Cleavage of extracellular matrix in periodontitis: Gingipains differentially affect cell adhesion activities of fibronectin and tenascin-C

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ABSTRACT

Gingipains are cysteine proteases that represent major virulence factors of the periodontopathogenic bacterium *Porphyromonas gingivalis*. Gingipains are reported to degrade extracellular matrix (ECM) of periodontal tissues, leading to tissue destruction and apoptosis. The exact mechanism is not known, however. Fibronectin and tenascin-C are pericellular ECM glycoproteins present in periodontal tissues. Whereas fibronectin mediates fibroblast adhesion, tenascin-C binds to fibronectin and inhibits its cell-spreading activity. Using purified proteins *in vitro*, we asked whether fibronectin and tenascin-C are cleaved by gingipains at clinically relevant concentrations, and how fragmentation by the bacterial proteases affects their biological activity in cell adhesion. Fibronectin was cleaved into distinct fragments by all three gingipains; however, only arginine-specific HRgpA and RgpB but not lysine-specific Kgp destroyed its cell-spreading activity. This result was confirmed with recombinant cell-binding domain of fibronectin. Of the two major tenascin-C splice variants, the large but not the small was a substrate for gingipains, indicating that cleavage occurred primarily in the alternatively spliced domain. Surprisingly, cleavage of large tenascin-C variant by all three gingipains generated fragments with increased anti-adhesive activity towards intact fibronectin. Fibronectin and tenascin-C fragments were detected in gingival crevicular fluid of a subset of periodontitis patients. We conclude that cleavage by gingipains directly affects the biological activity of both fibronectin and tenascin-C in a manner that might lead to increased cell detachment and loss during periodontal disease.

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1. Introduction

Periodontitis is considered to be the most common inflammatory disease in humans, and the role of bacterial infection in its etiology is well established [1]. Although hundreds of bacterial species are found in a periodontal plaque, only very few of these have been implicated as pathogens in severe periodontitis. One of the most important is *Porphyromonas gingivalis*, which expresses three cysteine proteases called gingipains that are known to be its major virulence factors [2].

Two arginine-specific gingipains possess practically identical catalytic domains, but differ in a C-terminal hemagglutinin domain that is present in HRgpA but not RgpB. The lysine-specific Kgp exhibits a hemagglutinin domain similar to HRgpA but a distinct catalytic domain. In mature HRgpA and Kgp, the hemagglutinin domains are cleaved but remain noncovalently complexed with the catalytic domains [3]. Gingipains are normally attached to the outer membrane of *P. gingivalis* via glycan moieties, but can be released into the environment. Although other virulence factors are present in *P. gingivalis*, the analysis of gingipain-defective strains has shown that these proteases are essential for survival, proliferation, and infectious potential of the pathogenic bacteria [3,4]. It has been shown recently that the concentration of Rgps in the periodontal pockets of periodontitis patients can exceed 1 μM [5].

Gingipains are versatile tools essential for a variety of processes that drive *P. gingivalis* infection, which explains their importance for disease progression during periodontitis [3]. In a first step, HRgpA and Kgp are required for bacterial adherence and colonization of host tissue, which are mediated by their hemagglutinin domains

binding specifically to major extracellular matrix (ECM) proteins such as laminin, vitronectin and fibronectin [6–8]. Second, the bacteria use gingipains to escape the host defense by degrading antimicrobial peptides and components of the complement system [7,9], and to manipulate the inflammatory response by deregulating the cytokine signaling network and by interfering with the blood clotting cascade [2]. Third, gingipains are able to agglutinate and lyse erythrocytes and to digest the released heme proteins, which are an essential nutrient source for the bacteria [10]. Finally, gingipains appear to specifically attack the interaction of gingival and periodontal fibroblasts with their extracellular matrix, thus promoting detachment, apoptosis and tissue destruction [7].

Fibronectin [11] is a large dimeric (2×230 kDa) pericellular matrix protein present in gingiva and periodontal ligament [12]. It mediates adhesion, spreading and motility of fibroblasts by interacting with specific cellular receptors, most notably integrins [13]. Interestingly, *P. gingivalis* appears to use gingipains to specifically attack the interaction of fibronectin with its receptors on fibroblast surfaces [14,15]. Incubation of cultured gingival fibroblasts with *P. gingivalis* culture supernatants or purified gingipains lead to a rapid loss of fibronectin and $\alpha5\beta1$ integrin from cell surfaces, and to cell detachment. A similar study reported a loss of integrin $\alpha2$-, $\alpha5$-, $\beta1$- and $\beta3$-chains in addition to fibronectin from the surface of gingival fibroblasts after treatment with *P. gingivalis* supernatants. Detached fibroblasts became committed to cell death. As expected, gingival crevicular fluid (GCF) samples from periodontitis patients were found to contain significantly increased amounts of fibronectin fragments [16,17]. Immunoblotting with domain-specific anti-fibronectin antibodies showed that fragments (120, 68 and 40 kDa) including the major cell- and/or heparin-binding region of fibronectin were enriched in periodontitis [16].

Tenascin-C is a large hexameric ECM protein known to modulate cell adhesion by inhibiting the spreading of fibroblasts on fibronectin [18,19]. Several splice variants of tenascin-C exist and are likely to be relevant for the periodontal apparatus. A "small" form with 200 kDa subunits is a normal constituent of tendons and ligaments [20] and presumably the one enriched in the attachment zones of the healthy periodontal ligament [12]. A large form (250 kDa subunits [20]) is known to be induced *de novo* and actively involved in many inflammatory processes [21]; mice deficient for tenascin-C show a reduced inflammatory response [22]. However, there are no reports yet whether tenascin-C levels are increased in periodontitis, and if this is the case, which splice variant is induced.

Fibroblast attachment and motility within the ECM are regulated by the balance between adhesive and anti-adhesive signals [19], and gingipains presented and released by *P. gingivalis* might disturb this balance in periodontitis. In this study, we therefore sought to address the following open questions: 1. What is the functional consequence of the reported cleavage of fibronectin by gingipains in terms of the cell adhesion-promoting activity of this ECM protein? This is not a trivial issue, since it is well known that many proteases can generate cell-binding fibronectin fragments that retain their full activity when still immobilized in the ECM [23]. To effectively destroy the adhesive activity of fibronectin, a protease has to cleave at specific sites within the cell-binding domain. 2. Is tenascin-C fragmented by gingipains, and is there a difference in susceptibility between large and small splice variants? 3. How does cleavage by gingipains affect the anti-adhesive activity of tenascin-C? 4. Are proteolytic cleavage products of tenascin-C found in GCF of periodontitis patients, as it has been reported before for fibronectin?

To this aim, human fibronectin was digested with purified gingipains, and fragments were tested in a standardized cell adhesion assay. Human recombinant tenascin-C was equally treated with the three enzymes, and its anti-adhesive activity was quantified before and after cleavage. In addition, gingival crevicular fluid of periodontitis patients was tested for the presence of fibronectin and tenascin-C fragments. Our data indicate that by simultaneously destroying the adhesion activity of fibronectin and generating tenascin-C fragments with increased anti-adhesive activity, gingipains might very rapidly and effectively induce detachment of gingival and periodontal fibroblasts from their ECM during periodontitis.

2. Materials and methods

2.1. Purification of fibronectin and recombinant cell-binding fragment

Fibronectin was purified from human serum (Gibco/Invitrogen, Basel, Switzerland) by affinity chromatography to gelatin-agarose (Sigma-Aldrich, Switzerland) as described [24]. Eluted fibronectin was dialyzed against 150 mM NaCl, 20 mM Na-phosphate (PBS), pH 7.4, and stored frozen in aliquots. A GST-tagged fibronectin fragment comprising fibronectin type III domains 7–11 (FNIII$^{7-11}$) was expressed in *Escherichia coli* as described [25]. Bacteria harboring the expression plasmid [26] were obtained from Dr. Gertraud Orend (University of Strasbourg, France) with permission from Dr. R. O. Hynes (MIT, Cambridge MA). The GST-tagged recombinant fragment was purified using glutathione-Sepharose beads (Qiagen, Basel, Switzerland), dialyzed against PBS, and frozen in aliquots.

2.2. Cloning and purification of recombinant tenascin-C variants

A plasmid (pCEP-huTNC-his [27]) containing the his-tagged human large variant of tenascin-C, cloned into pCEP-Pu vector [28] using *NotI*/*BamHI,* was obtained from Dr. Gertraud Orend (University of Strasbourg, France). This plasmid was further used to construct the his-tagged human small variant of tenascin-C. A *NotI*/*XhoI* fragment was subcloned into pSK(+) vector (Stratagene/Agilent Technologies, Basel, Switzerland). Splicing by overlap extension [29] was performed to excise the extra fibronectin type III repeats of the large tenascin-C variant using overlapping primers (5′-AGTGGATGCCTTCACAC-3′, 5′-GTGTGAAGGCATCCACTGCCATGGGCTCCCCAAA-3′). The spliced *NotI*/*XhoI* fragment was cloned back into the *NotI*/*XhoI* cut pCEP-huTNC-his plasmid, giving rise to pCEP-human small TNC-his. The construct was confirmed by restriction enzyme analysis and sequencing. After transfection of expression plasmids into HEK293-EBNA cells (obtained from Dr. Ruth Chiquet-Ehrismann, Friedrich Miescher Institute, Basel, Switzerland), secretion of human large or small tenascin-C variant, respectively, was determined by Western Blot analysis. Cells were grown to confluency in the presence of 1.5 µg/ml puromycin in DMEM/10% FCS, and starved for two days in serum-free DMEM without puromycin. Recombinant proteins were precipitated from collected conditioned medium with ammonium sulfate and dialyzed against PBS/0.01% Tween 20. After chromatography on a gelatin-agarose column (Sigma-Aldrich, Switzerland) to remove fibronectin, the eluate was loaded on a nickel column (HIS-Select HF Nickel Affinity Gel, Sigma-Aldrich, Switzerland). Bound large or small tenascin-C variants were eluted with 300 mM imidazole, 250 mM sodium phosphate (pH 7.5), 450 mM NaCl, and 0.01% Tween 20 and dialyzed against PBS/0.01% Tween 20.

2.3. Purification of gingipains

Arginine-specific (HRgpA and RgpB) and lysine-specific (Kgp) gingipains were obtained from the *P. gingivalis* HG66 strain culture fluid as described previously [30,31]. Briefly, Kgp and HRgpA were purified using gel filtration and affinity chromatography on arginine-Sepharose, whereas RgpB was purified using a combination of gel filtration and anion-exchange chromatography on Mono Q (GE Healthcare). The purity of each enzyme was checked by SDS-PAGE. The amount of active enzyme in purified gingipains was determined by active site titration using Phe–Pro–Arg–chloromethyl ketone and benzoyloxycarbonyl–Phe–Lys–CH$_2$OCO-(2,4,6-Me$_3$)phenyl–HCl (Z-FK-ck) (both from Bachem AG,

Bubendorf, Switzerland) as active site titrants for Rgps and Kgp, respectively [32,33].

2.4. ECM protein digestion by gingipains and trypsin

The purified gingipains (HRgpA, RgpB and Kgp) were diluted in activation buffer (10 mM cysteine-HCl, 0.2 M Tris–HCl, 1 mM $CaCl_2$, pH 7.6) to get a 1 μM solution, which was then activated for 15 min at 37 °C. From this, 100 nM, 10 nM and 1 nM solutions were prepared by diluting in activation buffer. The same concentrations including 0.1 nM were prepared for bovine pancreas trypsin (Serva, Heidelberg, Germany) in PBS. Digestion of fibronectin and tenascin-C was performed in 20 μl aliquots, taking 18 μl of 1 μM ECM protein in PBS and 2 μl of protease of the desired concentration. The reaction mixture as well as a negative control composed of 18 μl ECM protein and 2 μl activation buffer were incubated for 1 h at 37 °C. For analysis of fragments by SDS-PAGE, the reaction was stopped by adding reducing electrophoresis sample buffer and boiling. In digested samples later used for cell adhesion assays, proteases were inactivated by adding FFRck (Phe-Phe-Arg-chloromethylketone; Bachem AG, Bubendorf, Switzerland), an efficient inhibitor of gingipains [34], at a concentration of 50 nM for 15 min at 37 °C.

2.5. Western blot analysis

Digested samples were separated by SDS-PAGE and blotted to nitrocellulose membranes. After a blocking step in 1% milk, membranes were incubated with rabbit polyclonal anti-fibronectin antibody (1:500) [35], mouse monoclonal antibody B28-13 raised against the constant C-terminal domain of human tenascin-C (1:200) [36], mouse monoclonal antibody C18-13 against the alternatively spliced domain of tenascin-C (1:100) [36], or mouse anti-his antibody (1:100, Quiagen, Switzerland). They were then incubated for 1 h with anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (1:2000; Jackson ImmunoResearch, Suffolk, UK). Blots were developed using ECL reagent (GE Healthcare, Buckinghamshire, UK) and either exposed to Fuji X-ray films or scanned by a Storm 840 Phosphoimager (Glattbrugg, Switzerland).

2.6. Adhesion and anti-adhesion assay

Digestions of fibronectin and its cell-binding fragment (FNIII$^{7-11}$) were performed as described before and stopped by adding protease inhibitor FFRck (see above). For adhesion assays, intact or digested proteins were coated as a 20 μl drop on a non-adhesive plastic dish (bacteriological petri dish; Sterilin Life Sciences, Newport, UK) for 1 h at room temperature. After washing three times with PBS, 5×10^5 fibronectin-deficient mouse embryo fibroblasts (MEF FN$^{-/-}$ [25]) were seeded onto the dish in serum-free DMEM and allowed to attach at 37 °C in a CO_2-incubator. After 4 h, four randomly selected fields were photographed from each coated region, using a Leica inverted microscope with a $10\times$ phase contrast objective. Quantifications were done by counting round and spread cells on each image. The anti-adhesion assay with large tenascin-C variant was performed as published [37,38]. Briefly, intact or gingipain-digested tenascin-C (1 μM in 20 μl) was mixed 1:1 with fibronectin (1 μM in 20 μl) and coated as a 40 μl drop on non-adhesive dishes for 1 h at 4 °C. One hour after seeding MEF FN$^{-/-}$ as above, photographs were taken for quantification as described before. Data in figures represent the average and standard error of the mean of four measurements (ca. 4×80 cells) per sample from one representative experiment. Each adhesion and anti-adhesion assay was repeated at least twice using independently digested protein samples, with very similar results. Statistical significance was determined by one-way ANOVA and paired Student's t-test; differences with a value of $p<0.05$ were considered significant.

2.7. Analysis of gingival crevicular fluid samples

Gingival crevicular fluid (GCF) samples of periodontally healthy subjects and periodontitis patients were collected at the Iuliu Hatieganu University, Cluj-Napoca, Romania. The collection of human samples was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of the Iuliu Hatieganu University. Ten periodontitis patients (mean age 38.6 ± 9.7; 7 women, 3 men) and five periodontally healthy subjects (mean age 33.6 ± 6.2; 3 women, 2 men) from the University of Cluj-Napoca were enrolled in the study (Supplementary Table S1). In the periodontitis group, 5 patients (4 women, 1 man) were suffering from severe chronic periodontitis, and 5 patients (3 women, 2 men) from aggressive periodontitis [39]. In order to be included, periodontitis patients had to have probing pocket depths of at least 6 mm in each mouth quadrant, signs of radiographic bone loss, and no previous periodontal treatment. All periodontally healthy patients had probing pocket depths of ≤ 4 mm; two of the five (one male, one female) were diagnosed with plaque-induced gingivitis. The mean attachment loss (Supplementary Table S1) was 4.3 ± 2.3 mm in the periodontitis and 1.6 ± 0.9 mm in the control group (significant difference, $p<0.01$). All patients were systemically healthy and gave their informed written consent to participate in the study. Samples were taken for GFC and microbiological analysis at the deepest site in each quadrant. The sites were isolated with cotton rolls, air-dried, and supragingival plaque was carefully removed. A standard sterile paper strip (Periopaper, Oraflow Inc., Smithtown, NY, USA) was inserted for 30 s into the gingival crevice until mild resistance was felt (GFC sample). Afterwards, a sterile paper point was inserted into the same site and left in place for 15 s (microbial sample). Samples were collected in transportation plastic vials and stored at -70 °C (GCF samples) or -20 °C (microbial samples), respectively.

The GCF samples were eluted from paper strips in 100 μl PBS overnight at 4 °C and insoluble matter was removed by high-speed centrifugation for 5 min. Fifteen microliter aliquots of each supernatant were used for Western blot analysis with antibodies to fibronectin or tenascin-C, respectively (see above). Band intensities on blots were quantified by densitometric analysis from digital images using Image J software (http://rsbweb.nih.gov/ij/). For determining Arg-gingipain proteolytic activity, 2.5 μl GCF aliquots were diluted to 50 μl with assay buffer (10 mM cysteine-HCl, 1 M HEPES, pH 7.5) in microtiter plates. To each well, 100 μl substrate solution (0.5 mM benzoyl-arginine p-nitroanilide [Sigma-Aldrich, Buchs, Switzerland], 10 mM cysteine-HCl, 50 mM Tris–HCl, pH 7.5) was added. A standard curve with purified, activated RgpB was run on the same plate. After incubation for 16 h at 37 °C, the absorption at 405 nm was measured with an ELISA reader (Biotec EL808). Statistically significant differences ($p<0.05$) between the periodontitis and the control group were determined by Wilcoxon rank sum test with continuity correction.

For microbiological analysis of selected periodontopathogens, DNA was extracted by using the Chelex method [40]. Thereafter, the microIDent® assay (Hain Lifescience, Nehren, Germany) was performed according to the manufacturer's instructions. Of the periodontitis patients, 9/10 were positive for *P. gingivalis* and 10/10 for *Tannerella forsythia*, another important periodontopathogen. From the periodontally healthy control group, one of the subjects with gingivitis was positive for *P. gingivalis* and *T. forsythia*; the other four were negative for both species.

3. Results

3.1. Fragmentation of fibronectin by gingipains and effect on cell-spreading activity

It has been published before that *P. gingivalis* bacterial extracts eliminate fibronectin from the pericellular matrix of cultured fibroblasts

[15,14]. However, actual fragmentation of fibronectin by gingipains has not been demonstrated yet with the purified proteins. We therefore incubated intact fibronectin isolated from human serum with purified gingipains at different enzyme to substrate ratios for 1 h at 37 °C. For control, fibronectin was digested under the same conditions with purified trypsin. Proteolytic fragments were analyzed by immunoblotting using polyclonal anti-fibronectin antibody. As evident from Fig. 1, all three gingipains (HRgpA, RgpB and Kgp) cleaved fibronectin into distinct patterns of differently sized fragments. Expectedly, the proportion of smaller cleavage products was increased at higher enzyme to substrate ratios. Typical for HRgpA and RgpB at lower enzyme concentrations were intermediary fragments of about 100, 80 and 60 kDa, whereas at higher enzyme to substrate ratios peptides between ca. 60 and 40 kDa were prominently detected on the gel. Trypsin treatment generated similar cleavage patterns, however at ten times lower enzyme to substrate ratios. In contrast, digestion with Kgp produced a somewhat different peptide pattern with major fibronectin fragments in the range of 120–140 and 60–70 kDa, and the larger fragments persisted even with the highest enzyme concentration.

We then tested the effect of gingipain digestion on the major biological activity of fibronectin, namely its ability to promote cell adhesion and spreading of fibroblasts. When dishes were coated with intact fibronectin, cells assumed an elongated spindle-like or triangular shape, whereas they settled but remained rounded on uncoated dishes (Fig. 2). On dishes coated with fibronectin that had been previously treated with either HRgpA or RgpB at an enzyme to substrate ratio of 1:10, cells attached but at least half of them still stayed round even after 4 h (significant difference to untreated fibronectin control; $p < 0.01$). When the concentration of these two enzymes was lowered, cells spread on the treated fibronectin (data not shown), presumably because high molecular weight fragments (>180 kDa) were still present in the mixture (see Fig. 1). In contrast to what was found for HRgpA and RgpB, cells on fibronectin digested with Kgp at a 1:10 enzyme to substrate ratio spread almost as well as on dishes coated with untreated fibronectin (Fig. 2), although it was clearly fragmented. These results confirm previous findings with other proteases (e.g. chymotrypsin) that proteolytic fragmentation per se does not destroy the cell-spreading activity of fibronectin. The reason is that limited proteolysis can generate fibronectin fragments in which the cell-binding domain is still intact [24]. However, whereas Kgp digests of fibronectin apparently still retain active fragments even at the highest enzyme to substrate ratio, HRgpA and RgpB seem to effectively destroy the cell-binding domain of fibronectin under the same conditions.

3.2. Cleavage and inactivation of the cell-binding region of fibronectin by gingipains

For effective binding to fibronectin, the specific cellular receptor $\alpha5\beta1$ integrin requires an RGD peptide motif in the tenth and a synergy site in the ninth fibronectin type III (FNIII) domain; a recombinant fragment comprising FNIII domains 7 to 11 (FNIII[7-11]) was reported to retain the cell-binding activity of intact fibronectin [26]. Using a recombinant protein consisting of glutathione-S-transferase (GST; 25 kDa) fused to FNIII[7-11] (60 kDa) (Fig. 3A), we therefore asked whether gingipains were able to cleave fibronectin within its cell-binding region, and how this affected activity. Purified recombinant fusion protein (85 kDa) was incubated with any of the three gingipains at enzyme to substrate ratios of 1:100 and 1:10 for 1 h at 37 °C; trypsin was used for control. The digests were either analyzed directly by immunoblotting (Fig. 3B), or they were used for coating of plastic dishes and plating of fibroblasts as described above (Fig. 3C). For all enzymes, small stable fragments (ca. 35–40 kDa) reacting with anti-fibronectin antibody were visible after digestion with the higher enzyme to substrate ratio, indicating that some cleavage must have occurred within the 60 kDa tandem array of FNIII domains 7–11. At the lower enzyme concentrations, HRgpA, RgpB and trypsin generated a major intermediary fragment of 50 kDa, which was very faint in the Kgp digest compared to other bands (Fig. 3B). Thus, as with intact fibronectin, Kgp appeared to generate a fragmentation pattern distinct from that of the other proteases. We continued by exploring cell adhesion activity of the various digests. When coated onto plastic, intact FNIII[7-11] fusion protein mediated spreading of fibroblasts within 4 h similarly to full-length fibronectin (Fig. 3C); intact GST alone has no cell adhesion activity [26]. Digestion with HRgpA and RgpB at a 1:10 enzyme to substrate ratio substantially reduced the biological activity of FNIII[7-11] (Fig. 3C), since most of the cells remained rounded on these substrates (significant difference to undigested FNIII[7-11] control; $p < 0.01$). In obvious contrast, however, FNIII[7-11] treated with Kgp was similarly active as intact fusion protein in mediating spreading of fibroblasts (Fig. 3C). In conclusion, it was interesting to observe that although all three gingipains cleaved full-length fibronectin as well as its cell-binding region encoded in FNIII[7-11], HRgpA and RgpB treatment strongly affected the biological activity of both proteins, whereas Kgp digestion did not.

3.3. Digestion of large and small tenascin-C isoforms by gingipains

Tenascin-C is an ECM component that negatively regulates fibronectin-mediated cell adhesion [37]; the "anti-spreading" activity has been localized to the C-terminal domain of its subunits (Fig. 4A) [38,41]. We therefore asked whether tenascin-C is also attacked by gingipains, and how this would affect its anti-adhesive activity. Several splice variants of tenascin-C exist (Fig. 4A), of which the smallest (190 kDa subunits) is found in tendons and ligaments, whereas the largest (250 kDa) is associated with developmental and pathological processes. Purified recombinant large and small tenascin-C isoforms were incubated with gingipains at different enzyme to substrate ratios for 1 h at 37 °C as described above, or with trypsin for control
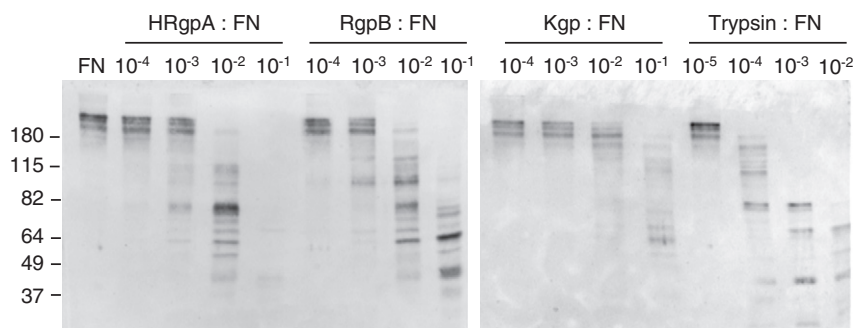

Fig. 1. Fragmentation of fibronectin by gingipains. Purified human serum fibronectin (FN; first lane; 3.6 μg per sample) was incubated with HRgpA, RgpB, Kgp, or with trypsin, respectively, at enzyme to substrate ratios indicated on top of each lane (e.g. $10^{-2} = 1:100$). After 1 h at 37 °C, the reaction was stopped by adding reducing electrophoresis sample buffer and boiling. Samples were run on a 7.5% polyacrylamide gel containing SDS, which was blotted to nitrocellulose and probed with polyclonal anti-fibronectin antibody.

A
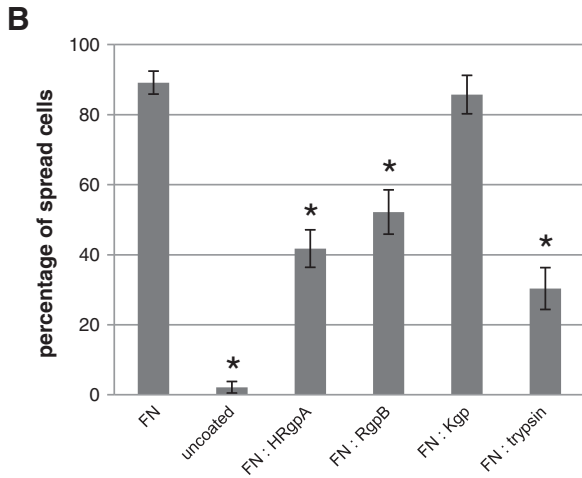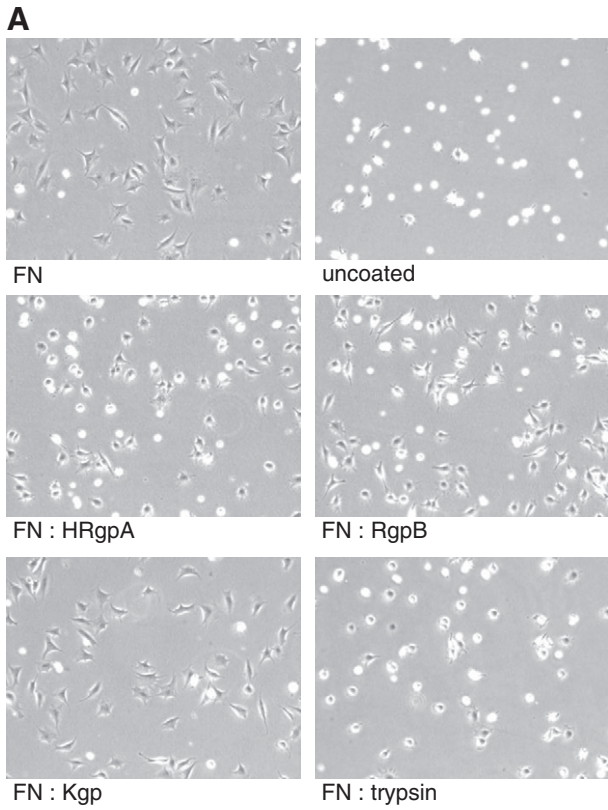
Fig. 2. Inhibition of cell-spreading activity of fibronectin by cleavage with HRgpA and RgpB but not Kgp. Purified human fibronectin was incubated with HRgpA (FN: HRgpA), RgpB (FN: RgpB), or Kgp (FN: Kgp) at an enzyme to substrate ratio of 1:10, or with trypsin (FN: trypsin) at 1:100 for 1 h at 37 °C. The digestion was stopped (see Materials and methods), and the fibronectin fragments were coated on plastic dishes. For positive and negative controls, dishes were either coated with intact fibronectin (FN) or left dry (uncoated). (A) Fibronectin-deficient fibroblasts were plated on the dishes in serum-free medium, allowed to attach for 4 h at 37 °C in a CO_2-incubator, and photographed with phase contrast optics. (B) The proportion of spread cells (with visible processes) versus round cells (exhibiting strong phase contrast) was quantified from digital images. *Significant difference to FN ($p < 0.01$) and to FN: Kgp ($p < 0.01$).

(Fig. 4B). The digests were run on SDS-PAGE, blotted to nitrocellulose, and probed with domain-specific anti-tenascin-C antibodies. Monoclonal antibody (mAb) B28 is directed against the constant C-terminal domain, mAb C18 against the alternatively spliced domain only present in the large isoform, and anti-His against the C-terminal poly-histidine tag of recombinant tenascin-C (Fig. 4A). As can be seen on Fig. 4B (top left), cleavage of large tenascin-C variant with all
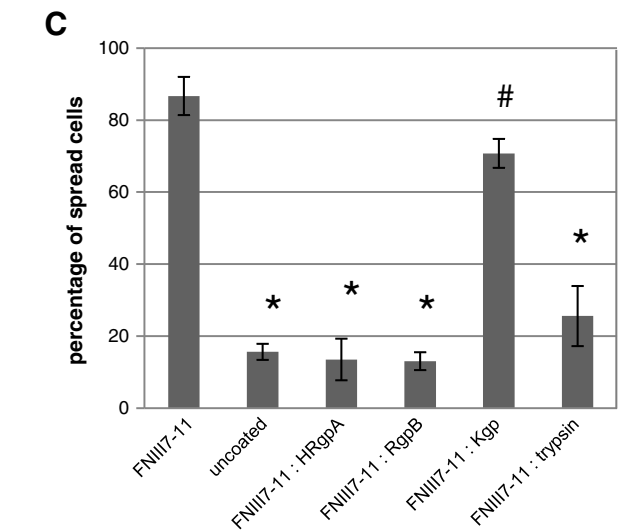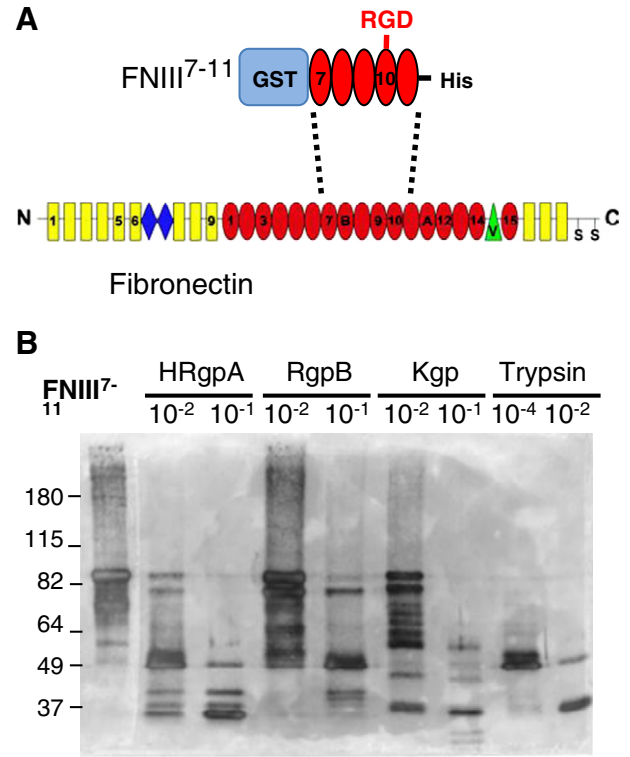
Fig. 3. Inactivation of the cell-binding region of fibronectin by cleavage with HRgpA and RgpB but not Kgp. (A) Top, model of recombinant fusion protein $FNIII^{7-11}$ (85 kDa) consisting of glutathione-S-transferase (GST; 25 kDa) linked to FNIII domains 7–11 (60 kDa) of fibronectin. The cell-binding RGD site is indicated. Bottom: model of the fibronectin subunit with type I (rectangles), type II (diamonds) and type III (ovals) domains. (B) Recombinant $FNIII^{7-11}$ was incubated with HRgpA, RgpB, Kgp, or with trypsin, respectively, at enzyme to substrate ratios indicated on top of each lane (e.g. $10^{-2} = 1:100$). After 1 h at 37 °C, the reaction was stopped and samples were run on SDS-PAGE. A blot of the gel was probed with polyclonal anti-fibronectin antibody. (C) $FNIII^{7-11}$ was digested with HRgpA ($FNIII^{7-11}$: HRgpA), RgpB ($FNIII^{7-11}$: RgpB), or Kgp ($FNIII^{7-11}$: Kgp) at an enzyme to substrate ratio of 1:10, or with trypsin ($FNIII^{7-11}$: trypsin) at 1:100. The digestion was stopped, and the reaction mixtures were coated on plastic dishes. For positive and negative controls, dishes were coated with intact fusion protein ($FNIII^{7-11}$) or left dry (uncoated). Fibronectin-deficient fibroblasts were plated on the dishes in serum-free medium, allowed to attach for 4 h at 37 °C in a CO_2-incubator, and photographed under phase contrast. The proportion of spread cells was quantified from digital images. #Significant difference to FN^{7-11} ($p < 0.05$). *Significant difference to FN^{7-11} ($p < 0.01$) and to FN^{7-11}: Kgp ($p < 0.01$).
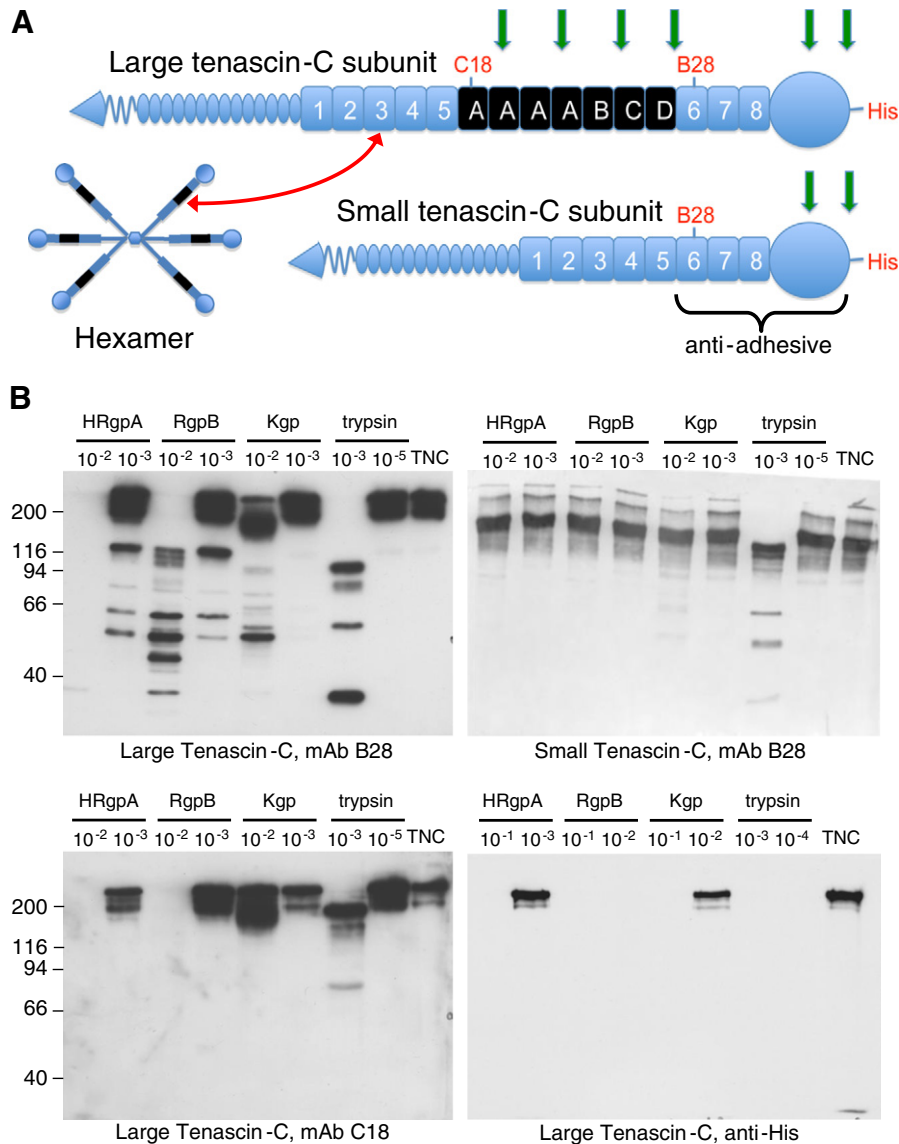
Fig. 4. Differential cleavage of tenascin-C variants by gingipains. (A) Domain model of large and small splice variants of tenascin-C. The N-terminal assembly domain (triangle at left) joins six identical subunits to a hexamer or "hexabrachion." In each subunit, this region is followed by EGF-like repeats (ovals) and a tandem array of fibronectin type III (FNIII) domains (rectangles). A globular domain homologous to fibrinogen is found at the C-terminus (large circle at right). The central FNIII domains (black) can be alternatively spliced, giving rise to large and small subunit variants. Labels C18 and B28 mark the epitopes of the respective anti-human tenascin-C mAbs, and His indicates the C-terminal polyhistidine tag of the recombinant subunits. Anti-adhesive activity is located in the C-terminal region [38,41]. Arrows indicate major gingipain cleavage sites. (B) Purified tenascin-C (TNC; last lane on each blot) was incubated with HRgpA, RgpB, Kgp, or with trypsin, respectively, at enzyme to substrate ratios indicated on top of each lane (e.g. $10^{-2} = 1{:}100$). After 1 h at 37 °C, the reaction was stopped, samples were run on SDS-PAGE, blotted to nitrocellulose, and probed with the antibodies indicated. The upper two blots depict proteolytic fragments generated from large (top left) and small (top right) tenascin-C variant, respectively, that are recognized by mAb B28 against the C-terminal domain (c.f. Fig. 4A). Note that gingipains release C-terminal fragments only from large tenascin-C variant. The two bottom panels show cleavage products of the large tenascin-C variant that are recognized by mAb C18 against the alternatively spliced region (bottom left), or by anti-His against the C-terminal polyhistidine tag (bottom right). Note that essentially no bands below 190 kDa are detected by these two antibodies.

proteases produced a ladder of fragments that reacted with mAb B28 against the C-terminal domain. Typical for gingipain digests were relatively stable fragments of 50–60 kDa; trypsin digestion in addition yielded a prominent 35 kDa fragment. This smallest fragment reacting with mAb B28 was also detected at the higher concentration of RgpB and very faintly with HRgpA (Fig. 4B, top left). Due to their antibody reactivity, all mAb B28-positive fragments must contain FNIII repeats 6–8 plus part or the entire C-terminal fibrinogen-like domain of tenascin-C (see Fig. 4A). When the same digests were probed with mAb C18 against the alternatively spliced domain, only intact tenascin-C and very large fragments (>190 kDa, with the exception of a weak 80 kDa band in the trypsinized sample) were recognized (Fig. 4B, bottom left). This indicated that many of the

gingipain (and trypsin) cleavage sites in the large tenascin-C isoform must be located within its alternatively spliced domain. This region of the molecule appeared to be degraded completely by higher concentrations of HRgpA and RgpB, since in this case only bands reacting with mAb B28 but none recognized by mAb C18 were detectable (Fig. 4B, left panels). An additional site highly susceptible to proteolysis by gingipains (and trypsin) must lie close to the C-terminus within the fibrinogen-like domain, since anti-His only recognizes full-length tenascin-C but none of the smaller fragments (Fig. 4B, bottom right). The major gingipain cleavage sites in large tenascin-C are indicated in the scheme of the molecule in Fig. 4A.

In contrast and very interestingly, the small tenascin-C variant appeared to be almost completely resistant to digestion by gingipains

even at the highest enzyme to substrate ratio (Fig. 4B, top right). This finding strongly supports the evidence described above that gingipains primarily cleave within the extra FNIII domains of large tenascin-C splice variant. Trypsin, on the other hand, produced some fragments (ca. 190, 70 and 50 kDa) from the small variant that were recognized by mAb B28. This points to the presence of tryptic cleavage sites in constant FNIII domains 1–5 and at the N-terminus of tenascin-C that are not attacked by gingipains (c.f. Fig. 4A).

3.4. Effect of fragmentation by gingipains on the anti-spreading activity of large tenascin-C variant

When a mixture of fibronectin and tenascin-C is used to coat plastic dishes, the spreading of seeded fibroblasts is considerably suppressed compared to plain fibronectin substrate ([37]; Fig. 5A). In the present experiments, over 40% of the cells remained rounded after 1 h on a substrate of fibronectin mixed with large tenascin-C variant (1:1 molar ratio), compared to only 10% on fibronectin alone (Fig. 5B). To test for the effect of gingipain digestion on anti-adhesive activity, fragmented large tenascin-C was mixed with intact fibronectin and coated onto dishes (see Materials and Methods). Surprisingly, fragmentation of large tenascin-C with either of the three gingipains lead to a significant increase in its anti-spreading activity in this assay ($p<0.05$), since 70-80% of the fibroblasts retained a round cell shape 1 h after plating them on the mixtures of fibronectin with digested large tenascin-C (Fig. 5B). For control, the cell-spreading assay was repeated by coating dishes with mixtures of just the cell-binding fibronectin domain (FNIII$^{7-11}$) with intact or gingipain-digested large tenascin-C. Because tenascin-C requires an adjacent but separate heparin-binding region to bind to fibronectin [42], the cell-spreading activity of FNIII$^{7-11}$ was neither inhibited by intact nor by fragmented tenascin-C, as was expected (data not shown). In addition, this control experiment demonstrated that inhibitor-treated gingipains do not interfere with the activity of fibronectin's cell-binding domain, and hence that anti-spreading activity in the experiment with full-length fibronectin (Fig. 5) must originate from tenascin-C and its fragments. We conclude that digestion of large tenascin-C by gingipains generates fragments that retain potent anti-spreading activity against full-length fibronectin.
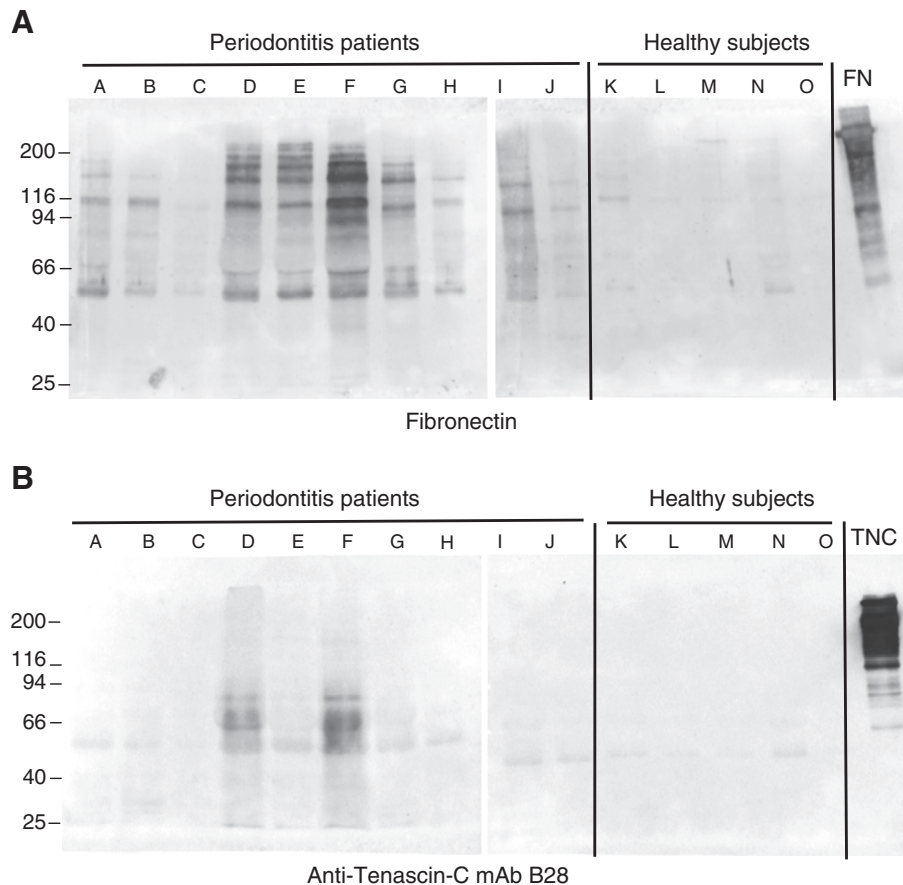
3.5. Fibronectin and tenascin-C fragments in gingival crevicular fluid of periodontitis patients

To test whether fibronectin and tenascin-C fragments are associated with periodontal disease, GCF of periodontally healthy subjects and periodontitis patients was analyzed by immunoblotting for the two proteins. In exudates from healthy people ($n=5$), a polyclonal antibody recognizing all fibronectin domains barely detected traces of full-sized or fragmented protein (Fig. 6A). In contrast, fibronectin fragments were clearly visible in eight out of ten patient samples, and very prominent in three of them. The pattern of fragments resembled those of fibronectin digested with purified gingipains, with major bands at ca. 180, 120, and 50–70 kDa (Fig. 6A). When an identical blot was probed with mAb B28 against the C-terminal domain of human tenascin-C, no signal was detected in GCF samples of all healthy subjects (Fig. 6B). In contrast, strong bands of 50–70 kDa were detected in two out of the ten patient samples; these two patients belonged to the group that also showed the highest signal for fibronectin fragments (Fig. 6A, B). Thus, whereas fibronectin and tenascin-C are not released into the gingival sulcus of healthy individuals, these two ECM proteins are highly enriched in fragmented forms in GCF fluid of a subset of periodontitis patients.

The mean Arg-gingipain activity in GCF samples from the periodontitis group was roughly 15 times higher than in the control group ($p<0.01$). However, there were large variations between patients, and values did not correlate directly with the amount of ECM

A

FN   TNC

uncoated   FN + TNC

FN + (TNC : HRgpA)   FN + (TNC : RgpB)

FN + (TNC : Kgp)   FN + (TNC : trypsin)

B
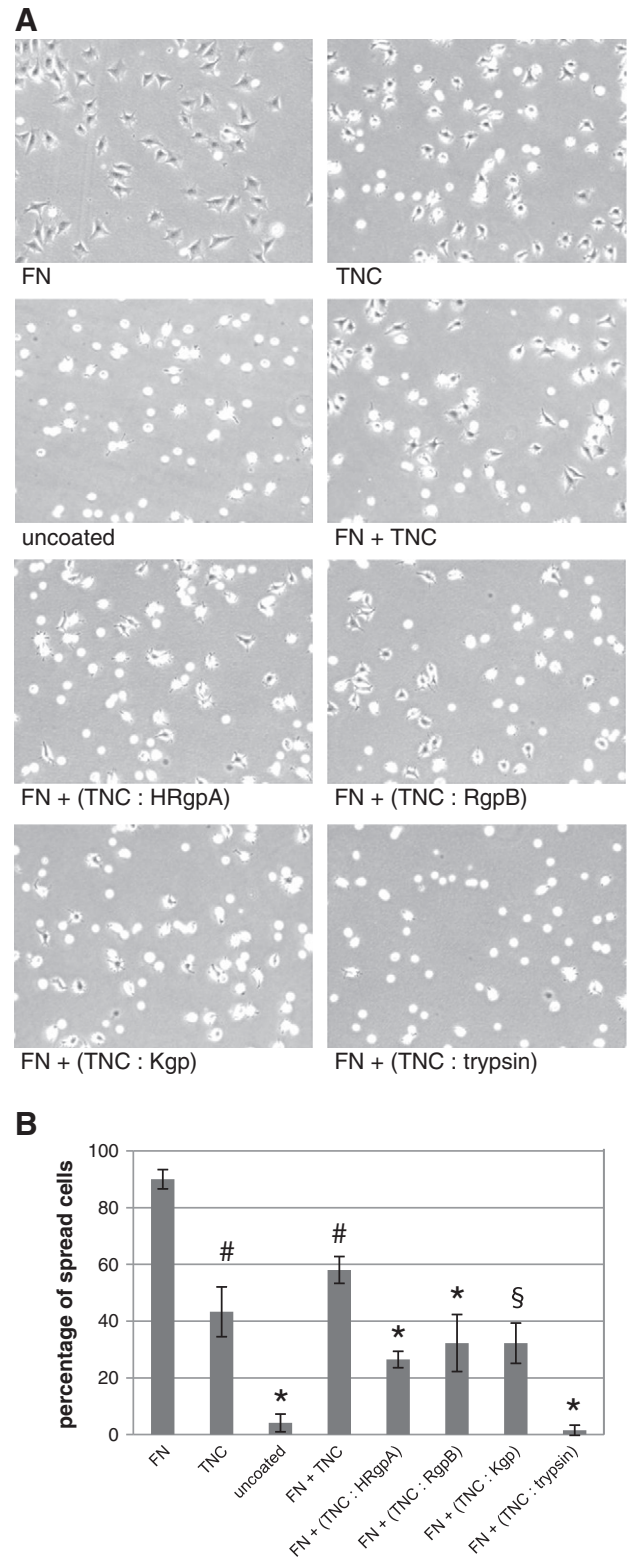
Fig. 5. Anti-spreading activity of intact and gingipain-digested large tenascin-C variant. (A) Large tenascin-C was digested with gingipains (enzyme to substrate ratio 1:10) or trypsin (1:100) and the reaction was stopped by adding protease inhibitor. Plastic dishes were either left uncoated for control, or coated with fibronectin alone (FN), large tenascin-C alone (TNC), a 1:1 mixture of fibronectin and intact large tenascin-C (FN + TNC), or 1:1 mixtures of fibronectin and protease-digested tenascin-C [FN + (TNC: Protease)]. Fibroblasts were plated in serum-free medium and photographed after 1 h. (B) The percentage of spread cells was quantified from digital images. Note the smaller proportion of spread cells on the fibronectin/tenascin-C mixtures compared to fibronectin alone. #Significant difference to FN ($p<0.01$). *Significant difference to FN ($p<0.01$) and to FN + TNC ($p<0.01$). §Significant difference to FN ($p<0.01$) and to FN + TNC ($p<0.05$).

A

B

Fig. 6. Presence of fibronectin and tenascin-C fragments in crevicular fluid of periodontitis patients. Sulcus fluid was collected from periodontally healthy subjects and from individuals suffering from periodontitis. Identical volumes were run on SDS-PAGE and immunoblotted with (A) polyclonal anti-fibronectin or (B) mAb B28 against the C-terminal domain of human tenascin-C. Note proteolytic fragments in patient samples.

fragments detected in the same sample (Supplementary Table S1; see Discussion).

4. Discussion

The important role of gingipains in tissue destruction during periodontitis has long been recognized, and several lines of evidence indicate that these bacterial proteases specifically attack the interaction of gingival and periodontal cells with their extracellular matrix [7,14,15,43–45]. The gingipain concentration in periodontal pockets of patients has been measured to be in the order of 1 μM [5], which is 10–100 times higher than what was used in the present or previous studies to digest relevant ECM substrates *in vitro*. Much research has focused on fibronectin, a major pericellular adhesion protein of fibroblasts [11]. Its most relevant cellular receptors are integrin α5β1, which recognizes an arg–gly–asp (RGD) peptide motif in the 10th fibronectin type III (FNIII) domain, and the cell surface proteoglycan syndecan-4, which engages with a heparin-binding site in the 13th FNIII domain of the fibronectin subunit [11]. Gingipains with a hemagglutinin domain were found to bind more effectively to fibronectin on the surface of gingival and periodontal fibroblasts than RgpB, apparently because this domain targets the protease to fibronectin fibrils, as was confirmed by colocalization experiments [14]. The catalytic domains then not only cleave fibronectin, but also its receptor α5β1 integrin, which colocalizes with fibronectin in fibrillar adhesions on the cell surface [46]. Interestingly, although Kgp does cleave fibronectin (c.f. Fig. 1), experiments with *P. gingivalis* mutants (ΔrgpA, ΔrgpB, Δkgp) and specific inhibitors indicated that the arginine-specific gingipains but not Kgp were primarily responsible for detaching fibroblasts from their ECM substrate [15].

Our present results might provide a rationale for this finding. First, we observed that although all three gingipains generated distinct fragment patterns from purified human fibronectin, only HRgpA and RgpB destroyed most of its cell-spreading activity in our *in vitro* assay, whereas a mixture of similarly sized fibronectin fragments (40–70 kDa) produced by Kgp cleavage and coated onto plastic dishes still promoted spreading of fibroblasts. Moreover, we were able to confirm these results with a recombinant fibronectin fragment (FNIII[7-11] [26]) that included the 10th fibronectin type III repeat with the RGD cell-binding site recognized by integrins. Again, all three gingipains cleaved this part of the fibronectin molecule, and the smallest stable fragments were of similar size (ca. 35 kDa). Nevertheless, FNIII[7-11] digested with HRgpA and RgpB, but not with Kgp, completely lost its cell-spreading activity. Thus, whether complex pericellular matrix [14,15], purified fibronectin, or its isolated cell-binding region is treated with gingipains, the arginine-specific proteases are much more effective in compromising cell adhesion function than the lysine-specific. It is of course tempting to speculate that HRgpA and RgpB might cleave the cell-binding region of fibronectin directly at the RGD motif, especially since this peptide sequence is known to be contained in an exposed loop at the surface of the 10th FNIII domain [47]. Thus, HRgpA and RgpB might very specifically target the binding of fibronectin to integrin α5β1, and thereby one of the most important interactions of fibroblasts with their ECM. For *P. gingivalis* this would be a very effective way to disturb homeostasis of the host tissue [7]; however, this interesting possibility needs to be explored further.

The extracellular matrix not only contains components that mediate firm attachment of embedded cells, such as fibronectin, laminins and many of the collagens, but also molecules that modulate and

tune interactions of cells with their ECM, and that can be "adhesive" or "anti-adhesive" depending on the context [48]. This is important for tissue homeostasis since certain conditions require cells to loosen their grip on the ECM, e.g. before mitosis, during migration, or under physical strain. We hypothesized that cleavage of such adhesion modulating proteins might be yet another mechanism by which gingipains disturb cell–ECM interactions in periodontal tissues. Among these "matricellular" ECM components [49] are the tenascins, which on the one hand can promote adhesion of certain cell types via specific integrins, and on the other hand are well known for interfering with fibronectin-dependent spreading of fibroblasts [19]. Mechanistically, the C-terminal region of tenascin-C recognizes the heparin-binding site in the 13th FNIII domain of fibronectin, thereby blocking the interaction of fibronectin with the cell surface proteoglycan syndecan-4 [42]. Integrin $\alpha5\beta1$ and syndecan-4 need to signal synergistically to activate the small GTPase RhoA, which in turn controls actin dynamics required for cell spreading [50]. This is the reason why isolated fibroblasts in fact attach to a mixture of fibronectin and tenascin-C, but remain rounded on such a substrate and are not able to extend processes [37,42]. We used this assay to test the effect of gingipain digestion on the anti-adhesive function of tenascin-C variants. First, we found that only "large" tenascin-C, which is the form induced in many inflammatory processes [18], is susceptible to cleavage by all three gingipains, whereas the "small" variant expressed in healthy ligaments and tendons [20] resists attack by these proteases. It can be concluded that gingipain cleavage sites are located mainly or exclusively in the alternatively spliced FNIII domains of tenascin-C. Surprisingly, digestion by any of the gingipains actually increased rather than decreased the anti-spreading function of "large" tenascin-C; i.e. fragments presumably from the C-terminal region [38,41] appeared to be more active in this assay than the intact protein. Although this seems paradoxical, there are many reports indicating that proteolytic cleavage of certain ECM proteins generates fragments ("matrikines") with activities that are cryptic in the intact molecules [51]; probably the best known is endostatin, a fragment of collagen XVIII with supposed anti-angiogenic activity [52]. Concerning fibronectin, fragments from the N-terminal half were reported to inhibit matrix assembly [53,54] and to increase cytokine secretion by macrophages [17], whereas different fragments even appear to exhibit metalloproteinase-like activity (reviewed in [55]). Fragments of tenascin-C containing N-terminal EGF-like repeats have been reported to bind to the EGF receptor with moderate affinity and to trigger cell proliferation [56], whereas a fragment consisting of FNIII repeats 1–8 inhibits fibronectin assembly [57]. Thus, one might speculate that the anti-adhesive activity in the C-terminal domain [38,41] is partially self-inhibited in the large tenascin-C variant by the alternatively spliced region, and that proteolytic cleavage by gingipains generates fragments with higher activity than the intact large subunit. It should be noted that not all proteases act on tenascin-C in this manner. We have shown earlier that in contrast to gingipains, which cleave in the extra repeats, the metalloproteinase meprin-β destroys the anti-adhesive activity of tenascin-C by cutting at a distinct site, namely within the constant C-terminal domain [58].

Similar to fibronectin fragments, we detected tenascin-C cleavage products in gingival crevicular fluid obtained from periodontitis patients. They were of similar size as those generated by gingipains in vitro, and likewise reacted with an antibody to the constant C-terminal domain of tenascin-C, which has been reported to harbor the anti-adhesive activity [38,41]. Earlier, we reported similar cleavage products in sulcus fluid of peri-implantitis patients [59]. However, tenascin-C fragments were detected only in a small subset of periodontitis cases, and so far we have not been able to correlate their appearance with clinical parameters such as the severity of the disease or the presence or absence of certain bacterial species. The individuals with high amounts of tenascin-C fragments in their GCF were positive for P. gingivalis as well as for T. forsythia, another highly proteolytic periodontopathogen [60], but so were all other periodontitis patients in our sample (with one exception for

P. gingivalis). When comparing groups, the mean proteolytic activity was found to be several fold increased in patient GCF compared to controls, whereas values for individual patients did not correlate directly with the level of ECM fragments in the same sample (Supplementary Table S1). However, the amount of proteolytic products also depends on the available substrate. In the periodontal pockets of most patients, the gingipain concentration is in excess of what is required to cleave ECM substrates in vitro [5]. Assuming that the fragments found in patient samples have been generated by gingipain cleavage, they must originate from "large" tenascin-C variant, i.e. from the form that is known to be induced during inflammatory processes [18]. Thus, the appearance of fragments in a specific patient sample is likely to reflect an increase in tenascin-C expression during the course of the disease, and must not necessarily correspond to a higher concentration of bacterial proteases in periodontal tissue. Such high tenascin-C expression might be due to a different inflammatory response in these specific patients [18,21], but a prospective study will be required to elucidate the exact mechanism behind this observation.

5. Conclusion

Our present results provide strong evidence that P. gingivalis can use gingipains to specifically interfere with cell adhesion in their host tissue in a dual way: first, by cleaving fibronectin in its major cell-binding domain, and second, by increasing the anti-adhesive activity of tenascin-C, a major modulator of contacts between fibroblasts and their ECM. Further studies are required to address the clinical relevance of these findings.

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Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgments

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