

The cardiovascular effects of proteolysis of high molecular weight basic fibroblast growth factor by inflammatory serine proteases

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Abstract

Cardiovascular disease is characterized by structural and functional changes of blood vessel's wall that lead to reduced blood flow and eventually occlusion. The integrity of the vascular wall is maintained by homeostatic mechanisms controlled by the endothelium. Stress caused by age, oxidants, mechanical injury, and inflammation can result in endothelial dysfunction leading to remodeling of the vessel wall. We found that thrombin, a key protease of the coagulation cascade and inflammatory response, cleaves the high molecular weight (HMW) forms of basic fibroblast growth factor (FGF-2), a ubiquitous protein with trophic effects on vascular cells. The C-terminal fragment of FGF-2 generated by thrombin is similar to low molecular weight (LMW; 18 kDa) FGF-2, and induces vascular cell activation of the mitogen-activated protein kinases ERK-1/2, migration and proliferation. The N-terminal fragment generated by thrombin cleavage of HMW FGF-2 contains a sequence rich in asymmetric-dimethyl-arginine (ADMA) residues. In free form ADMA inhibits key reactions for blood vessel homeostasis such as nitric oxide synthesis, and its serum levels, elevated in diabetes, renal failure, hypertension, and hypercholesterolemia, correlate with a poor prognosis in cardiovascular patients. We found that thrombin cleavage of HMW FGF-2 dramatically upregulates intracellular ADMA levels in cultured cells. Thus, the C-terminal cleavage product of HMW FGF-2 can activate intracellular signaling and control vascular cell functions, while the N-terminal fragment of FGF-2 generates ADMA, a powerful inhibitor of nitric oxide synthesis. We hypothesize that upon vascular injury HMW FGF-2 is processed by inflammatory serine proteases such as thrombin generating molecules that accelerate the development of intimal hyperplasia. This novel mechanism implicates human HMW FGF-2 in the pathogenetic mechanisms of vascular injury occurring in hypertension, diabetes, and dyslipidemia, conditions that are all characterized by elevated serum levels of ADMA. The elucidation of these mechanisms will foster the development of new pharmacological tools for the treatment of the cardiovascular disorders associated with these diseases.

Fgf-2 gene expression

Basic fibroblast growth factor (FGF-2) is the prototypic member of a large family of proteins with pleiotropic effects initially identified as an 18 kDa protein [1]. Analysis of the human cDNA sequence upstream of the 5' AUG revealed the existence of at least three (two in rodents) additional CUG initiation sites located on the same mRNA. These alternative initiation codons originate 22, 22.5, and 24 kDa (20.5 and 21 kDa in rodents) forms of FGF-2 referred to as high molecular weight (HMW) FGF-2. An additional 34 kDa form, generally poorly translated, is

synthesized under cellular stress conditions [2; 3; 4]. HMW FGF-2 forms are, therefore, colinear extensions of 18 kDa, or low molecular weight (LMW) FGF-2. The various FGF-2 forms are differentially distributed in the cell, which might explain in part their differential biological activity [5]. LMW FGF-2 is mostly cytosolic whereas HMW FGF-2 forms predominately localize to the nucleus; independent of their intracellular localization, all FGF-2 forms are released and associate with the extracellular matrix despite the lack of a classical signal peptide that directs secretion through the ER-Golgi pathway [6; 7; 8; 9]. LMW FGF-2 promotes

murine HMW FGF-2 [38] presents substantial differences from the human counterpart (Fig. 1) [5]. To test whether thrombin cleaves HMW FGF-2 directly we also added thrombin to affinity-purified HMW FGF-2. The results (Fig. 2) showed that thrombin cleaves HMW FGF-2 also in a cell-free system. Using an antibody specific for HMW FGF-2 (a gift from Dr. Daniel B Rifkin, NYU), we also identified the ~ 6 kDa N-terminal peptide generated by thrombin cleavage of HMW FGF-2 (Fig. 2).

Therefore, our findings showed that cleavage of HMW FGF-2 (e.g., by thrombin) generates an 18 kDa-like form of FGF-2 (ELF-2) and an N-terminal peptide. However, unlike the observation in the test tube, such N-terminal peptide could not be detected when thrombin was directly added to cultured cells suggesting that it likely undergoes rapid intra- or extra-cellular degradation by other proteases. Degradation of proteins methylated on arginine residues is known as the major mechanism that generates high intracellular and extracellular levels of mono-methylated (MMA) and dimethylated arginine (DMA) [39]. DMA can occur as a modified amino acid with either symmetric (SDMA) or asymmetric

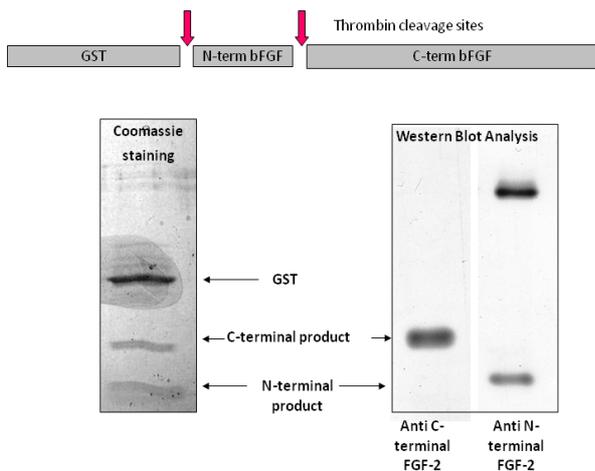


Fig. 2. A GST-human HMW FGF-2 fusion protein purified from *E. coli* was incubated with human thrombin for 15 min at 37 °C. Thrombin frees HMW FGF-2 from GST plus cleaves it at its putative site(s) (red arrows) generating three major fragments: GST, a C-terminal product (ELF-2), and an N-terminal product (see Coomassie staining, left). ELF-2 was recognized by Western blotting using a monoclonal antibody to 18 kDa FGF-2, whereas a polyclonal antibody to HMW FGF-2 identified a ~ 6 kDa fragment corresponding to the cleaved N-terminal fragment.

(ADMA) configuration of the methyl groups [39]. In eukaryotic cells HMW FGF-2 forms contain MMA, SDMA, and ADMA [29]. ADMA is to date the major

endogenous inhibitor of nitric oxide (NO) synthase (NOS) [40]. Elevated ADMA levels have been detected in peripheral blood of patients with atherosclerosis, diabetes mellitus, hypertension or heart failure [39].

High ADMA levels are also associated with endothelial dysfunction, a condition that predisposes to cardiovascular disease. ADMA has been implicated not only in the reduction of NO levels in the dysfunctional vessel wall, but also in the induction of angiotensin-converting enzyme, a finding that further supports the involvement of ADMA in hypertension [39; 41]. Locally elevated ADMA levels appear to alter its ratio with L-arginine, the substrate used by NOS to generate NO. L-arginine supplementation ameliorates the general conditions of patients with elevated blood levels of ADMA, an effect known as “the arginine paradox” [42]. While SDMA has no effect on NOS activity, MMA can competitively inhibit cellular uptake of L-arginine, further preventing NO generation. Therefore, a combined elevation of MMA and ADMA levels will synergistically reduce overall NO production. Enzymes that directly demethylate methyl-arginine residues have not been described. However, ADMA can be catabolized to methylamines by dimethylamine dimethylarginine hydrolases (DDAHs). Two forms of DDAH have been described (DDAH-1 and -2) that afford intracellular clearance of ADMA [43]. Elevated ADMA blood levels of clinical significance could therefore result from increased degradation of methylated proteins and/or reduced DDAH activity [44]. Reduced renal excretion such as in end stage renal disease (ESRD), a severe complication of diabetes, also contributes to elevating ADMA levels [45].

Physiological significance of HMW FGF-2 cleavage

We postulated that proteolysis of HMW FGF-2, which may well occur upon tissue injury when growth factors are released from damaged vascular cells and thrombin is generated via the coagulation cascade, could be a contributing mechanism that controls ADMA generation. If such a mechanism occurs *in vivo*, what could its implications be in both physiological and pathological conditions?

We investigated the biological significance of this mechanism of proteolysis using a cultured cell model. The FGF-2^{-/-} endothelial cells we had generated in our laboratory appeared to be the most suitable model; these cells, for instance, migrate very poorly when injured and this effect is caused by lack of

wound-induced ERK-1/2 activation, which is controlled by endogenous FGF-2 [46]. Using these cells as such (FGF-2^{-/-} cells) or stably transfected with human HMW FGF-2 we demonstrated that thrombin induces their migration and proliferation only when HMW FGF-2 is expressed and cleaved [37]. These results showed that, in addition to alternative translation initiation, expression of the *fgf-2* gene can be controlled through a novel mechanism: Cleavage of (precursor) HMW forms into a (mature) LMW form. Furthermore, 18 kDa FGF-2 is not degraded by thrombin [37; 47] and the LMW FGF-2 generated upon thrombin cleavage of HMW FGF-2 has an apparent MW slightly higher than human recombinant 18 kDa FGF-2, a difference in size consistent with the addition of a few amino acids to 18 kDa FGF-2. We identified the actual cleavage sites by means of site-directed R to A mutagenesis: one cleavage site is at the R-P bond at -7 R upstream of the AUG initiation codon (methionine, M), one at the R-G bond at -10 R, and a third one at the R-A at -16 R, which also presents a P residue in P₂ position (Fig. 1) [37]. Interestingly, the R-A bond followed by a P residue in P₂ position is missing in the sequence of murine HMW FGF-2 [38]. Mutation of single cleavage sites did not block HMW FGF-2 cleavage by thrombin; however combined mutations of two or all three sites blocked HMW FGF-2 cleavage by thrombin completely [37]. Supporting our hypothesis that thrombin needs to cleave HMW FGF-2 in order to induce endothelial cell migration and proliferation, we found that when cells expressing a mutated, cleavage-resistant form of HMW FGF-2 were treated with thrombin neither cell migration nor proliferation was induced [37].

Our quest for the fate of the N-terminal peptide gave encouraging results, though not conclusive. Data generated independently and in a blinded fashion by ELISA (Cardiovasics, Inc, Palo Alto, CA) indicated that while ADMA was not detected in cell extracts from cultured NIH 3T3, a concentration of about 0.2 $\mu\text{moles/L}$ was measured in extracts from NIH 3T3 overexpressing human HMW FGF-2; this concentration doubled when cells were treated with thrombin for 15 min. The difference in ADMA levels observed in our cells ($\sim 0.2 \mu\text{moles/L}$) is consistent with changes observed in patients and in experimental systems with diminished catabolism of ADMA [42; 44]. Although assessing the content of HMW FGF-2 in the vascular wall may prove to be complicated by lack of ELISA specific for the HMW forms these preliminary results suggest that the differences observed with cultured cells may predict a physiological relevance in the vascular wall. These results, however, have not been confirmed yet in our

endothelial cell model. We sought to investigate the potential effect of human HMW FGF-2 when expressed in FGF-2^{-/-} mouse endothelial cells on NO production. To this purpose, we measured NOS activity indirectly in cultured mouse endothelial cells obtained from FGF-2-null mice and engineered to express the HMW forms of human FGF-2, or their respective FGF-2-null cells used as a control, via determination of the nitrates/nitrites present in the conditioned media (CM). In order to elicit NO production ECs were treated overnight with a combination of bacterial endotoxin or lipo-polysaccharide (LPS, 100 ng/ml) and interferon gamma (IFN- γ , 50 U/ml) which are known to increase the levels of inducible nitric oxide synthase (iNOS) [48]. Under these conditions cells produce higher levels of NO than control, untreated cells. Figure 3 represents measurements obtained from control cells (zeo, clones N2 and N4) and HMW FGF-2-expressing cells (365, clone N1). As illustrated in the figure, the presence of HMW FGF-2, and presumably its turnover, was sufficient to decrease the levels of nitrates/nitrites in the cells' conditioned media using the Griess reagent (Total Nitric Oxide, Assay Designs, Stressgen, Ann Arbor, MI).

These results, although very preliminary (hence no statistical analysis), suggested that expression and/or

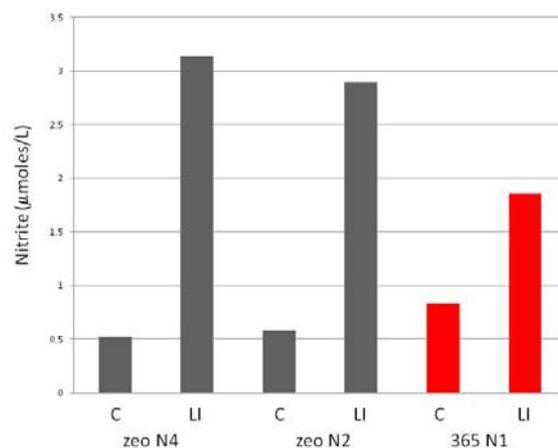


Fig.3. Conditioned media obtained from HMW FGF-2-expressing cells (365, clone N1) or from control cells (zeo, clones N4 and N2) were filtered through Centricon tubes (10,000 MW cutoff, Millipore) and assayed for nitrite content as per manufacturer's instructions. While in both control cell clones overnight treatment with LPS and IFN- γ (LI) resulted in a robust (~ 6 -fold) induction of nitrite over control cells (C), the same treatment (LI) gave a very modest (~ 2 -fold) response in HMW FGF-2-expressing cells, suggesting an inhibitory effect on NOS activity.

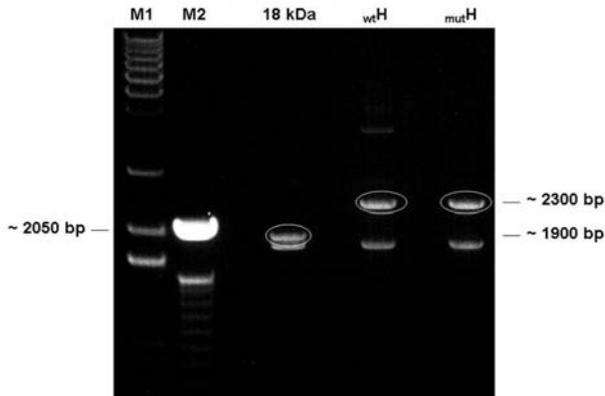


Fig. 4. Preparation of constructs containing different FGF-2-encoding cDNAs. cDNAs encoding for 18 kDa, wild-type HMW (wtH), or cleavage-resistant HMW (mutH) FGF-2 were excised from pcDNA 3.1 plasmid using digestion with Bgl II/ Bsp HI followed by Pvu II digestion. Inserts of ~ 1900 bp, 2300 bp, and 2300 bp were excised, respectively, and visualized onto an agarose gel upon electrophoresis (circled bands). M1, M2: molecular weight ladders.

degradation of human HMW FGF-2 may result in decreased NOS activity, possibly by increasing the levels of free ADMA.

Encouraged by these findings and by our preliminary data on ADMA generation and NO production, we decided to generate transgenic mice expressing human 18 kDa FGF-2, or HMW FGF-2 or, alternatively, its mutant, cleavage-resistant form. Transgenic mice were generated at Cold Spring Harbor Laboratories (Cold Spring Harbor, NY, USA) using three constructs under the control of a cytomegalovirus (CMV) strong, global promoter in an FVB/N background. These constructs were obtained by excision from pcDNA 3.1 plasmid using digestion with Bgl II/ Bsp HI followed by Pvu II. Inserts of ~ 1900 bp, 2300 bp, and 2300 bp were excised, respectively, and visualized on agarose gel electrophoresis (Fig. 4): wt HMW FGF-2 (wtH), mut HMW FGF-2 (mutH), or 18 kDa FGF-2.

For genotyping genomic DNA was extracted from tail tips using QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Mice positive for the transgene were identified by Titanium Taq PCR (Clontech, Mountain View, CA, USA) using the following primers:
 FGF2_488_{For} 5'-
 AGCGGCTGTACTGCAAAAACGG-3' and
 pcDNA3.1_1158_{Rev} 5'-
 ACACCTACTCAGACAATGCGATGC-3'.

Six to eight founder mice, of FVB/N background, from each of the three lines were mated with wild-type mice to form F1 lines.

Proteolysis of HMW FGF-2 and cardiovascular implications

Our laboratory has had a long-term interest in vascular remodeling and development of intimal hyperplasia secondary to vascular injury. We are therefore resorting to an established murine model of arterial injury in which ligation of the carotid artery at the carotid bifurcation leads to the development of intimal lesion, or hyperplasia [49]. Female mice, 8 weeks old, of each transgenic strain expressing comparable amounts of human FGF-2 (wt HMW, or mut HMW, or 18 kDa) as well as non-transgenic FVB/N mice will be used for these studies. After anesthesia and carotid injury the specimens, i.e. the ligated left common carotid and the control, uninjured right common carotid artery, will be prepared for staining with hematoxylin and eosin (HE), as well as with van Gieson's. This will allow us to calculate the intima to media ratio (I/M) and determine whether the expression and/or catabolism of human HMW FGF-2 has an impact on the development of arterial lesion secondary to injury.

A number of observations support our hypothesis that human HMW FGF-2 post-translational processing plays a relevant role in vascular remodeling secondary to injury. First, growth factors as well as inflammatory mediators have been long implicated in the development of the vascular lesions that typically follow injury [31]. Of these, FGF-2 plays a prominent role in stimulating VSMCs, usually quiescent, to proliferate and migrate into the disrupted intima thus generating a neointima with thrombogenic characteristics [50; 51]. This effect has been almost exclusively ascribed to the canonical 18 kDa form, or LMW FGF-2, that interacts with its own receptor(s) and is also quite conserved throughout the animal kingdom. The generation of an 18 kDa-like form of FGF-2 (ELF-2) by proteolysis of HMW FGF-2 would lead to a molecule that is both biochemically and biologically similar to LMW FGF-2 thus triggering phosphorylation of the FGF-receptor(s) (FGF-R) and the related intracellular signaling pathways (Fig. 5). The remaining N-terminal fragment of this proteolytic reaction could then undergo complete degradation by proteolytic enzymes other than thrombin, ultimately generating a locally elevated concentration of mono- and dimethylated arginine residues. These would then inhibit NOS activity thus reducing NO bioavailability. NO is a known anti-thrombotic

molecule, a vasodilator, and an efficient inhibitor of VSMCs [39]. In the absence or reduced availability of NO, VSMC proliferation and migration induced by vascular injury would then be unrestricted thus accelerating the formation of intimal lesions. The observation that altering NO production and availability results in exacerbated vascular remodeling secondary to injury support the possibility that degradation products derived from HMW FGF-2 have an impact on the development of intimal hyperplasia. In summary, HMW FGF-2 post-translation processing by thrombin could affect vascular remodeling secondary to injury in a two-fold manner by generating locally elevated levels of 1) 18 kDa-like forms of FGF-2 and 2) endogenous NOS inhibitors (Fig. 5).

Our preliminary results obtained using a limited number of transgenic and control animals indicate that mice expressing human HMW FGF-2 display an accelerated development of intimal hyperplasia based on their increased (about two-fold) I/M ratios over control mice as measured at 1 and 2 weeks after surgery (Pintucci, unpublished data). Even more intriguing appears the preliminary observation that in mice expressing the mutated, cleavage-resistant form of HMW FGF-2 no intimal hyperplasia could be

detected, similar to what we observed in control, uninjured arteries (although the blood vessel's anatomy appears disrupted by injury with likely loss of the original intima) (Pintucci, unpublished data).

If confirmed, these data would indicate that *per se* (i.e., uncleaved) HMW FGF-2 may function as an inhibitor of vascular cell migration and proliferation as suggested by our studies in cultured cells [37], whereas its cleavage by thrombin and/or other serine proteases could unearth molecular components that favor the progression of intimal hyperplasia.

Conclusions

We have recently shown that HMW FGF-2 is cleaved by thrombin to generate an 18 kDa-like form of FGF-2 (ELF-2) which mediates the proliferative and migratory effects of thrombin on mouse endothelial cells [37]. This mechanism is remarkable not just because it appears to be independent of the thrombin receptor PAR-1, but also because it highlights the possibility that a precursor molecule(s), HMW FGF-2, with inhibitory functions on cell migration and proliferation, is converted to a C-

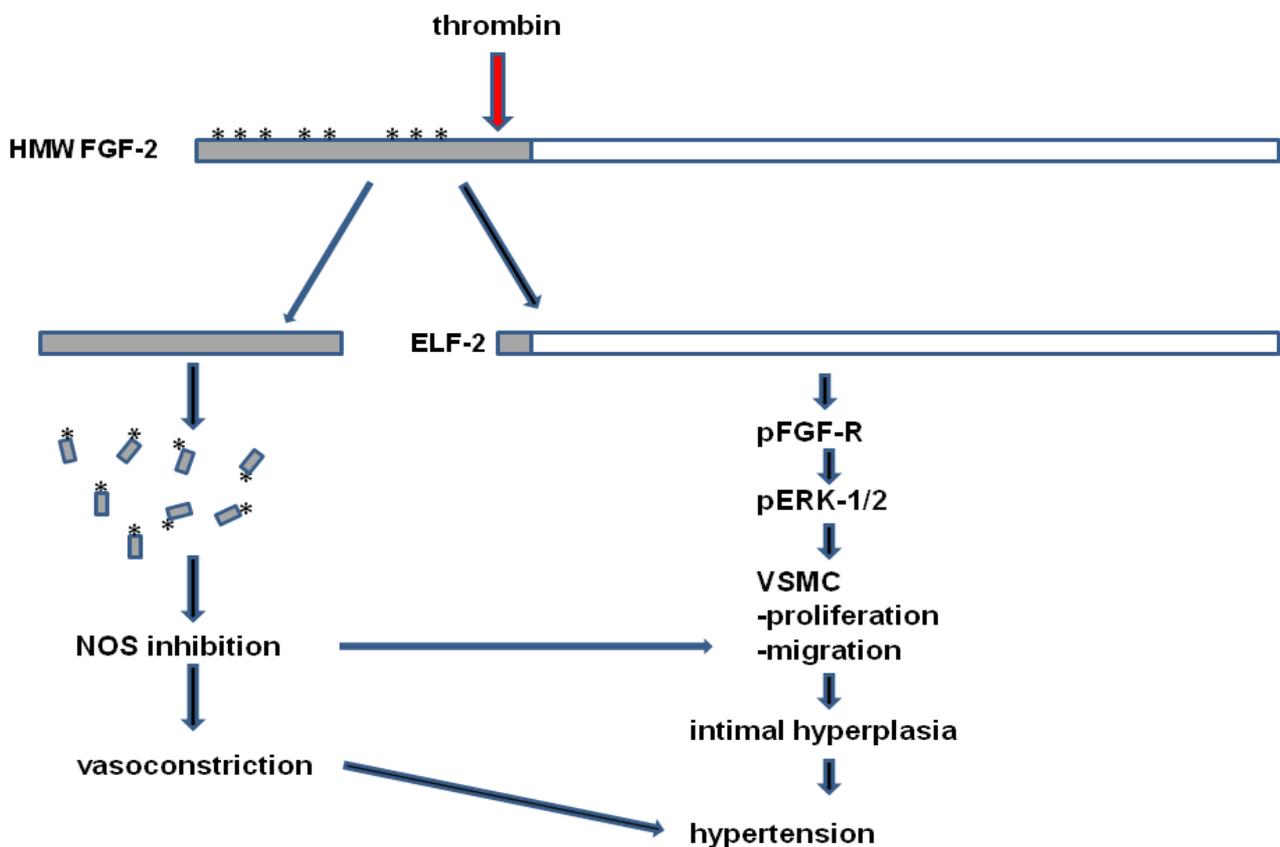


Fig. 5. Schematic of HMW FGF-2 proteolysis and expected impact on vascular remodeling secondary to injury. Upon vascular damage, released HMW FGF-2 is cleaved by thrombin into an 18 kDa-like form (ELF-2) that triggers FGF-receptor phosphorylation (pFGF-R) and MAPK activation (pERK-1/2) with consequent VSMC

proliferation and migration. This will thicken the vessel wall inward (intimal hyperplasia) thus reducing its lumen with consequent local blood flow restriction. Complete proteolysis of the N-terminal fragment frees methyl-arginine residues (grey blocks with asterisk) that function as endogenous NOS inhibitors. Reduced NO bioavailability leads to vasoconstriction and hypertension directly and also contributes to intimal hypertension by lack of restriction on medial VSMCs.

terminal protein fragment with proliferative and pro-migratory activities on vascular cells similar to 18 kDa FGF-2. The fate of the N-terminus resulting from this cleavage has not been fully elucidated; however, the presence in it of a number (up to eight) arginine residues that undergo methylation has prompted us to hypothesize that its complete degradation leads to free methyl-arginines that in turn could inhibit the formation and bioavailability of NO. Given the impact of both FGF-2 and NO on the homeostasis of blood vessels we have begun to test the hypothesis that human HMW FGF-2 has an impact on vascular remodeling secondary to injury. To do so we have generated mice that overexpress human HMW FGF-2 in its wild-type form or mutated in that it is resistant to cleavage by serine proteases. Endothelial damage and/or dysfunction are early events of vascular remodeling secondary to injury. Remodeling of the vessel wall upon arterial angioplasty or vein graft arterialization represents a significant clinical issue as loss of the endothelial layer integrity, activation of medial VSMCs, and deposition of abundant extracellular matrix lead to intimal hyperplasia and restenosis, ultimately causing conduit failure [31]. Growth factors and inflammatory proteases and mediators participate in injury-induced vascular remodeling [36]. On the other hand, the impact of NO bioavailability, or its absence, has been well studied in the remodeling of injured vessels [44; 52]. These considerations support the idea that HMW FGF-2 cleavage by thrombin may result in accelerated progression of intimal hyperplasia and vascular lesions.

Our preliminary data indicate that when overexpressed in FVB/N mice human HMW FGF-2 affects vascular remodeling exacerbating the VSMC response. Whether this is the direct consequence of its expression or of its cleavage remains to be demonstrated; and if HMW cleavage is the culprit of an accelerated progression of intimal hyperplasia it will be interesting to see whether this is an effect caused by ELF-2 or increased levels of ADMA, or both. The availability of transgenic mice expressing human HMW FGF-2 should provide the necessary tools to investigate these mechanisms. Of great importance are the preliminary data obtained with a yet small number of transgenic mice that express a mutated form of human HMW FGF-2 that cannot be cleaved by inflammatory serine proteases as they

indicate that 1 week after vascular injury no intimal hyperplasia could be measured in these mice. These and other results will need to be confirmed by using a larger number of animals per each group, and also analyzing the vascular remodeling response in mice overexpressing human 18 kDa FGF-2 (although its mouse ortholog, unlike HMW FGF-2, presents a great homology to the human counterpart, and very similar biological activities).

Thus, it may well be possible that FGF-2 in its different forms derived from a unique mRNA affects the progression of vascular remodeling by an array of mechanisms involving its cell membrane receptor(s) and also via novel pathways that interfere with another molecule, NO, highly implicated in blood vessels' homeostasis. The occurrence of this mechanism of cleavage of the HMW forms of FGF-2 operated by serine proteases relevant to the inflammatory response [36] could have consequences in other physiological and/or pathological settings in the organism. The fact that FGF-2 is a ubiquitous and pleiotropic growth factor certainly authorizes to hypothesize that the interaction of its HMW forms with proteases such as thrombin, plasmin, plasminogen activators, elastase, and cathepsin G is far from imaginary and in fact might have important biological repercussions. The consideration that cancer is sustained by growth factors such as FGF-2 and by a number of inflammatory mediators, and that vascular remodeling, including angiogenesis, is one of the hallmarks of its progression suggests that the HMW FGF-2 cleavage could also play a role in this important pathology. Furthermore, the discovery that FGF-2 is implicated in the development of pulmonary artery hypertension [53] could prompt future investigations aimed at clarifying whether or not HMW and its cleavage play any role in it. The demonstration of elevated amounts of ADMA levels in patients with pulmonary hypertension [54] appears as a supporting data in this direction.

We have presented here preliminary results in support of the hypothesis that proteolysis of human HMW FGF-2, by thrombin and/or other proteolytic enzymes with similar substrate affinity, contributes to the development and progression of intimal hyperplasia secondary to vascular injury. Ultimately, a complete study will be needed to determine whether a link exists between HMW FGF-2-derived ELF-2, ADMA, and NO reduction in controlling the

progression of intimal lesion. The elucidation of this mechanism(s) will also afford the design of molecular decoys that mimic the HMW FGF-2 sequence containing the cleavage sites but devoid of methylated arginines. Such decoys could then be used as a competing inhibitor to block the cleavage of HMW FGF-2 without affecting thrombin functions in maintaining haemostasis.

Our preliminary results obtained using an established mouse model of arterial injury indicate that the presence of human HMW FGF-2 in FVB/N mice results in doubling the I/M ratio over their control in just one week. If confirmed, such a result will strongly demonstrate that human HMW FGF-2 and possibly its cleavage (since the cleavage-resistant form appear to be inhibitory) by proteases activated during and/or after injury can modulate the development of intimal hyperplasia towards a more pathological phenotype. We hypothesize that this could be the result of ELF-2 generation possibly combined with release of harmful methyl-arginine residues that inhibit NO synthesis. That this is the case remains to be demonstrated since we have not analyzed yet in detail whether or not ELF-2 is present at any given point after injury and whether or not ADMA levels are

indeed increased in injured arteries of HMW FGF-2 transgenic animals over their control counterparts (with consequently decreased NO bioavailability). All of these parameters have to be assessed very carefully, and this task might prove a very challenging one: ELF-2 detection might prove elusive given the relative small quantities that are generated and the unknown kinetics of their generation, and a similar issue could be true for ADMA levels, particularly if their biological effect is a reflection of a temporary and locally limited increase. Nonetheless, our preliminary data appear very encouraging in attributing a relevant role to the human form of HMW FGF-2 and its post-translational processing in a number of physiological and pathological conditions.

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