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Increased expression of bFGF is associated with carotid atherosclerotic plaques instability engaging the NF-κB pathway

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Abstract

Unstable atherosclerotic plaques of the carotid arteries are at great risk for the development of ischemic cerebrovascular events. The degradation of the extracellular matrix by matrix metalloproteinases (MMPs) and nitric oxide induced apoptosis of vascular smooth muscle cells (VSMCs) contribute to the vulnerability of the atherosclerotic plaques. Basic fibroblast growth factor (bFGF) through its mitogenic and angiogenic properties has already been implicated in the pathogenesis of atherosclerosis. However, its role in plaque stability remains elusive. To address this issue, a panel of human carotid atherosclerotic plaques was analysed for bFGF, FGF-receptors-1 and -2 (FGFR-1/-2), inducible nitric oxide synthase (iNOS) and MMP-9 expression. Our data revealed increased expression of bFGF and FGFR-1 in VSMCs of unstable plaques, implying the existence of an autocrine loop, which significantly correlated with high iNOS and MMP-9 levels. These results were recapitulated *in vitro* by treatment of VSMCs with bFGF. bFGF administration led to up-regulation of both iNOS and MMP-9 that was specifically mediated by nuclear factor-κB (NF-κB) activation. Collectively, our data demonstrate a novel NF-κB-mediated pathway linking bFGF with iNOS and MMP-9 expression that is associated with carotid plaque vulnerability.

Keywords: bFGF • carotid atherosclerotic plaques • NF-κB • plaque instability • iNOS • MMP

Introduction

Stroke remains a great socioeconomic burden for developed and developing countries. Strokes are currently classified into two broad categories: ischemic and haemorrhagic. Ischemic attacks, provoked by either thrombosis or embolism, account for 85% of all stroke cases and are closely related – among other risk factors [1] – with the existence of complicated atherosclerotic plaques of carotid arteries [2, 3]. Clinical and experimental data suggest that plaques with rupture or ulceration and intraplaque haemorrhage – defined as 'vulnerable' or 'unstable' plaques – are at high risk of producing thromboembolic events in cerebral circulation [4, 5]. Therefore, it remains crucial to unravel the critical factors that determine the progression of atherosclerotic plaques to instability.

Basic fibroblast growth factor (bFGF/FGF-2) – the prototypic member of a family comprising over 20 related polypeptides [6] – has been detected in human atherosclerotic plaques and is synthesized by three cell types: endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages [7]. bFGF, along with other growth factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), has been implicated in the pathogenesis of atherosclerosis through its mitogenic [8] and angiogenic [9] role, the control over smooth muscle cells migration [10] and the suppression of collagen synthesis [11]. The two latter properties of

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bFGF contribute also to plaque rupture through either the gradual expansion of the plaque [12], or by promoting its weakening due to decreased collagen synthesis by VSMCs [13]. Plaque rupture is also facilitated by the increased activity of matrix metalloproteinases (MMPs) detected in atherosclerotic lesions [14] and the nitric oxide mediated apoptosis of VSMCs [15]. Recent studies have linked bFGF to plaque vulnerability through regulating MMP-2 and MMP-9 activity [16]. However, there are no evidence regarding the status of bFGF in human carotid atherosclerotic plaques, and the bFGF-triggered molecular mechanisms that contribute to plaque vulnerability.

To address this question, we examined the expression of bFGF in VSMCs of carotid atherosclerotic plaques in relation to plaque stability and the expression of two vital molecules related to plaque rupture, namely inducible nitric oxide synthase (iNOS) and MMP-9. The functional basis of the observed relationships in the clinical samples was recapitulated *in vitro* in human VSMCs treated with recombinant bFGF. This cellular model was also used to identify nuclear factor-κB (NF-κB) as the mediator of the bFGF-dependent pathway that induces iNOS and MMP-9 expression. Finally, we assessed our clinical samples for the expression and subcellular localization of NF-κB as well as its relationship with plaque stability and with the expression of the other molecules investigated.

Table 1 Clinicopathological data

Age	53–82 years (mean 69.1years)
Sex (Male/Female)	31 (86.1%)/5 (13.9%)
Smoking	30 (83.3%)
Hypertension	30 (83.3%)
Diabetes mellitus	10 (27.7%)
Hyperlipidaemia	27 (75%)
Ischemic heart disease	19 (52.7%)
Peripheral arterial occlusive disease	11 (30.5%)
Symptoms	22 (61.1%)
Carotid artery stenosis (70–79%/80–89%/>90%)	10 (27.7%)/8 (22.2%)/18 (50.1%)
Plaque echogenicity (hypoechoic/hyperechoic)	15 (41.7%)/21 (58.3%)
Plaque histology (grade V/VI)	17 (47.2%)/19 (52.8%)

Materials and methods

Tissue samples

Formalin-fixed and paraffin-embedded carotid plaque tissues from 36 patients were analysed. Patients' clinicopathological data are presented in Table 1. Patients were pre-operatively evaluated by a neurologist and assigned as symptomatic or asymptomatic, as previously described [17]. Symptomatic patients were classified based on the presence of stroke, transient ischemic attacks and amaurosis fugax. A cerebral CT scan was performed for identification of brain infarcts. All patients underwent endarterectomy during 3 and 6 weeks after exhibiting symptoms. The degree of stenosis was determined according to the North American Symptomatic Carotid Endarterectomy Trial criteria [17].

Histology

Haematoxylin and eosin staining was performed for histological evaluation of the specimens. Two pathologists, blinded to the clinical data, examined each specimen to assess atherosclerotic plaque morphology, using the American Heart Association classification of atherosclerotic plaques [18]. According to this classification, carotid plaques were assigned as fibroatherotic (type V) and complicated (type VI). The latter type included plaques with intraplaque haemorrhage, ulcer or thrombus, which were considered unstable.

Immunohistochemistry (IHC)

IHC was performed according to a previously published protocol [19] and the results were evaluated by two independent observers. The following antibodies were used: anti-FGF-2 (1:25) (#147; Santa-Cruz, Bioanalytica, Athens, Greece), anti-MMP-9 (1:50) (Neomarkers-LabVision, AntiSel, Athens, Greece), anti-p65 (1:250) (F-6; Santa-Cruz, Bioanalytica), anti-iNOS (1:25) (Neomarkers-LabVision, BioAnalytica), anti-FGFR-1 (1:100) (ab10646, Abcam, AntiSel) and anti-FGFR-2 (1:1000) (ab10648, Abcam, AntiSel). Macrophages, smooth muscle cells and endothelial cells were identified, respectively, by: anti-CD-68 (Dako, Kalifronas, Athens, Greece), anti-α-smooth muscle actin (1A4; Dako) and anti-CD34 (QBE; Dako). IHC scoring was performed as previously described [20, 21].

Immunofluorescent (IF) analysis

IF analysis was performed according to a previously published protocol [19] and the results were evaluated by two independent observers. The anti-p65 (1:250) (F-6; Santa-Cruz, Bioanalytica) antibody was used.

Immunoblot analysis

Protein extraction

Total protein extraction from cells was performed according to previously described protocols [19].

SDS-PAGE

Thirty micrograms of protein from total extracts from each sample were adjusted with NuPAGE LDS sample buffer (Invitrogen, AntiSel) and loaded on 4–12% gradient NuPAGE precast gels (Invitrogen, AntiSel), according to the manufacturer's instructions. Gel electrophoresis, transfer to PVDF membrane (New England Nuclears [NEN]; AlterChem, Athens, Greece), blotting, immunodetection and signal development were performed as previously described [19].

Antibodies

The antibodies employed were: anti-NOS-2 (1:100) (C-11; Santa Cruz, Bioanalytica), anti-FGF-2 (1:200) (#147; Santa-Cruz, Bioanalytica), anti-MMP-9 (1:200) (Neomarkers-LabVision, Bioanalytica), anti-p65-pS536 (1:1000) (Cell Signalling, Bioline, Athens, Greece) and anti-β-actin (1:1000) (AC-15; Abcam, AntiSel).

Cell lines

The conditionally immortalized human VSMC line HVTs-SM1 (kindly provided by Dr. D. Kletsas) was cultured as previously described [17]. For the stimulation experiments, cells at confluence were stimulated after 24 hrs of cultivation in a serum-free medium with 100 ng/ml of recombinant human bFGF (CN 233-FB/CF; RD Systems, AntiSel). Following treatment, cells were harvested at predetermined time-points (see 'Results' section) for the subsequent immunoblotting analysis and for the luciferase assay. The tumour necrosis factor treated HeLa cell line was used as a positive control.

NF-κB activation assay

For the luciferase reporter assay the HVTs-SM1 VSMCs were transfected with the NF-κB Luc plasmid [20] (a generous gift from Dr. E. Andreakos) and incubated in serum-free medium in the presence or absence of 100 ng/ml recombinant human bFGF (CN 233-FB/CF; RD Systems, AntiSel). Luciferase activity was measured using a luciferase assay system (Promega, SB Biotechnology, Athens, Greece), according to the manufacturer's instructions.

Statistical analysis

Spearman correlation and Mann-Whitney tests were employed for the statistical analysis. All calculations were performed with the SPSS 10.0 software.

Results

bFGF expression-dependent up-regulation of iNOS and MMP-9 is associated with atherosclerotic plaque instability

The expression of bFGF was assessed immunohistochemically in a panel of human carotid atherosclerotic plaques. bFGF was present in the VSMCs and macrophages surrounding the lipid core (cap), as well as in the cells located at the shoulders of the plaque. The staining was predominantly cytoplasmic and was noted both in stable and unstable atherosclerotic plaques (Fig. 1a). However, the fraction of bFGF-expressing VSMCs was significantly higher in unstable carotid plaques than stable ones ($P = 0.032$, Mann-Whitney test) (Fig. 1b), as well as in symptomatic patients compared to asymptomatic ones ($P = 0.034$, Mann-Whitney test) (Fig. 1b). These results were also quantitatively confirmed by immunoblotting analysis (data not shown). On the contrary no difference in bFGF expression in macrophages (recognized in atherosclerotic plaques by CD68 staining) between stable and unstable plaques was noted (Fig. S1).

bFGF is known to exert its action by binding to cell surface detected FGF receptors (FGFRs). The existence of an autocrine pathway in VSMCs, where the secreted bFGF binds to the membranous FGFRs, has been shown *in vitro* [21–23]. To examine whether an analogous loop exists *in vivo,* we assessed the expression of FGFR-1 and FGFR-2 – the main FGFRs expressed in VSMCs [24] – in our panel of specimens. Immunohistochemical analysis revealed membranous and cytoplasmic staining in VSMCs for both receptors (Fig. 1c). However, only FGFR-1 expression statistically correlated with that of bFGF ($P = 0.001$, Spearmann's $\rho = 0.652$) and was higher in unstable atherosclerotic plaques ($P = 0.016$, Mann-Whitney test) (Fig. 1c and d).

We next analysed the same tissue panel for MMP-9 and iNOS expression. Our IHC analysis revealed cytoplasmic staining for both molecules in VSMCs (Fig. 1e) that was statistically higher in unstable plaques ($P = 0.045$ for MMP-9 and $P = 0.031$ for iNOS, Mann-Whitney test) in accordance with our previously published data [25]. Analogous differences in expression were also detected by immunoblot analysis (Fig. 1f). Importantly, bFGF expression correlated in a statistically significant manner both with MMP-9 and iNOS expression in VSMCs ($P = 0.046$ and $P < 0.001$, respectively, Spearman correlation), providing primary evidence that bFGF could regulate molecules associated with plaque vulnerability (Fig. 1g).

The above-described *in vivo* association was subsequently recapitulated *in vitro* by treatment of HVTs-SM1 VSMCs with recombinant bFGF, mimicking the *in vivo* autocrine action of bFGF. bFGF addition in the culture media led to up-regulation of iNOS and MMP-9 (Fig. 1h), supporting the *in vivo* observed statistical correlation.

NF-κB mediates the bFGF effects related to plaque instability

To decipher the exact molecular pathway triggered by bFGF, HVTs-SM1 VSMCs were treated *in vitro* with this growth factor. Subsequently, the activation of two established bFGF-downstream pathways, namely the mitogen-activated protein kinase (MAPK) pathway and the NF-κB pathway, was monitored. bFGF administration led to a robust increase in NF-κB transcriptional activity as documented by a luciferase reporter assay (Fig. 2a). Immunoblot analysis also revealed that the phosphorylated form of the RelA/p65 subunit of NF-κB (p65-pSer536) – contributing to the enhancement of its transcriptional activity [26] – was present 30 min. after bFGF addition and was maintained up to 3 hrs after administration (Fig. 2b). In contrast, only subtle differences in the p42/p44 (phosphorylated extracellular signal-regulated kinase, pERK) expression were noted (Fig. 2b). To the best of our knowledge,

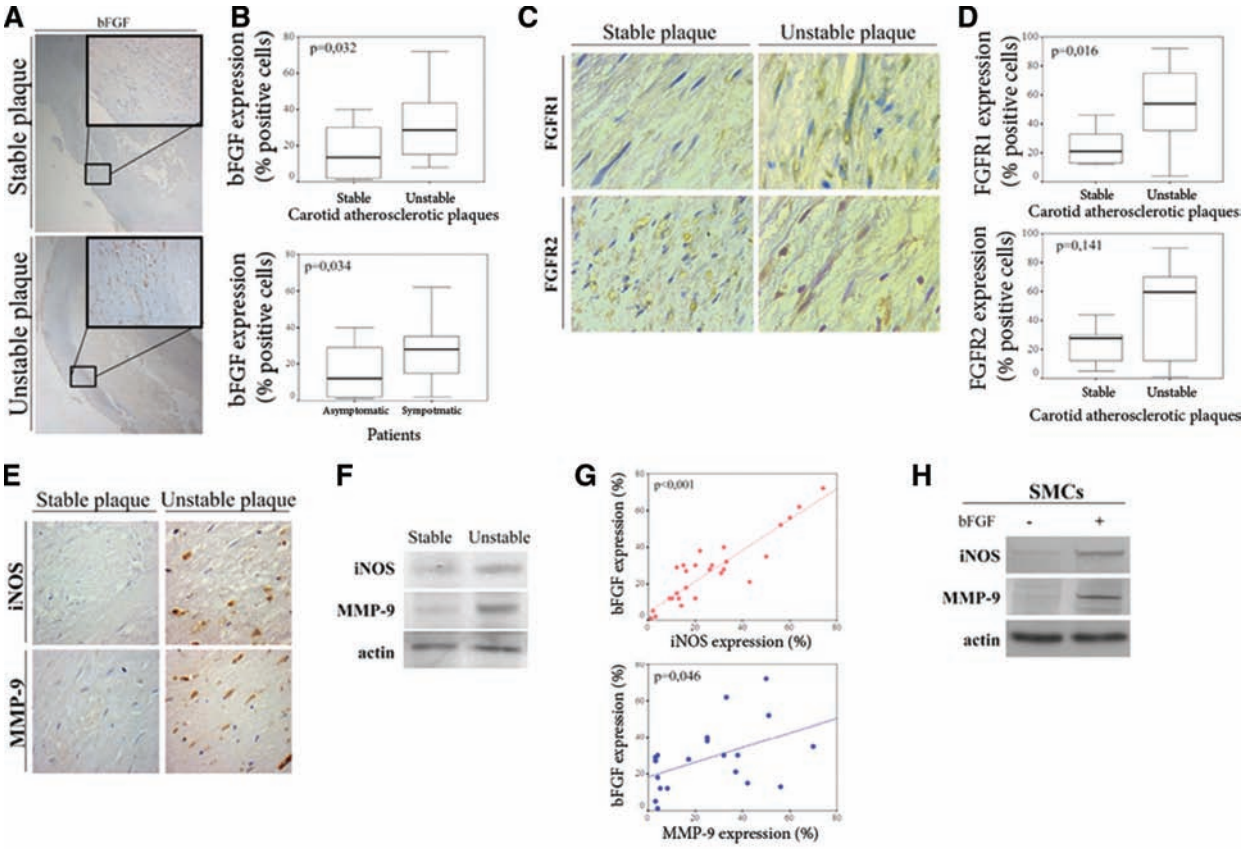

Fig. 1 bFGF expression is increased in unstable carotid atherosclerotic plaques and correlates with FGFR-1, MMP-9 and iNOS expression. (**A**) Representative pictures of bFGF immunohistochemical expression in stable and unstable plaques. Insets represent higher magnification of the selected areas. (**B**) Boxplots depict differences in bFGF expression according to plaque stability and patients' symptoms. (**C**) Representative pictures of FGFR-1/2 expression in stable and unstable carotid atherosclerotic plaques. (**D**) Boxplots showing differences in FGFR-1 and FGFR-2 expression between stable and unstable carotid plaques. (**E**) Representative pictures from two cases with low and high expression levels of iNOS and MMP-9, respectively. (**F**) Representative immunoblotting analysis for iNOS and MMP-9 in carotid specimens. (**G**) Scatterplots showing the correlation among bFGF and iNOS and MMP-9 expression. (**H**) Immunoblotting analysis of human VSMCs for iNOS and MMP-9 expression, before and after the addition of recombinant bFGF in the culture medium.

this is the first demonstration that bFGF can induce activation of NF-κB in human VSMCs. Furthermore, iNOS and MMP-9 up-regulation by bFGF was specifically mediated *via* NF-κB, because the expression of both these molecules returned to control levels after RelA/p65 gene silencing. In contrast, their expression levels remained unaffected by the addition of the MAPK pathway inhibitor PD98059 (Fig. 2c). Therefore, the MAPK pathway that mediates the mitogenic effects of bFGF seems to have no implication in the induction of MMP-9 and iNOS in VSMCs.

The above-described mechanism was also supported by our *in vivo* experiments. The carotid atherosclerotic plaques from our database were immunohistochemically analysed for NF-κB expression. VSMCs stained positive for NF-κB and the staining was either solely cytoplasmic or concomitantly cytoplasmic and nuclear (Fig. 2d). The nuclear localization of NF-κB, as detected by IHC analysis, is indicative for its activity [27]. This subcellular distribution was further confirmed by detection of RelA phosphorylated at Ser536 (p65-pSer536) employing IF analysis (Fig. 2e) and immunoblotting in extracts from carotid clinical specimens displaying sole cytoplasmic *versus* cytoplasmic/nuclear IHC staining (Fig. 2f). In the latter cases, with IHC nuclear localization of NF-κB, p65-pSer536 levels were markedly increased (Fig. 2f). Notably, higher fraction of cells with concomitant cytoplasmic and nuclear NF-κB nuclear distribution was observed in unstable plaques *versus* stable ones ($P = 0.025$, data not shown). Subsequently, the expression of bFGF, FGFR-1/-2, MMP-9 and iNOS was stratified according to NF-κB subcellular distribution. The statistical analysis revealed significant increase in their expression levels – with the exception of FGFR-2 – when NF-κB localization was nuclear, confirming our *in vitro* observations (Fig. 2g).

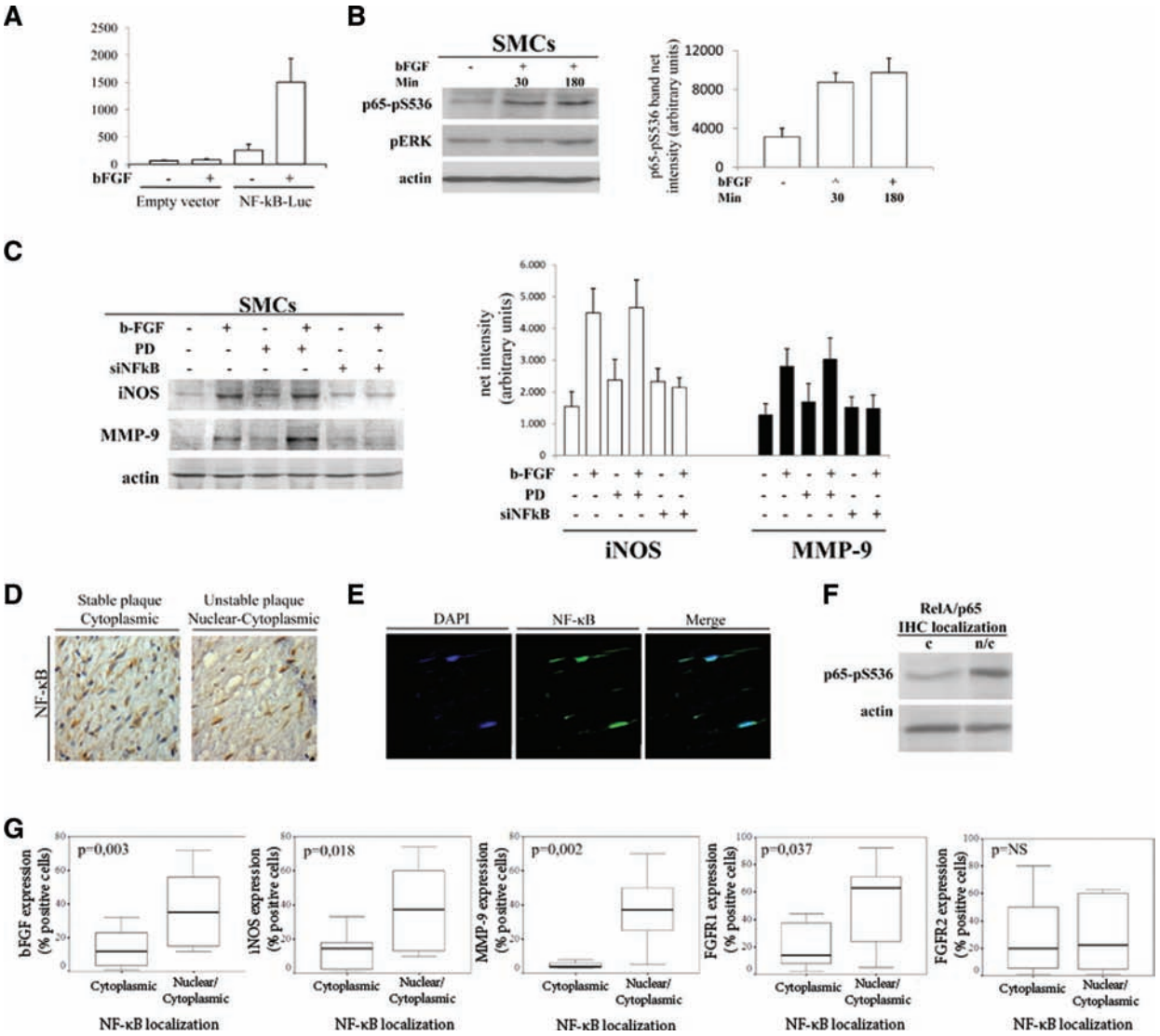

Fig. 2 NF-κB specifically mediates the up-regulation of MMP-9 and iNOS by bFGF treatment. (**A**) NF-κB luciferase reporter assay of VSMCs transfected either with empty vector or firefly luciferase reporter plasmid driven by five consecutive artificial NF-κB-binding sites [NF-κB–luc). (**B**) Immunoblotting analysis of p65-pSer536 and pERK in VSMCs at 30, 60 and 180 min. after addition of bFGF in the culture medium. Actin serves as loading control. The histogram depicts the quantitative estimation of p65-pSer536 expression levels after densitometric analysis. (**C**) iNOS and MMP-9 immunoblotting analysis in VSMCs treated with bFGF along with either MAPK inhibitor PD98059 or siRelA. The histogram depicts the quantitative estimation, after densitometric analysis, of iNOS and MMP-9 expression levels with several treatments. (**D**) Representative cases depicting sole cytoplasmic (stable plaque) and cytoplasmic/nuclear (unstable plaque) NF-κB IHC staining in carotid atherosclerotic plaques. (**E**) IF analysis of case 1 depicting cytoplasmic/nuclear NF-κB localization. (**F**) Immunoblotting analysis for total p65-pSer536 levels in two representative cases expressing only cytoplasmic (stable plaque) and both cytoplasmic and nuclear (unstable plaque) IHC staining of p65, respectively. (**G**) Boxplots depicting differences in bFGF, iNOS, MMP-9, FGFR-1 and FGFR-2 expression, respectively, according to NF-κB localization. NS, non-significant.

Discussion

Several growth factors have been implicated in the pathogenesis of atherosclerosis. bFGF is one of these and according to several animal models enhances VSMC proliferation and neointima formation as well as vasa vasorum proliferation through its mitogenic and angiogenic activity [8, 9, 28, 29]. Studies in patients have shown the presence of high serum levels of bFGF, suggesting a potential relation connecting bFGF with active atheromatosis [30] and plaque instability [16]. The latter is of major clinical

importance, because it may lead to detrimental effects such as acute coronary and cerebrovascular ischemia [28].

To clarify this issue we examined bFGF expression directly in human carotid atherosclerotic plaques and in relation to their stability that was evaluated by histological classification. Complicated atherosclerotic plaques (grade VI) exhibited significantly higher expression of bFGF in VSMCs, in comparison to those classified as stable ($P = 0.032$, Mann-Whitney test) (Fig. 1b). Furthermore, bFGF expression in VSMCs was also higher in patients who presented symptomatic carotid disease (stroke, transient ischemic attacks, amaurosis fugax) ($P = 0.034$, Mann-Whitney test) (Fig. 1b). In addition, bFGF seems to exert its action in an autocrine manner *via* FGFR-1, as depicted by the significantly higher levels of FGFR-1 in the VSMCs of the unstable atherosclerotic plaques. Studies in apolipoprotein E deficient mice have underscored the significance of this autocrine system in atherosclerosis progression [31]. On the contrary, the percentage of bFGF expressing macrophages remains unchanged during atherosclerotic plaque progression (Fig. S1), indicating an additional paracrine action of bFGF on VSMCs, unrelated to the stability of the plaque.

The above data suggest a role of bFGF produced by VSMCs in the progression of atherosclerotic plaques to instability. Although bFGF may directly facilitate plaque weakening and rupture by inhibiting collagen synthesis in VSMCs [13], it may also affect major mechanisms controlling plaque rupture such as MMP-mediated cleavage of the extracellular matrix [14] and/or nitric oxide induced apoptosis of SMCs [15].

Regarding the MMPs, several metalloproteinases have been detected in atherosclerotic plaques [32], but MMP-9 along with MMP-3 are the most abundantly expressed [32]. Furthermore, MMP-9 exerts its proteolytic activity against major components of the extracellular matrix of atherosclerotic plaques – including collagen IV, elastin and proteoglycans – and previous studies have correlated the increase in MMP-9 activity in carotid atherosclerotic plaques with cerebral ischemic events [25, 33]. Similarly, iNOS is the major nitric oxide synthase in VSMCs and produces large amounts of nitric oxide for long time periods upon cytokine stimulation [34]. Increased amounts of nitric oxide are known to cause DNA damage through hydroxyl radicals' formation [35] and to induce apoptosis in VSMCs [15].

In concert with the above, our analysis revealed that both MMP-9 and iNOS expression levels were higher in unstable atherosclerotic plaques ($P = 0.045$ for MMP-9 and $P = 0.031$ for iNOS, Mann-Whitney test) (Fig. 1e and f). Moreover, their expression significantly correlated with bFGF expression ($P = 0.046$ and $P < 0.001$, respectively, Spearman correlation) (Fig. 1g), while *in vitro* bFGF treatment led to up-regulation of MMP-9 and iNOS (Fig. 1h) enhancing the notion that bFGF bears a nodal role in atherosclerotic plaque instability.

bFGF exerts its activity by binding to the FGF receptors at the cellular membrane and triggering intracellular signalling cascades. In SMCs the mitogenic effects of bFGF are mediated through activation of the MAPK pathway [36]. However, it was recently shown in cancer cell lines and rat VSMCs that bFGF could also potentiate the transcription factor NF-κB [37, 38] that is a known modulator of both MMP-9 [39] and iNOS [40]. Indeed, our study revealed that MAPK pathway, although modestly triggered by bFGF (Fig. 2c), plays no role in MMP-9 and iNOS up-regulation that is instead specifically mediated by NF-κB activation (Fig. 2a and b), as validated also by the corresponding inhibition and silencing experiments performed (Fig. 2c). Additionally, we showed that bFGF treatment leads to phosphorylation of the RelA/p65 subunit of NF-κB at Ser536 (Fig. 2d–f), which enhances NF-κB transcriptional activity [26]. In agreement, the *in situ* analysis demonstrated nuclear localization of NF-κB and augmented nuclear levels of p65-pSer536 in unstable atherosclerotic plaques that were significantly correlated with increased bFGF, FGFR-1, iNOS and MMP-9 expression (Fig. 2g).

Collectively, the above data pose an important role for bFGF in the progression of atherosclerotic plaques to instability. bFGF expression is up-regulated in VSMCs of unstable carotid atherosclerotic plaques along with FGFR-1, enhancing this autocrine signalling system that leads to NF-κB potentiation and the subsequent increase in MMP-9 and iNOS expression. Hence, this novel pathway uncovers possible molecular targets that may facilitate the development of pharmacological inhibitors of the atherosclerotic process.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 bFGF expression in macrophages is independent of plaque stability. (a) Representative photos of the immunohistochemical analysis for CD68 and bFGF in serial sections from stable and unstable atherosclerotic carotid plaques. (b) Boxplots depict differences in bFGF expression in macrophages according to plaque stability. NS = non-significant.

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