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Preservation of Rabbit Aorta Elastin From Degradation by Gingival Fibroblasts in an Ex Vivo Model

Bruno Gogly, Adrien Naveau, Benjamin Fournier, Nicoleta Reinald, Eric Durand, Camille Brasselet, Bernard Coulomb, Antoine Lafont

Objective—Embryo-like gingival healing properties are attributed to the gingival fibroblast (GF) and could be used as a model for other types of healing dysfunctions. Abdominal aortic aneurysm (AAA) formation is associated with elastin degradation and increase in matrix metalloproteinase (MMP)-9 activity. We aimed to validate the concept of using GF healing properties in arteries.

Methods and Results—We evaluated MMP-9 and its tissue inhibitor (TIMP-1) in rabbit aortic rings cultured in collagen gels with or without GFs and observed throughout 21 days. We also performed cocultures of human smooth muscle cells (hSMCs) with either gingival, dermal, or adventitial fibroblasts, and alone (control). In control arteries, elastic fibers became spontaneously sparse. In presence of GFs, elastic fibers were preserved. There was a dramatically reduced protein level of MMP-9 in coculture of aorta and GFs, in contrast with control aorta. MMP-9 expression was unaffected by GFs. MMP-9 inhibition was related to increased TIMP-1 secretion, TIMP-1 forming a complex with MMP-9. Cell cocultures of hSMC with GFs showed similar results. Dermal and adventitial fibroblasts did not affect MMP-9.

Conclusions—Elastic fiber degradation was specifically preserved by GFs via reduction of MMP-9 protein level by increasing TIMP-1 synthesis. Vascular transfer of gingival fibroblasts could be a promising approach to treat AAA. (*Arterioscler Thromb Vasc Biol.* 2007;27:000-000.)

Key Words: gingival fibroblast ■ elastin ■ MMP-9 ■ TIMP-1 ■ aneurysm

Aortic aneurysms represent a common and severe disease, and their management does not specifically target the mechanisms of the disease. Expansive remodeling may result in aortic aneurysms defined by a loss of cellular and extracellular structures, ie, smooth muscle cells (SMCs), elastin, and collagen. Moreover, the media and the adventitia are infiltrated by macrophages and T lymphocytes producing matrix metalloproteinases (MMP) and inducing cell apoptosis.¹ The consequences of these alterations may result in extreme cases in aortic rupture. Extracellular matrix destruction is clearly expressed by an increase of MMP-2 and 9 in the aneurysmal wall secreted by inflammatory cells and decreased levels of TIMPs.^{2,3} In a model of experimental aneurysm, neither MMP-2 nor MMP-9 knockout mice developed aneurysmal lesions.⁴ Plasma levels of MMP-9 may predict the severity of AAA.^{5,6} Hence, MMP-9 may reasonably be considered as a target for a therapy.⁷ The gold standard treatment of AAA is surgery: it excludes the AAA by bypassing the aneurysm. This therapy has shown to be effective, although it does not treat the underlying disease. Endovascular prostheses aim to obviate surgery, but may malfunction, and fail to prevent aneurysm rupture.⁸ Allaire et

al have proposed to treat the aortic aneurysmal disease by extracellular matrix production via SMC transfer.⁹ Indeed, smooth muscle cells contribute to arterial extracellular matrix homeostasis, and decrease the experimental AAA. However, in humans, SMCs fail to limit the extracellular matrix loss in AAA. Moreover, SMCs undergo apoptosis in aneurysmal human lesions.¹⁰ Our approach was to identify the ideal cell type issued from a tissue subjected to permanent injury and able to adapt adequately. The gingiva daily responds to mechanical, thermal, bacteriological, and chemical injuries. Gingival fibroblasts (GFs) have the fascinating ability to restore ad integrum the tissue without scar. The healing process of gingiva is not comparable to cutaneous and muscular healings and resembles embryological repair.¹¹

GFs interact with the environment: through the membrane-anchored integrins, GFs perceive the state of the surrounding macromolecules, and according to the informations received from the matrix (collagens, proteoglycans, glycoproteins and elastin) and from the other cells (hormones, cytokines, growth factors) or from bacteriae (lipopolysaccharides and lipoteichoic acid) they respond by proliferating, migrating, synthesizing matrix components, or matrix-related en-

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zymes.^{12–14} Moreover, GFs can in turn influence the surrounding cells, namely polymorphonuclear cells, osteocytes/blasts, mastocytes, macrophages, endothelial cells, and epithelial cells in the gum. The multitude of responses developed by GFs probably refers to their capacity of differentiation into multiple cell types.¹¹ Indeed, GFs are now considered as progenitor cells^{15,16} thanks to their high healing potentialities, and therefore their plasticity could be evaluated in other organs. Our study aimed to evaluate the ability of GFs to adequately respond to elastolysis.

Materials and Methods

For detailed methods, please see <http://atvb.ahajournals.org>.

Human Cell Culture

Five human gingival and 3 dermal fibroblasts (hGF and hDF) cultures were obtained from gingival and dermal explants as previously described¹⁷ and incorporated in collagen lattices.¹⁸

Ex Vivo Aorta Culture

Ex vivo rabbit artery culture models have been already described^{19–21} as explants surrounded with liquid medium. Here we incorporated calibrated sections of rabbit aorta within a 3D collagen lattice that also permitted coculture of aorta with fibroblasts in an environment mimicking tissue organization. To recognize GFs they could be labeled using a colloidal suspension of iron oxide nanoparticles.²²

MMP-9 and TIMP-1 Secretion Analysis

MMP-2 and MMP-9 were analyzed by using gelatin zymographies.^{23–26} Dot Blot²² and ELISA were also used for TIMP-1 MMP-9 analysis.

MMP-9/TIMP-1 Complexes Determination

Total human MMP-9/TIMP-1 complexes were quantified, using ELISA and Western blotting.^{25,26}

Histology and Immunohistochemistry

Elastin fiber integrity was evaluated after orcein staining and paraffin sections were analyzed as previously described^{24,27} to evaluate the relationship between MMP-9/TIMP-1 complex formation and elastin fragmentation (statistical analysis by two-way ANOVA).

Results

Gingival Fibroblasts Prevent Elastin Degradation in an Ex Vivo Aorta Culture Model

In our ex vivo aorta culture model, elastin network became progressively disorganized and trended to disappear (Figure 1). More than a decrease in elastin fiber density, this was mainly attributable to a severe fragmentation of elastin fibers. Presence of rGF prevented elastin network degradation. Taking into account the 5 experiments, the elastin fiber density was maintained in coculture ($41.5 \pm 18.4\%$ at day 3; $41.1 \pm 4.6\%$ at day 21) as compared with the decrease observed in aorta culture only ($38.4 \pm 14.6\%$ at day 3; $30.4 \pm 6.2\%$ at day 21). Furthermore, rGF significantly prevented elastic fiber fragmentation as soon as the 7 day time point of analysis (Figure 4c).

Gingival Fibroblasts Reduce MMP-9 Activity

At each time point of analysis (3, 7, 14, and 21 days), degradation of the elastin network in the ex vivo aorta culture

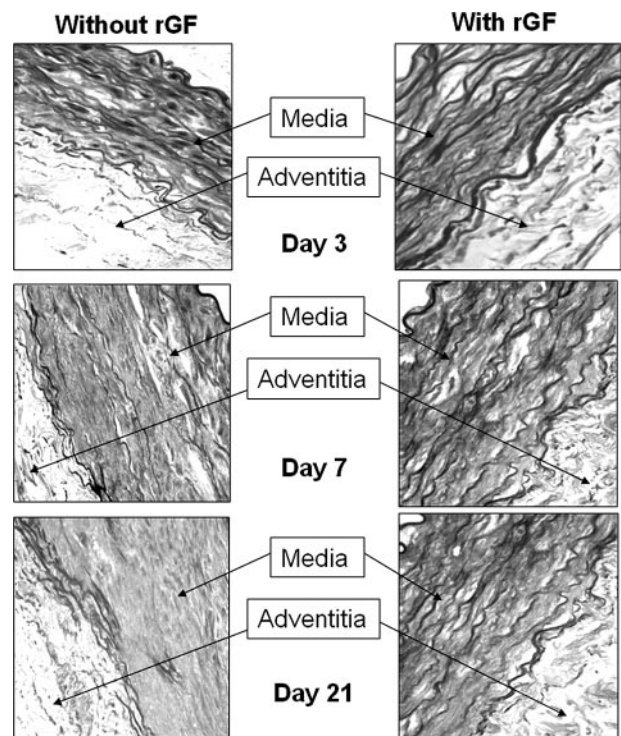


Figure 1. Gingival fibroblasts protect elastin network degradation. Ex vivo aorta culture in the presence or not of rabbit gingival fibroblasts (rGF) at day 3, 7, and 21 stained by orcein (magnification $\times 10$). Media elastin fibers were protected from degradation in the presence of gingival fibroblasts. Representative experiment out of 5.

was associated with an increase in MMP-9 protein level (Figure 2).

In aorta coculture, rGF inhibited MMP-9 protein levels (Figure 2). Inhibition of secreted free forms of MMP-9 was progressive (more intense from day 14, as compared with day 3 and 7) as shown by Dot Blot analysis (Figure 2a). Zymographic analysis showed that hGF inhibited both pro- and active forms of MMP-9 secreted by arteries (Figure 2b and 2c), while hAF and hDF were without effect (supplemental Figure I, available online at <http://atvb.ahajournals.org>).

By immunohistochemistry, an important MMP-9 labeling was observed in adventitia and media from day 3 to day 21 when aorta was cultured alone. In presence of rGF, MMP-9 labeling was clearly decreased both in media and adventitia (Figure 2d).

Cocultures of hGF and hSMC yielded similar results. In single cell culture within a collagen network, only hSMC secreted the proform of MMP-9 (Figure 2e) in contrast with any of the studied fibroblasts (ie, hDF, hAF, and hGF). In hGF/hSMC cocultures, the lysed band corresponding to pro-MMP-9 decreased as soon as day 3, which persisted throughout the study time of the experiments. In contrast, in hDF/hSMC and hAF/hSMC cocultures pro-MMP-9 secretion by hSMC was not significantly affected (Figure 2e).

In contrast with results on MMP-9 protein level, we did not observe in rGF/aorta or hGF/hSMC cocultures a significant decrease of MMP-9 RNA (supplemental Figure IIa and IIb).

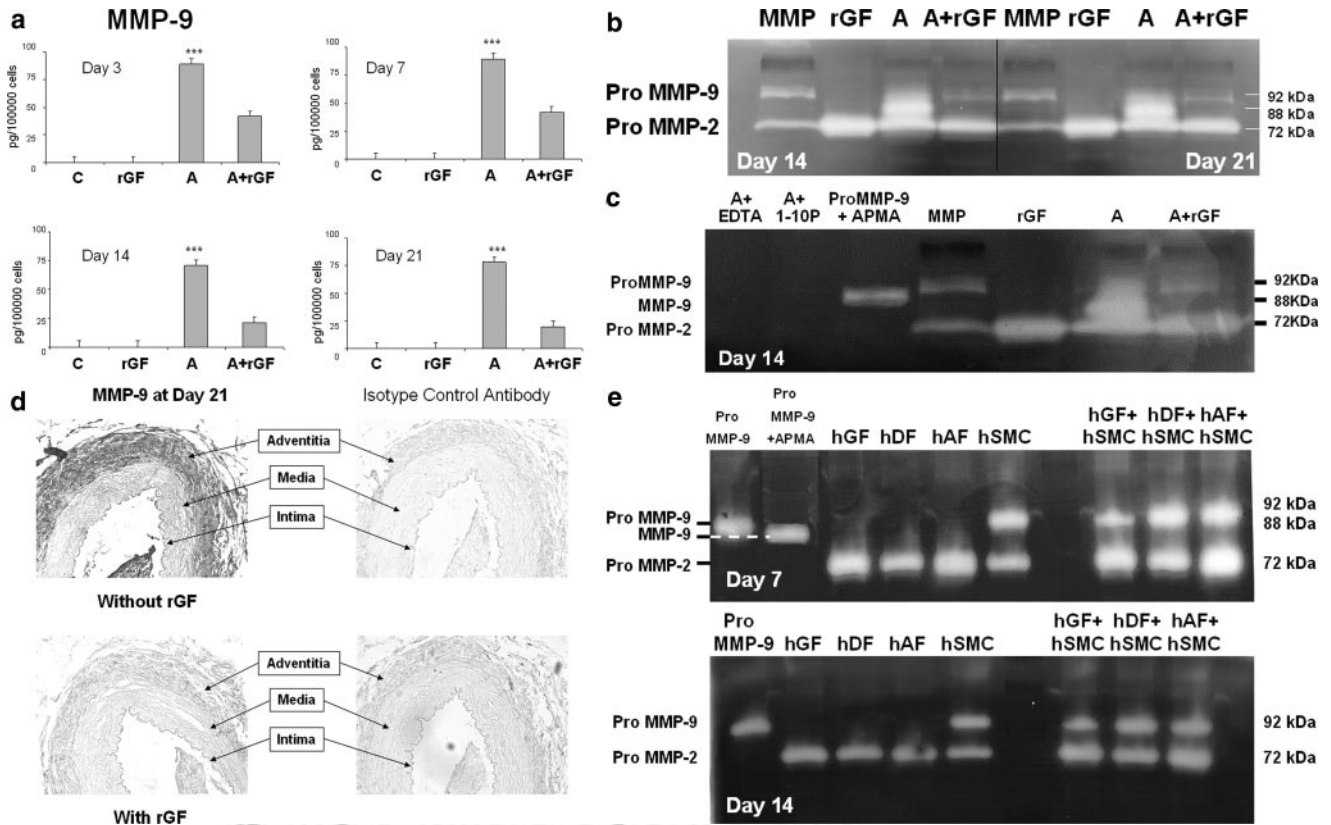


Figure 2. Gingival fibroblasts decrease MMP-9. a, From day 3 to 21, dot blots analysis showed a progressive decrease of MMP-9 protein level in the medium conditioned by aorta cultured in the presence of rGF as compared with aorta cultured alone. For each culture condition results are expressed as mean \pm SEM of 5 independent experiments. *** $P < 0.05$ Student *t* test. C indicates control (collagen gel without cells); rGF, gingival fibroblasts; A, aorta culture; A+rGF, aorta+rabbit gingival fibroblasts. b, Gelatin zymogram of medium conditioned by aorta/rGF cocultures at day 14 and 21. Gelatinolytic activity corresponding to pro-MMP-9 and MMP-9 clearly decreased in presence of rGF as compared with aorta culture alone. Representative experiment out of 5. MMP indicates standard pro-MMP9 (92 kDa) and pro-MMP-2 (72 kDa); rGF, rabbit gingival fibroblasts; A, aorta culture; A+rGF, aorta+rabbit gingival fibroblasts. c, Gelatin zymogram of medium conditioned by aorta/rGF cocultures at day 14. The use of inhibitors (EDTA 10 mmol/L and 1 to 10 phenantroline 2 mmol/L) in the sample buffer led to a complete disappearance of the bands testifying that the corresponding bands of the other samples are MMPs. Activation of pro-MMP-9 with 2 mmol/L APMA permits to visualize MMP-9 position and to differentiate the pro and active forms (respectively 92 and 88 kDa) in the other samples. These controls permit to specify that the presence rGF strongly inhibits the secretion by aortae of active MMP-9. MMP indicates standards pro-MMP-9 (92 kDa)+pro-MMP-2 (72 kDa); Pro-MMP-9+APMA, standard pro-MMP-9+2 mmol/L APMA; rGF, rabbit gingival fibroblasts; A, aorta culture; A+rGF, aorta+rabbit gingival fibroblasts; A+EDTA, aorta+EDTA; A+1 to 10P, aorta+1 to 10 phenantroline. d, MMP-9 immunohistochemistry on ex vivo aorta culture in the presence or not of rabbit gingival fibroblasts at day 21 (magnification $\times 10$). MMP-9 labeling decreased in the presence of rGF. Representative experiment of 5. e, Gelatin zymogram of medium conditioned by cocultures of hGF/hSMCs, hDF/hSMCs, and hAF/hSMCs at day 7 and 14. Gelatinolytic activity corresponding to pro-MMP-9 decreased only in hGF/hSMC coculture as compared with the others cocultures underlying the specificity of hGF. Representative experiment out of 5. Pro-MMP-9+APMA indicates MMP-9 (88 kDa) resulting in standard pro-MMP-9 (92 kDa) activation by 2 mmol/L APMA; hGF, human gingival fibroblast; hSMC, smooth muscle cells; hDF, dermal fibroblast; hAF, adventitial fibroblast.

To further explore the mechanisms, we analyzed the secretion of TIMP-1 and the formation of TIMP-1/MMP-9 complexes.

Gingival Fibroblasts Increased TIMP-1 Expression

Both rGF and aorta grew separately within a collagen matrix-secreted TIMP-1. In rGF/aorta coculture, TIMP-1 release was significantly increased from day 3 to day 21 in a synergic way (Figure 3a).

In parallel, immunohistochemistry showed that TIMP-1 labeling was clearly increased within the different layers of the artery in the presence of rGF (Figure 3b).

Results were similar in hGF/hSMC cocultures (Figure 3c). Both hGF and hSMC grown separately within a collagen matrix secreted TIMP-1 whatever the time point of analysis

(comprised between 5 and 30 pg/10⁵ cells). hGF/hSMC cocultures led to an important synergic effect on the secretion of TIMP-1 (up to 150 pg/10⁵ cells), corresponding to a 5- to 24-fold increase (Figure 3c).

Increase of TIMP-1 protein release in rGF/Aorta or hGF/hSMC cocultures corresponded with an increase of TIMP-1 mRNA expression (supplemental Figure IIc and IId).

Detection of the free forms of MMP-9 by Elisa secreted in the medium was at least 6-fold less when 1 μ g/mL of TIMP-1 was added in the medium of ex vivo cultured aorta (Figure 3d). This inhibitory effect by 1 μ g/mL of TIMP-1 was in the same range as the one observed in presence of 1.5 $\times 10^5$ rGF grown within the collagen matrix surrounding the cultured aorta. In parallel, addition of TIMP-1 led to inhibition of the elastin network (Figure 3d) as rGF did (Figure 1).

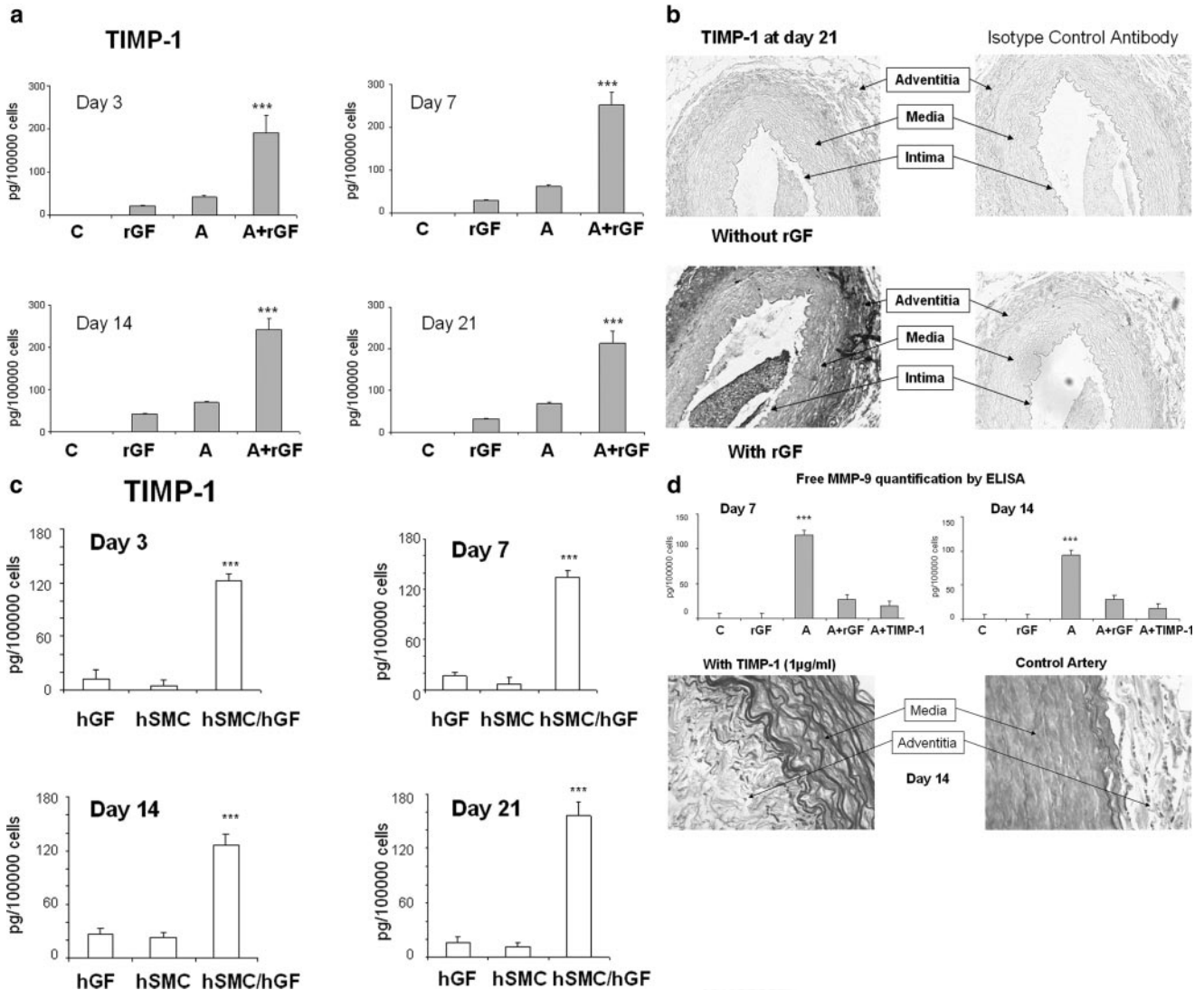


Figure 3. Gingival fibroblasts increase TIMP-1. **a**, From day 3 to 21, TIMP-1 dot blots analysis showed that the basic level of TIMP-1 secreted by rGF or aorta was increased in a synergic way in rGF/aorta coculture. For each culture condition results are expressed as mean±SEM of 5 independent experiments. *** $P < 0.05$ Student *t* test. C indicates Control (collagen gel without cells); rGF, rabbit Gingival Fibroblasts; A, aorta culture; A+rGF, aorta+rabbit Gingival Fibroblasts. **b**, TIMP-1 immunohistochemistry on ex vivo aorta culture with or without gingival fibroblasts at day 21 (magnification ×10). TIMP-1 labeling increased in the presence of rGF. Representative experiment of 5. **c**, TIMP-1 dot blots analysis from day 3 to 21 showed a synergic effect of hGF/hSMC coculture on TIMP secretion. For each culture condition results are expressed as mean±SEM of 5 independent experiments. *** $P < 0.05$ Student *t* test. hGF indicates human gingival fibroblasts; hSMC, smooth muscle cell; hGF+hSMC, Gingival Fibroblasts+smooth muscle cells. **d**, Addition of TIMP-1 (1 µg/mL) in the culture medium of aorta mimic the presence of rGF. Free MMP-9 measured by Elisa was reduced both at day 7 and 14, and the elastin network analyzed at day 14 after orcein staining was maintained as compared with control aorta.

Increase of TIMP-1 release could thus explain the decrease of free MMP-9 protein level and consequently the preservation of the aortic elastin network. We therefore verified MMP-9/TIMP-1 complex formation.

MMP-9/TIMP-1 Complexes Detection

In hGF/hSMC cocultures, the amount of MMP-9/TIMP-1 complexes detected by Elisa was about 3-fold higher as compared with hSMC cultured alone (Figure 4a). This increase was observed whatever the time point of analysis. As expected, because of the absence of MMP-9 secretion by hGF, no significant detection of MMP-9/TIMP-1 complexes was observed in single hGF cultures. Similar results were observed in rGF/aorta cocultures (Figure 4c).

At all the time points of analysis, alkylation/reduction treatment of the samples before Western blot analysis led to a complete disappearance of the areas corresponding to MMP-9/TIMP-1 complexes (ie, 110 kDa) in aorta/rGF cocultures (Figure 4b), confirming Elisa MMP-9/TIMP-1 complexes analysis (Figure 4c). Simultaneously, TIMP-1 labeling increased in aorta/rGF cocultures as compared with aorta or rGF alone.

Complex formation of MMP-9/TIMP-1 was statistically related to elastic network conservation ($P \leq 0.05$; Figure 4c).

Discussion

This work aimed to study the gingival fibroblast repair potential by the use of coculture and an ex vivo artery model.

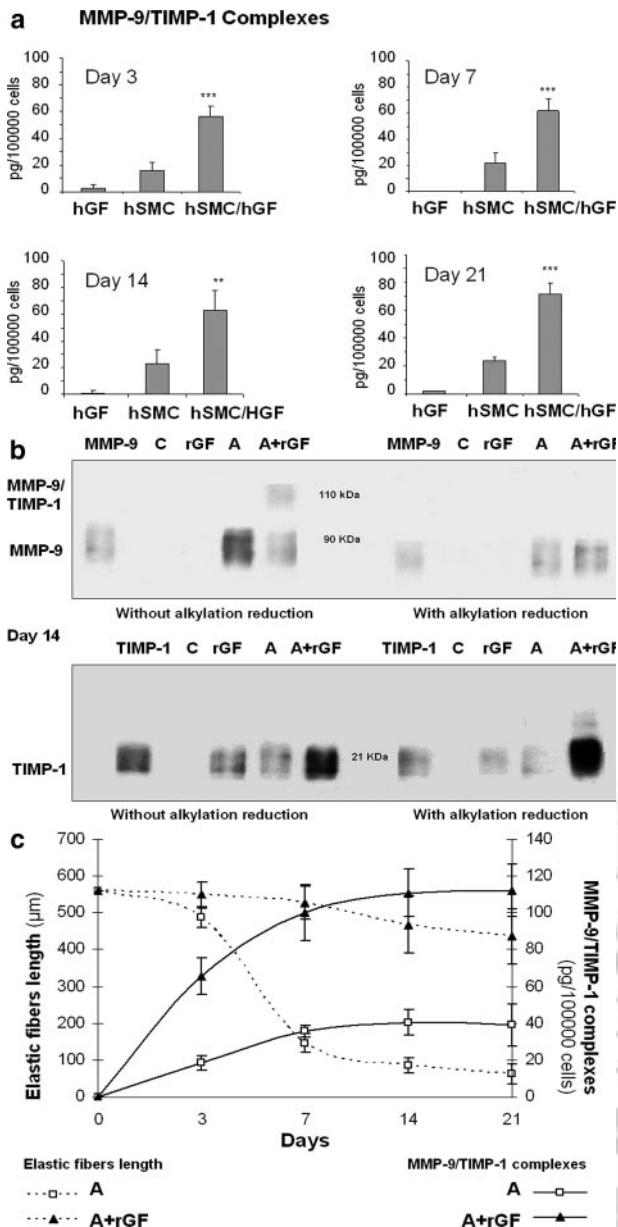


Figure 4. MMP-9/TIMP-1 complexes. a, Elisa quantification of MMP-9/TIMP-1 complexes in hGF/hSMC coculture from day 3 to 21. MMP-9/TIMP-1 complexes increased in hGF/hSMC cocultures. For each culture condition results are expressed as mean \pm SEM of 5 independent experiments. ** $P < 0.05$; *** $P < 0.005$ Student *t* test. hGF indicates human gingival fibroblasts; hSMC, smooth muscle cell; hGF+hSMC, Gingival Fibroblasts+smooth muscle cells. b, MMP-9 and TIMP-1 Western blots analysis at day 14 with or without alkylation/reduction treatment of the samples. Reduction/alkylation conditions of A+rGF samples medium led to the disappearance of MMP-9/TIMP-1 complexes and to an increase in the amount of MMP-9 as compared with reduction/alkylation samples of aorta cultured in the absence of rGF. In parallel, reduction/alkylation increased TIMP-1 detection in A+rGF. MMP-9, or TIMP-1=10 pg of MMP-9 or TIMP-1 standards. c, Correlation between elastic network fragmentation and MMP-9/TIMP-1 complex levels. Complex formation of MMP-9/TIMP-1 was statistically related to elastic network conservation (Anova, $P \leq 0.05$). A indicates aorta culture; A+rGF, aorta+gingival fibroblasts.

The most salient finding of this study is that gingival fibroblast increases the expression and secretion of TIMP-1 which correspondingly decreases MMP-9 protein level and translates, at least in an ex vivo setting, into preservation of the aorta elastic network.

In aneurysms, the media elastic network is impaired, SMC number diminishes, and inflammatory cells invade the expanding vascular wall. Elastin alteration in the aortic wall depends on elastase production by resident cells (SMCs and adventitial fibroblasts) and inflammatory cells. Lower elastin/collagen ratio is the most significant finding in AAA.²⁸ Matrix metalloproteinases participate in vascular connective tissue remodeling,²⁹ and among them, MMP-9 plays an important role in elastic fiber degradation and is highly involved in aneurysmal lesions.³⁰ MMP-9 is the most abundant elastase secreted by human AAA tissue explants in vitro.² MMP-9 plays a direct role in the degradation of extracellular matrix of AAA.^{2,5} Moreover, plasma level of MMP-9 may mark the severity in patients with AAA, being directly related to its size and enlargement.^{7,31,32} MMP-9 is preferentially localized in adventitia infiltrated by macrophages and in areas adjacent to adventitial vasa vasorum of aneurysmal aortic wall.³³ We previously showed that this enzyme released from SMCs and inflammatory cells was not secreted by GF except in particular conditions.³⁴ To explain the release of MMP-9 by hSMCs, we performed an ELISA test to quantify the level of interleukin (IL)-1 β , a proinflammatory cytokine that stimulates MMP-9 synthesis.^{29,35,36} IL-1 β concentration in our cocultures (data not shown) was not less than 60pg/mL, which could account for the hSMC synthesis of MMP-9. Furthermore, IL-1 β stimulates the synthesis and expression of TIMP-1 by GF³⁷ and arteries,^{29,35,36} a possible explanation for the TIMP-1 overexpression in our model. We therefore studied vascular MMP-9 activity modulation potential by GF cellular coculture and vascular organoid coculture.

In cell coculture, we showed that hGF could inhibit MMP-9 released by hSMCs. Moreover, hDF or hAF cultured with hSMCs did not modify MMP-9 protein level. In contrast to what we expected, RNA levels of MMP-9 in hGF/hSMC coculture did not change. So, GF acted on translation or posttranslation steps. Its tissue inhibitor, TIMP-1, was increased in synthesis and transcription. Simultaneously, MMP-9/TIMP-1 complex level measured out by ELISA was increased by 3-fold. We focused on TIMP-1, but we cannot exclude the involvement of other TIMPs. TIMP-1 binds active or latent forms of MMPs in a molecular 1:1 ratio.³⁸ Pro and active MMP-9 can form 2 types of complexes with TIMP-1: Pro-MMP-9 complexes with the C terminus of TIMP-1 whereas active MMP-9 has the possibility for additional complex formation with the N terminus of TIMP-1. Only the latter complex inhibits activity. Moreover, complexes of MMP-9/TIMP-1 enhanced endocytic clearance of MMP-9 by the LRP pathway and probably participate in inducing MMP-9 activity decrease.³⁹ Thus, MMP-9 reduction was not attributable to synthesis inhibition but to an increase of its inhibitor resulting in MMP-9/TIMP-1 complex formation, as confirmed by reduction/alkylation treatment on Western blots. Similar results were shown in rGF and aorta

coculture. The rGF inhibited MMP-9 by increasing TIMP-1 secretion at every time point of measurement. GF in hSMC or aorta coculture behaved like in inflammatory gum: MMP-9 secretion from inflammatory cells was inhibited by TIMP-1 secreted by GF.⁴⁰

Our ex vivo model allowed us to study the elastic fiber network degradation in vessels. In fact MMP-9 synthesis promotes elastin degeneration within the outer aortic layers of AAA.⁴¹ We showed by histology and immunohistochemistry an elastic network protection in aorta cocultured with rGF. The elastic network integrity protection statistically correlated with a MMP-9 protein level reduction in relation with a TIMP-1 increase. In a previous study, mouse lacking the MMP-9 gene did not suffer aortic dilatation and contributed to elastin preservation, despite the presence of inflammatory cells within the aortic wall.⁴² In addition, TIMP-1 overexpression correlated with the protection and prevention of the aneurismal aorta rupture.⁴³ The factors controlling TIMP1/MMP-9 imbalance still remain unknown. Although the MMP-9/TIMP1 ratio is elevated in aneurysms, the production of TIMP-1 does not exceed the increased MMP-9 secretion.⁴⁴

In the present study, rGF reversed the balance of elastin degradation. The gingival fibroblast acted as a healing cell and reproduced in our aorta ex vivo model its strong potential of wound healing which is naturally expressed in the gum. The gingival fibroblast develops a high potential of plasticity. The numerous responses developed by gingival fibroblasts probably result from its capacity to differentiate into multiple cell types.¹¹ More recently, multipotent postnatal stem cells were described in human periodontal ligament.⁴⁵ These results represent a first step toward a potential gingival fibroblast cell therapy in AAA. Further studies are warranted to evaluate in vivo the healing potential of the gingival fibroblast in an aneurysmal artery model.

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Disclosures

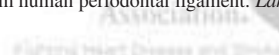
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