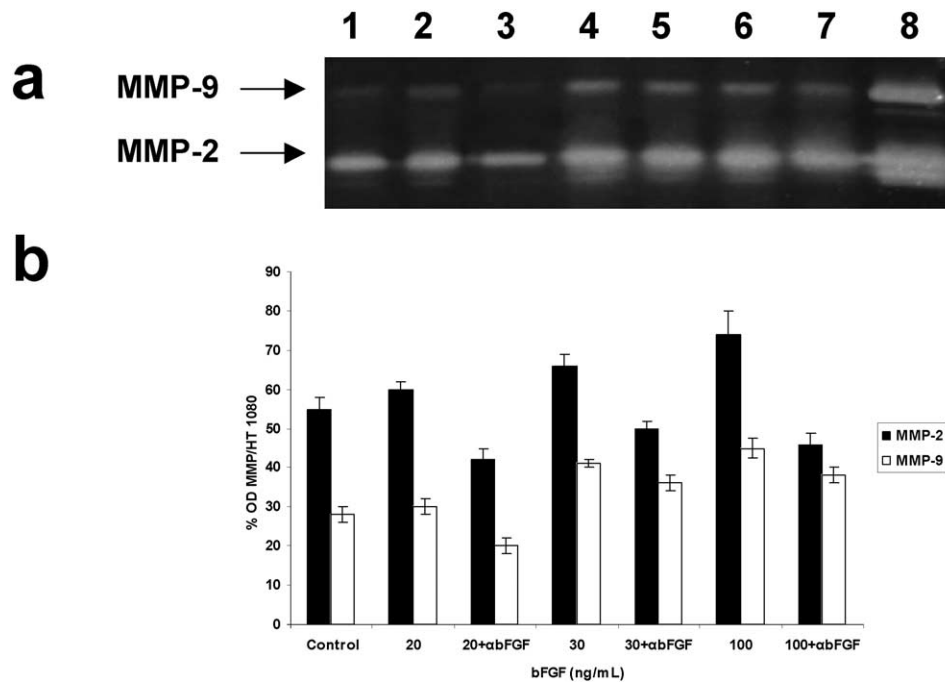
Fig. 1. bFGF effects on MMP-2 and MMP-9 gelatinase activity in HUASMC. 72-kDa MMP-2 and 92-kDa MMP-9 bands were detected (a). Conditioned medium obtained from HUASMC not exposed to bFGF (lanes 1), exposed to bFGF at concentrations of 20 ng/ml (lanes 2), 30 ng/ml (lanes 4) and 100 ng/ml (lanes 6) and exposed to bFGF with the addition of monoclonal anti-bFGF antibody at the same concentrations (lanes 3, 5, 7, respectively). HT1080 cells were used as positive controls (lanes 8). The ratios between optical density (OD) of MMP and HT1080 are shown in the bar graph (b).

manner with the increase of bFGF concentration from 20 to 100 ng/mL when compared to controls [MMP-2: 1.4 (1.1–1.6) $p < 0.001$, 2 (1.6–2.4) $p < 0.001$ and 2.3 (1.9–2.6) $p < 0.001$, respectively; MMP-9: 1.9 (1.5–2.2) $p < 0.001$, 2.3 (2.1–2.7) $p < 0.001$ and 3 (2.5–3.4) $p < 0.001$, respectively] (Fig. 3). The addition of monoclonal anti-bFGF antibody to the medium of HUASMC exposed to bFGF at every concentrations, significantly decreased the mRNA expression of MMP-2 and MMP-9 when compared to controls [MMP-2: 1.2 (0.9–1.4) $p < 0.001$, 0.3 (0.1–0.5) $p < 0.001$ and 1.4 (1.1–1.7) $p < 0.001$, respectively; MMP-9: 1 (0.8–1.3) $p < 0.001$, 0.4 (0.1–0.6) $p < 0.001$ and 0.5 (0.2–0.9) $p < 0.001$, respectively] (Fig. 3).

Effect of nonspecific antibody for bFGF
The addition of monoclonal anti-bFGF and anti-PDGF antibody to the medium of HUASMC exposed to bFGF at every concentrations from 20 to 100 ng/mL not significantly decreased the activity, production and expression of MMP-2 and MMP-9 when compared to controls (p = NS).

Discussion

The atherosclerotic plaque is a dynamic structure that undergoes continuous remodeling of the extracellular matrix on which its structural integrity depends. The matrix degrading MMP promote physiological turnover of all extracellular matrix components. Deregulated degradation of basement membrane may lead to plaque instability and acute plaque changes immediately precede the onset of clinical symptoms. Each phase of this process is mediated by a series of MMP, secreted as latent pro-enzymes which require activation by limited proteolysis. The events leading up to this activation remain unclear.

Few studies have examined the interactions between connective tissue mitogens and MMP, despite the fact that both are present in atherosclerotic plaques. Increased levels of connective tissue mitogens at the site of inflammation have been described and are able to mediate various biological responses.[12,13] The site of plaque rupture is characterized by an intense inflammatory infiltration,[14] which consists predominantly of macrophages, foam cells, and T-lymphocytes playing a role in the destabilization of the plaque.[15] This infiltrate undergoes a period of activation at the time of acute coronary syndromes and the associated release of proteolytic enzymes may lead to plaque instability.[16]

In the present study, we demonstrated that there was an increased plasmatic concentration of MMP-2
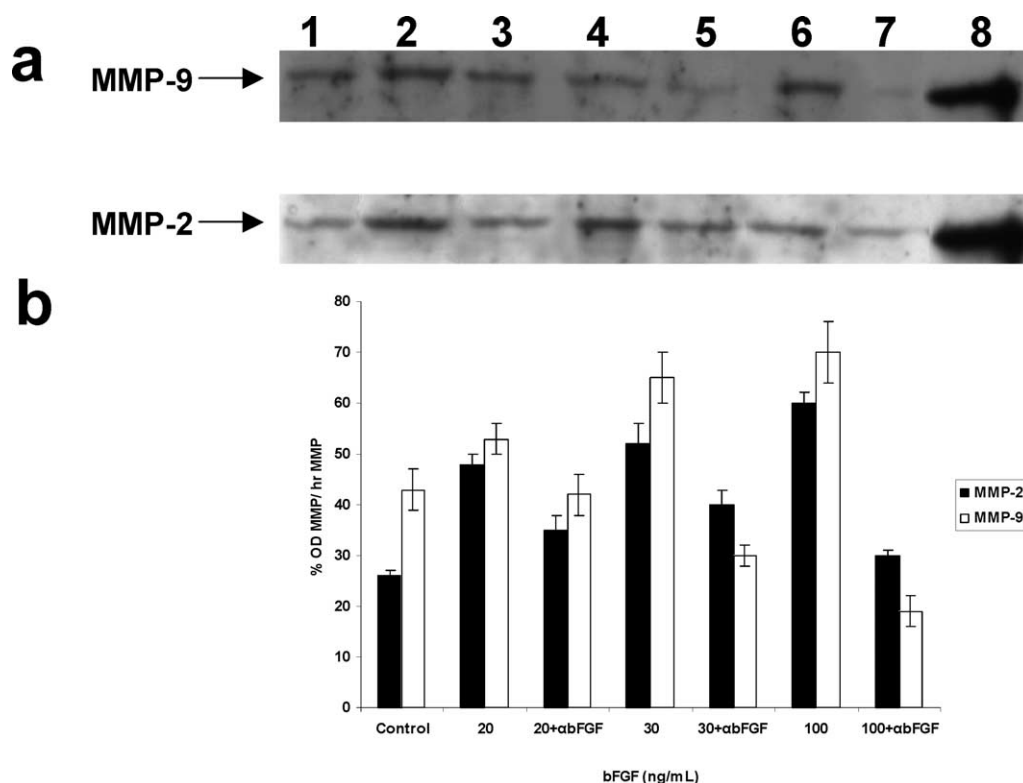
Fig. 2. Western blotting of MMP-2 and -9. Samples derived from HUASMC not exposed to bFGF (lanes 1), exposed to bFGF at concentrations of 20 ng/ml (lanes 2), 30 ng/ml (lanes 4) and 100 ng/ml (lanes 6) and exposed to bFGF with the addition of monoclonal anti-bFGF antibody at concentrations of 20, 30 and 100 ng/ml (lanes 3, 5 and 7, respectively) (a). The identity of the bands as MMP-2 and -9 was confirmed with a human recombinant MMP-2 and -9 (150 ng/ml) electrophoresed as a positive control (lane 8) (a). The ratios between optical density (OD) of MMP and hrMMP-2 and -9 are reported in the bar graph (b).

and -9 in patients affected with unstable carotid plaques. Recently, Loftus *et al.*[17] have observed that plasma MMP-9 levels are elevated in patients with embolizing carotid plaques. The Authors also tested other MMP but did not found any differences between stable and unstable carotid plaques. Kai *et al.*[18] demonstrated an early increase in circulating levels of both MMP-2 and -9 in patients with acute coronary syndromes. Our findings have also shown that there is an increase in the gelatinase activity, production and gene transcription of MMP-2 and -9 in the unstable plaques. Both active and latent MMP-2 and -9 forms had a significantly higher gelatinase activity in unstable plaques when compared to stable plaques. However, other Authors[9] have found a significantly higher intraplaque level, activity and expression of MMP-9 but no differences in the level of MMP-2 or other MMP in carotid specimens from patients affected with embolizing plaques.

The plasmatic level of bFGF in patients affected with soft carotid plaques were higher than in those with hard plaques and both were higher as compared to healthy subjects. Interestingly, 24 h after surgery, the plasma levels of bFGF returned to normal values. Katinioti *et al.*[19] reported that bFGF circulating levels were higher in patients affected with coronary artery diseases as compared to normal subjects. The plasmatic levels of bFGF returned to normal after coronary angioplasty and remained stable for 6 months after the procedure. Since the atherosclerotic process affects several arterial districts, this phenomenon is not easy understandable. In our population bFGF serum levels seem to be related to the presence of carotid artery stenosis and decrease to normal values after endarterectomy.

Our study pointed out that the stimulation of HUASMC with bFGF determined a significant increase in the release, activity, production and expression of MMP-2 and -9. This increase was dose-dependent and the inhibition with monoclonal anti-bFGF antibodies significantly decreased the release, activity, production and expression of MMP-2 and -9.

The effect of bFGF on MMP-2 and -9 should be also viewed under various aspects. bFGF is normally found in extracellular matrix[20,21] because of its strong interaction with heparin which reflects the affinity for
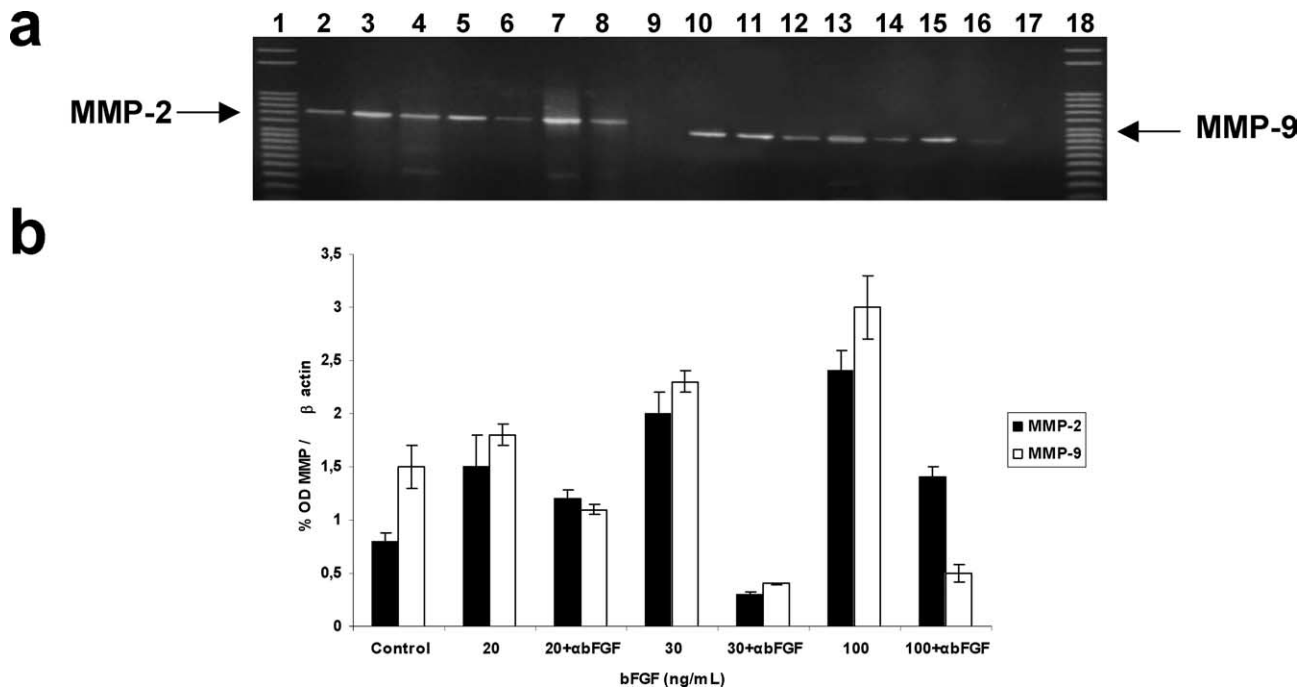
Fig. 3. RT-PCR of MMP-2 and MMP-9 mRNA expression. RT-PCR products of MMP-2 and MMP-9 were obtained from HUASMC not exposed to bFGF (lanes 2 and 10), exposed to bFGF at concentrations of 20 ng/ml (lanes 3 and 11), 30 ng/ml (lanes 5 and 13) and 100 ng/ml (lanes 7 and 15) and exposed to bFGF with the addition of monoclonal anti-bFGF antibody at concentrations of 20, 30 and 100 ng/ml (lanes 4, 6, and 8 for MMP-2 and lanes 12, 14 and 16 for MMP-9) (a). The molecular size was determined using φX/HaeIII DNA standard molecular marker (MM) (lanes 1 and 18) (a). Lanes 9 and 17 are the negative controls (B) (a). The ratios between optical density (OD) of MMP and β-actin are described in the bar graph (b).

extracellular matrix heparan sulfate proteoglycans and glycosaminoglycans and it is stored in the extracellular structures and mobilized when needed by remodeling of the basement membrane or extra-cellular matrix.[21,22] Furthermore, bFGF is able per se to suppress collagen synthesis thus contributing to plaque instability.[23]

Kenagy *et al.*[24] observed that part of the stimulatory effect on primate SMC migration caused by bFGF is mediated by MMP-2 and -9. However, while MMP-2 is constitutively expressed by vascular SMC and is physiologically activated by the membrane-bound MMP, which contains a trans-membrane domain within the structure, the stimulus for an increased MMP-9 production, not constitutively expressed, may depend on activation by several growth factors simultaneously.

Bond *et al.*[25] have found in fact that MMP-9 is synergistically upregulated by growth factors and inflammatory cytokines in rabbit and human fibro-blasts. Increased expression of MMP-9 occurs in response to IL-1 and tumor necrosis factor-α in a number of cell types[26,27] and human SMC do not make MMP-9 constitutively or in response to bFGF.

Conversely, in our study we found that MMP-9 in HUASMC may respond differently to bFGF stimulation than other type of cells.[26] Although we cannot exclude the interaction with other growth factors or cytokines normally secreted by HUASMC, we believe that bFGF may exert a key role in the upregulation of MMP-9 on HUASMC. Our results are also consistent with the observation that bFGF seems to regulate MMP-2. The increase of bFGF in the plasma of patients affected with carotid artery stenosis which is even higher in the plasma of patients with unstable plaques, may lead to the hypothesis that this growth factor is able to upregulate MMP-2 and -9. Further studies are required to better understand the under-lying mechanisms at the basis of this phenomenon.

In conclusion, our results suggest that bFGF plays a key role in the instability of the carotid plaque upregulating the expression of MMP-2 and -9 capable of degrading collagen type IV which is the major component of the basement membrane.

References

1 Tunstall-Pedoe H, Kuulasmaa K, Amouyel P, Arveiler D, Rajakangas AM, Pajak A. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38

populations from 21 countries in four continents. *Circulation* 1994; **90**:583–612.

2 LIBBY P. Molecular bases of the acute coronary syndromes. *Circulation* 1995; **91**:2844–2850.

3 WIGHT TN. The extracellular matrix and atherosclerosis. *Curr Opin Lipidol* 1995; **6**:326–334.

4 GALIS ZS, SUKHOVA GK, LARK MW, LIBBY P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994; **94**:2493–2503.

5 WOESSNER JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**:2145–2154.

6 MATRISIAN LM. The matrix-degrading metalloproteinases. *Bioessays* 1992; **14**:455–463.

7 BROWN DL, HIBBS MS, KEARNEY M, LOUSHIN C, ISNER JM. Identification of 92-kD gelatinase in human coronary atherosclerotic lesions. Association of active enzyme synthesis with unstable angina. *Circulation* 1995; **91**:2125–2131.

8 LI Z, LI L, ZIELKE R, CHENG L, XIAO R, CROW M, STETLER-STEVENSON WG, FROEHLICH J, LAKATTA EG. Increased expression of 72-kD type IV collagenase (MMP2) in human aortic atherosclerotic lesions. *Am J Pathol* 1996; **148**:121–128.

9 LOFTUS IM, NAYLOR R, GOODALL S, CROWTHER M, JONES L, BELL PRF, THOMPSON MM. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. *Stroke* 2000; **31**:40–47.

10 CORDON-CARDO C, VLODAVSKY I, HAIMOVITZ-FRIEDMAN A, HICKLIN D, FUKS Z. Expression of basic fibroblast growth factor in normal human tissues. *Lab Invest* 1990; **63**:832–840.

11 CONNOLLY DT, STODDARD BL, HARAKAS NK, FEDER J. Human fibroblast-derived growth factor is a mitogen and chemoattractant for endothelial cells. *Biochem Biophys Res Commun* 1987; **144**:705–712.

12 Ross R. Atherosclerosis. An inflammatory disease. *N Engl J Med* 1999; **340**:115–126.

13 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**:801–809.

14 MORENO PR, FALK E, PALACIOS IF, NEWELL JB, FUSTER V, FALLON JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 1994; **90**:775–778.

15 BUJA LM, WILLERSON JT. Role of inflammation in coronary plaque disruption. *Circulation* 1994; **89**:503–505.

16 MANN JM, KASKI JC, PEREIRA WI, ARIE S, RAMIRES JA, PILEGGI F. Histological patterns of atherosclerotic plaques in unstable angina patients vary according to clinical presentation. *Heart* 1998; **80**:19–22.

17 LOFTUS IM, NAYLOR AR, BELL PR, THOMPSON MM. Plasma MMP-9. A marker of carotid plaque instability. *Eur J Vasc Endovasc Surg* 2001; **21**:17–21.

18 KAI H, IKEDA H, YASUKAWA H, KAI M, SEKI Y, KUWAHARA F, UENO T, SUGI K. Peripheral blood levels of matrix metalloproteinases-2 and -9 are elevated in patients with acute coronary syndromes. *J Am Coll Cardiol* 1998; **32**:368–372.

19 KATINIOTI AA, TOUSOULIS D, ECONOMOU E, STEFANIDIS C, TRIKAS A, TENTOLOURIS C, PITSAVOS C, ANDROULAKIS A, TOUTOUZAS P. Basic fibroblast growth factor changes in response to coronary angioplasty in patients with stable angina. *Int J Cardiol* 2002; **84**:195–199.

20 BAIRD A, LING N. Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem Biophys Res Commun* 1987; **142**:428–435.

21 VLODAVSKY I, FOLKMAN J, SULLIVAN R, FRIDMAN R, ISHAI-MICHAELI R, SASSE J, KLAGSBRUN M. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc Natl Acad Sci* 1987; **84**:2292–2296.

22 FOLKMAN J, KLAGSBRUN M, SASSE J, WADZINSKI M, INGBER D, VLODAVSKY I. A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. *Am J Pathol* 1988; **130**:393–400.

23 MAJORS A, EHRHART LA. Basic fibroblast growth factor in the extracellular matrix suppresses collagen synthesis and type II procollagen mRNA levels in arterial smooth muscle cell cultures. *Arterioscler Thromb* 1993; **13**:680–686.

24 KENAGY RD, HART CE, STETLER-STEVENSON WG, CLOWES AW. Primate smooth muscle cell migration from aortic explants is mediated by endogenous platelet-derived growth factor and basic fibroblast growth factor acting through matrix metalloproteinases 2 and 9. *Circulation* 1997; **96**:3555–3560.

25 BOND M, FABUNMI RP, BAKER AH, NEWBY AC. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB. *FEBS Lett* 1998; **435**:29–34.

26 GALIS ZS, MUSZYNSKI M, SUKHOVA GK, SIMON-MORRISSEY E, UNEMORI EN, LARK MW, AMENTO E, LIBBY P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 1994; **75**:181–189.

27 UNEMORI EN, EHSANI N, WANG M, LEE S, MCGUIRE J, AMENTO EP. Interleukin-1 and transforming growth factor-alpha: synergistic stimulation of metalloproteinases, PGE2, and proliferation in human fibroblasts. *Exp Cell Res* 1994; **210**:166–171.