A Secreted Collagen- and Fibronectin-binding Streptococcal Protein Modulates Cell-mediated Collagen Gel Contraction and Interstitial Fluid Pressure*

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Fibroblast-mediated collagen gel contraction depends on collagen-binding β1 integrins. Perturbation of these integrins reveals an alternative contraction process that is integrin αVβ3-dependent and platelet-derived growth factor (PDGF) BB-stimulated. Connective tissue cells actively control interstitial fluid pressure (IFP), and inflammation-induced lowering of IFP provides a driving force for edema formation. PDGF-BB normalizes a lowered IFP by an αVβ3-dependent process. A potential modulation of IFP by extracellular matrix-binding bacterial proteins has previously not been addressed. The fibronectin (FN)-binding protein FNE is specifically secreted by the highly virulent Streptococcus equi subspecies equi. FNE bound FN and native collagen type I with K_d values of ~20 and ~50 nM determined by solid-phase binding assays. Rotary shadowing revealed a single FN binding site located at on average 122 nm from the C terminus of procollagen type I. FNE induced αVβ3-mediated contraction by C2C12 cells in a concentration-dependent manner having a maximal effect at ~100 nM. This activity of FNE required cellular FN, and FNE acted synergistically to added plasma FN or PDGF-BB. FNE enhanced binding of soluble FN to immobilized collagen, and conversely the binding of collagen to immobilized FN. Marked bell-shaped concentration dependences for these interactions suggest that FNE forms a bridge between FN and collagen. Finally, FNE normalized dermal IFP lowered by anaphylaxis. Our data suggest that secreted FNE normalized lowering of IFP by stimulating connective tissue cell contraction.

Species of pathogenic staphylococci and streptococci express several cell wall-bound or released proteins that specifically interact with host components involved in e.g. the blood clotting and the complement system or with extracellular matrix (ECM) proteins (1, 2). Streptococcus equi subspecies equi and subspecies zooepidemicus are both important horse pathogenic bacteria (3). Subspecies equi causes the serious horse respiratory disease called strangles. Subspecies zooepidemicus is considered as an opportunistic commensal, often occurring in the upper respiratory tract of horses. In subspecies zooepidemicus, two cell wall-anchored fibronectin (FN)-binding proteins have been identified, FNZ (4) and FNZ2 (5). In subspecies equi the analogues of these proteins are denoted FNE (6) and FNEB (7), respectively. The different FN-binding proteins expressed by both subspecies display distinct binding specificities and affinities to FN (7). A major difference between FNE and FNZ is that a frameshift mutation in the fne gene results in a truncated protein that is secreted to the growth medium, i.e. FNE. We have previously shown that a recombinant protein representing the N-terminal half of FNZ, denoted FNZN, binds both FN and native collagen type I and in addition, stimulates collagen gel contraction (8).

Loose interstitial connective tissues surround all peripheral blood vessels that are involved in the exchange of solutes from plasma to tissues (for a review see Ref. 9). Connective tissue cells participate actively in the fluid balance by controlling the IFP that is normally around zero but lowered during anaphylactic and inflammatory reactions (10). A reduction in IFP provides a driving force for edema formation during inflammation and burn injuries. Edema formation is one of the classical signs of inflammation induced by e.g. tissue reactions to invading bacteria. The pathophysiological relevance of edema formation is most likely to increase drainage from the tissue, as well as to facilitate for phagocytes and soluble antimicrobial proteins to reach the infectious foci.

Data from our laboratories suggest that control of IFP and edema formation depends on integrins (10). The collagen-binding integrin α2β1 maintains IFP during homeostasis in rat dermis (11). A lowered dermal IFP resulting from anaphylaxis or blockade of collagen-binding β1 integrins can be normalized

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§ This abbreviation is used for: ECM, extracellular matrix; PDGF-BB, platelet-derived growth factor BB; IFP, interstitial fluid pressure; FN, fibronectin; TEM, transmission electron microscopy; CB, cyanogen bromide; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
by instilments of PDGF-BB (11, 12). PDGF-BB exerts its effect on IFP by a process dependent on the specific Arg-Gly-Asp directed αvβ3 integrin (13). Fibroblast-mediated collagen gel contraction can serve as an in vitro model for control of IFP in vivo (11, 13–18). Contraction depends on collagen-binding β1 integrins but αvβ3 can also mediate contraction when β1 integrins are absent or perturbed and then only after stimulation of cells by PDGF-BB (13, 19, 20).

Because the recombinant FNZN stimulates collagen gel contraction and, especially, the fact that the homologous FNE is secreted we hypothesized that FNE could modulate edema formation in infected tissues. If so, it could function as a novel virulence factor during subspecies S. equi infection by rendering bacteria less accessible for the innate immune system. Here we have investigated the mechanism by which FNE stimulates collagen gel contraction in vitro and its possible effect on IFP in vivo.

**EXPERIMENTAL PROCEDURES**

**Cells**—The murine C2C12 myoblast cell line was provided by Dr. Anna Starzinski-Powitz (Goethe-Universitaet, Frankfurt am Main, Germany). FN-deficient mouse embryonic fibroblasts (clone 4D) (21, 22) were donated by Dr. Staffan Johansson (Uppsala University, Sweden). Both C2C12 and 4D cells lack expression of collagen-binding β1 integrins (22, 23). C2C12 cells were stably transfected with either integrin α2 or α11, therefore only expressing collagen-binding integrins α2β1 and α11β1, respectively (C2C12-α2 and C2C12-α11). Cells were propagated in DMEM with Glutamax (Invitrogen), supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and 50 μg/ml Gentamicin (Invitrogen) at 37 °C with 5% CO₂.

**Reagents**—Bovine dermal collagen type I (Purecol, 3 mg/ml) was from Inamed (Fremont, CA). Calf nasal collagen type II, rat skin collagen type III, Engelbreth-Holm-Swarm (EHS)-sarcoma collagen type IV and collagen α1(I) cyanogen bromide (CB) peptides 3, 7 and 8, were produced as described previously (24–26). Human plasma FN was purified as described by Miekka et al. (27). Recombinant platelet-derived growth factor BB (PDGF-BB) was purchased from Invitrogen. The anti-human FN polyclonal IgG has been described elsewhere (28). The specific integrin αvβ3 inhibitor cyclo-(Arg-Gly-Asp-d-Phe-Val) was obtained from Bachem (Bubendorf, Switzerland) and the specific monoclonal anti-murine β3 integrin antibody (HMβ3) was from BD PharMingen (San Diego, CA).

**Recombinant S. equi Proteins**—The proteins used in this study were recombinant forms of the S. equi subspecies equi and zooepidemicus proteins FNZ, FNE, FNEB, and SFS. FNZ is a cell surface-bound FN-binding protein expressed by subspecies zooepidemicus (4), and the recombinant 34-kDa protein, FNZN, represents the N-terminal part of FNZ (6). The 32-kDa protein FNE, the truncated FN-binding FNE homologue found in subspecies equi and SFS, are special in this panel of proteins because they are secreted proteins (6, 29). The following primers were used to PCR-amplify the fne gene using genomic DNA from subspecies equi strain 1866 as template: FNE F2: 5’-CATGGCTAGCCTTTATATTGGTGATGATGGA-3’, FNE R: 5’-CATGCCGGGATCTGGAAAGGATCTCGTTT-3’. The underlined nucleotide sequences of the primers hybridize to the fne gene, and the introduced restriction endonuclease cleavage sites are written in bold. The PCR products were digested with NheI and Smal and ligated into the pTYB4-vector (New England Biolabs) previously digested with the same restriction endonucleases. After ligation, the plasmid was electroporated into Escherichia coli ER2566. FNEB is a cell surface-bound FN-binding protein of subspecies equi, and the characterization and production of the recombinant 40 kDa protein FNEB L, used in this study, was described in Ref. 7. SFS is an extracellular FN-binding protein (29), and the recombinant 36-kDa protein, corresponding to the mature protein, used in this study is described in Ref. 7. All constructions and purifications of the recombinant proteins were performed using the IMPACT™ system (New England Biolabs, Ipswich, MA). A schematic representation of the streptococcal proteins is given in Fig. 1.

**Microwell Assay for Determination of Protein Interactions**—125I-labeled collagen or FN in the presence of BSA (100 μg/ml) was added to the wells together with different concentrations of FNE for 2 h at 37 °C. The wells were washed two times with PBS supplemented with 0.05% Tween 20 (PBS-T), and binding was measured using a γ-counting device (25).
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ter (Searle). As a control, the streptococcal protein SFS was used instead of FNE. To measure unspecific binding, wells were coated with 0.05 M NaHCO₃ only, and the obtained cpm values were subtracted from the values of the tested ligands.

For determining binding of FNE to different collagen types and to cyanogen bromide (CB) fragments 3, 7, and 8, microwells were coated overnight at 4 °C with 10 μg of the collagens or CB fragments diluted in 100 μl of 0.05 M NaHCO₃. For denaturing, collagens were heated at 55 °C for 20 min, and microwells were coated overnight at the same temperature. The wells were incubated with 200 μl of PBS supplemented with BSA (20 mg/ml) overnight at 4 °C. Different concentrations of biotinylated FNE were added in the presence of BSA (100 μg/ml) and wells incubated for 2 h at 37 °C. The wells were further incubated with 100 μl of alkaline phosphatase streptavidin (1:500) (Vector, Burlingame, CA) to detect binding.

Rotary Shadowing of the Procollagen I/FNEZN Complex and Negative Staining of the Procollagen I/FNE Complex—For Transmission Electron Microscopy (TEM) involving rotary shadowing, procollagen I, isolated as described before (30), and FNZN were dialysed against 200 mM ammonium bicarbonate, pH 7.9. Both binding partners were allowed to react for 3 min before they were mixed with an equal volume of 80% (v/v) glycerol and squeezed between two freshly cleaned mica pieces. Specimens were then dried for 2 h under high vacuum. Subsequently, they were decorated with 3-nm platinum/carbon at a low angle on a rotating table, followed by coating with 10-nm carbon at a 90° angle. Replicas were floated off on distilled water and picked up on 400 mesh copper grids. Finally, complexes were visualized in a Jeol JEM 1230 electron microscope operated at an accelerating voltage of 60 kV. Images were recorded with a Gatan Multiscan 791 CCD camera.

For the negative staining a mixture of both protein solutions dissolved in TBS were allowed to react for about 1 min before 5-μl aliquots were adsorbed for 1 min on carbon-coated copper grids, which had been rendered hydrophilic by glow discharge at low pressure beforehand. Adsorbed samples were washed twice with water and stained with 0.75% (w/v) uranyl formate. Specimens were visualized as described above.

Cell Adhesion—24-well plates were coated overnight at 4 °C with human plasma FN (2 μg/ml), FNE (10 μg/ml), or collagen type I (50 μg/ml) in Buffer 3 consisting of (in mM): NaCl 140, KCl 4.7, MgSO₄ 0.65, CaCl₂ 1.2, HEPES 10, pH 7.4. Plates were washed three times with Buffer 3 and 200 μl of Buffer 3 were added per well. Cells were trypsinized and washed twice with ice-cold Buffer 3 and diluted to 500,000 cells/ml in the same buffer. 100,000 cells were allowed to adhere at 37 °C and when indicated, FNE was added together with the cells at a concentration of 500 nM. Non-adherent cells were removed, and the wells were washed gently two times with prewarmed Buffer 3. The number of adhered cells was quantified using a hexosaminidase assay, as described elsewhere (31).

Binding of 125I-FNE to Cells—24-well plates were blocked in 2% BSA overnight at 37 °C. Cells were trypsinized and washed twice in cold PBS with BSA (1 mg/ml) and diluted to a final concentration of 5 × 10⁶ cells/ml in PBS with BSA. In each well, 100 μl of cells was mixed with 100 μl of 125I-labeled FNE (~270,000 cpm and final concentration at 2 nM) without or with 100 μl human plasma FN to a final concentration of 5 or 110 nM. FNE was labeled with 125I as described above. The final volume was adjusted to 500 μl using PBS with BSA (1 mg/ml). Plates were incubated at 37 °C on a rocking table; the latter to prevent cell sedimentation. At indicated time points, the cell suspension was transferred to tubes containing a preformed 10% Percoll gradient (GE Healthcare, Uppsala, Sweden). Tubes were centrifuged for 10 min at 209 × g. Percoll was aspirated and pelleted viable cells were lysed in 1 M NaOH overnight at 4 °C. Radioactivity was measured in a γ-counter (COBRA auto-γ, Packard), and values represent the percentage of total radioactivity added.

Collagen Gel Contraction—Collagen gel contraction was quantified as described elsewhere (28). Briefly, 96-well plates were blocked in 2% BSA, and a collagen solution was prepared from double concentrated DMEM, HEPES, and collagen type I (Purecol). One part cell suspension (10⁶ cells/ml) was mixed with 9 parts collagen solution. When indicated, bacterial proteins, FN, antibodies, and/or inhibitors were added to the cell-collagen solution. Cell-collagen gels (100 μl) were allowed to form, and the gels were detached by ejection of 100 μl of Dulbecco’s modified Eagle’s medium, with or without PDGF-BB, into the wells. The relaxed, free-floating gels were further incubated at 37 °C, and gel diameters were measured microscopically at indicated time points. The degree of contraction is presented as the gel area in percentage of the original area.

Reverse Transcription and PCR—C2C12 and 4D cells were seeded in 60-mm dishes and grown to ~90% confluency. mRNA was extracted using TRIzol® reagent (Invitrogen) and according to the manufacturer’s instructions. cDNA was generated with the Reverse Transcription System (Promega), and PCR fragments for FN and cyclophilin-B (CP-B) were generated with 30-cycle PCR using the following primers: FN forward, 5'-CCTGTTTCAAATCGAGTA-G-3'; FN reverse, 5'-ACCCGGTAGCCAGTGAGCTG-3'; CP-B forward, 5'-GGAGATGAATCTGTAGGACGAGTC-3'; CP-B reverse, 5'-CGTACCACATCCCCGCCTCT-3'. Samples were run on a 1% agarose gel, and DNA fragments were visualized with UV light.

Metabolic Labeling with [35S]Cysteine/Methionine to Detect FN Synthesis—24-well plates were blocked in 2% BSA overnight at 37 °C. Collagen gels were made as described above, but with a total gel volume of 500 μl and a total of 100,000 cells. [35S]Redivue Promix (GE Healthcare) was added to the cell-collagen suspension (1:100), and gels were allowed to form for 1.5 h. Gels were detached with EMEM (supplemented with 2 μM methionine and 45 mM HEPES) containing [35S]Redivue Promix (1:100) and where indicated, PDGF-BB was added at a final concentration of 20 ng/ml. Gels were incubated at 37 °C for 2 and 4 h. Collagen gels were centrifuged at 1877 g for 10 min and subsequently heated to 55 °C for 15 min. Collagen/cell suspensions were lysed in a final concentration of 1× radiomunoprecipitation assay buffer consisting of (in mM): Tris/HCl 50, NaCl 150, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, pH 8.0. Supernatants were cleared with preimmune rabbit IgG (Sigma) for 1.5 h at 37 °C, and cleared lysates were immunoprecipitated with a polyclonal anti-human FN antibody for 1.5 h at 37 °C. Immunoprecipi-
tated proteins were separated on a 5% polyacrylamide SDS-gel, and the gel was fixed for 30 min in fixing buffer (25% isopropyl alcohol, 10% acetic acid, 65% H2O). Then the gel was dried in Amplify (GE Healthcare) for 15 min and dried on a gel dryer before it was exposed to x-ray film (Fujifilm Corporation, Tokyo, Japan).

**Knockdown of FN with siRNA**—C2C12 cells were seeded in 24-well plates at 90,000 cells per well and grown for 24 h in antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cells were transfected with a final concentration of 20 nM siRNA (Sigma-Proligo) directed against murine FN mRNA (Pubmed accession number: NM_010233) or control (scrambled) siRNA that has no binding interaction with any known mRNA. Transfection agent was N-TERTM Nanoparticles (Sigma) that was used according to the manufacturer’s instructions. Cells were harvested at 24–48 h, and FN protein levels were assessed by immunoprecipitating 35S-labeled FN as described above. Transfected cells were used in collagen gel contraction assays.

**In Vivo Measurement of Interstitial Fluid Pressure (IFP)**—Male and female BALB/c mice were used. Mice were fed ad libitum before experiments and anesthetized by a s.c. injection of Ketamin (Ketalar, Pfizer, New York, NY; 12.2 mg/ml) combined with Medetomidin (Domitor, Orion Pharma, Espoo, Finland; 24.3 μg/ml) (0.1 ml/10 g body weight). The mice were catheterized in the external jugular vein for i.v. injections. Circulatory arrest was induced by an i.v. injection of saturated KCl. Experiments were performed with the approval of and in accordance with the recommendations laid down by the Norwegian State Commission for Laboratory Animals.

IFP was measured using sharpened glass capillaries (tip diameter 3–5 μm) filled with 0.3 m NaCl colored with Evans Blue and connected to a servo-controlled counter pressure system. The punctures were performed through intact skin using a stereomicroscope (Wild M5, Heerbrugg, Switzerland). Care was taken not to cause any compression or retraction of the skin while puncturing. The animal was placed in a supine position and the left hind paw was carefully fixed to the table with surgical tape. Control IFP was measured with the circulation still intact. 200 μg C48/80 (Sigma) in 0.1 ml 0.9% NaCl was injected i.v. and allowed to circulate for 2 min before circulatory arrest was induced. C48/80 causes a lowering of the IFP through degradation of mast cells. Circulatory arrest was induced to prevent a potential underestimation of the lowered IFP due to an increase in interstitial fluid volume as a result of increased transcapillary fluid flux. The lowering of the IFP was monitored for 30 min and 1 μl FNE (−40 μm) or control vehicle (0.9% NaCl) was injected subdermally using a 10-μl chromatography syringe (Hamilton, Bonaduz, Switzerland) with a 33-gauge needle. Measurements were continued for another 60 min. The pressure measurements were averaged in the following periods: 0–10, 11–20, 21–30, 31–40, 41–50, 51–60, and 61–90 min after C48/80 injection. For a measurement to be accepted, the following criteria had to be fulfilled: (1) feedback gain could be changed without changing the pressure; (2) applying suction to the pipette by the pump increased the resistance in the pipette. This ensured contact between the pipette and the interstitial fluid, i.e. the pipette was open; and (3) zero pressure did not change during the measurement.

**Statistical Analyses**—For collagen gel contractions, adhesion assays, solid phase assays, FN-production, and binding of 125I-labeled FNE to cells, statistical analyses were performed using Student’s t test, and data are mean ± S.E.; each experimental group was compared for control and 21–30 min and 61–90 min using one-way repeated measure analysis of variance (ANOVA) followed by Bonferroni-corrected t-tests. For all experiments p < 0.05 was considered statistically significant.

**RESULTS**

**FNE Bound to Collagens and Stimulated the Interaction between Native Collagen Type I and FN**—FNE bound with equal affinity to native collagen types I and II, less effectively to collagen type III and not at all to collagen type IV (Fig. 2A). FNE did not bind heat-denatured collagens or CB fragments (data not shown). Furthermore, binding of FNE was not affected by additions of EDTA, Ca2+, or Mg2+ at concentrations up to 10
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mM, or by a 20-min pretreatment with 20 mM NaIO4 to remove carbohydrate residues from the collagen substrate (data not shown). Adhesion to collagen type I by cells expressing only either α2β1 or α11β1 as collagen-binding integrins was not affected by addition of 500 nM FNE (Fig. 2B). Rotary shadowing experiments demonstrated that the FNE homologue FNZN binds to a single distinct binding site of the procollagen type I monomer (Fig. 2C). The inset in Fig. 2C shows the appearance of FNZN in the absence of procollagen. Negative staining showed that also FNE binds to a single distinct binding site of the procollagen molecule (Fig. 2D). Measurements on the location of the FNZN/FNE binding site on procollagen revealed it to be situated at 122 ± 6 nm (mean ± S.D.) from the C terminus (Fig. 2E).

FN binds to denatured collagen type I with high affinity whereas binding to native collagen type I has a several orders of magnitude lower affinity (32). Because FNE binds FN and, in addition, bound to procollagen we investigated whether FNE could increase binding of soluble FN to immobilized native collagen type I and, conversely if FNE increased binding of soluble collagen type I to immobilized FN. Binding of 125I-labeled FN to immobilized native collagen type I was negligible in the absence of FNE but increased with increasing concentrations of FNE up to around 50 nM whereafter binding declined (Fig. 3A). 125I-labeled native collagen type I bound to immobilized FN in the presence of added FNE, again with a bell-shaped concentration curve showing maximal binding at around 5 nM of FNE (Fig. 3B). The secreted streptococcal FN-binding protein SFS had no significant effect on the interaction between collagen and FN (data not shown). Scatchard plot analyses of solid-phase binding assays with 125I-labeled FNE to immobilized FN and collagen showed Kd values of ~20 and ~50 nM, respectively. Bound 125I-labeled FNE was displaced by a 100 times molar excess unlabeled FNE, validating the obtained Kd values (data not shown). Our results show that FNE stimulated the interaction between native collagen type I and FN in a saturable fashion. A plausible mechanism for this stimulation is that FNE, bound to a single binding site on the collagen monomer simultaneously can bind FN. At high concentrations of FNE the protein will saturate binding sites on both collagen and FN leading to a diminished interaction between collagen and FN.

Cells Bound Soluble and Immobilized FNE by an FN-dependent Process—We performed cell adhesion and molecular binding experiments to investigate a possible interaction between cellular FN and FNE in living cells. Both C2C12 and 4D cells adhered to immobilized human plasma FN with similar kinetics (Fig. 4A). However, adhesion of 4D cells to immobilized FNE was significantly lower compared with C2C12 cells (Fig. 4A). These findings point to a need for FN expression to adhere to FNE. Furthermore, we performed binding studies of 125I-labeled FNE to suspended C2C12 and 4D cells. Binding was investigated at 37 °C over a 60-min time period, longer incubation times led to prohibitive cell death. C2C12, but not 4D cells...
bound $^{125}$I-labeled FNE, suggesting that cell surface-associated FN was the major binding partner for FNE (Fig. 4B). Added soluble plasma FN at a concentration of $\sim$5 nM had no effect on binding to any of the two cell types, whereas the presence of $\sim$110 nM reduced binding to C2C12 cells to background levels (data not shown). The latter observation is in line with the saturable character of FNE-FN interaction. In summary, these results suggest that cell surface-bound FN served as a binding site for FNE.

FNE-induced Cell-mediated Collagen Gel Contraction Is Dependent on FN—FNE-induced C2C12 cell-mediated collagen gel contraction (Fig. 5A). The ability to stimulate C2C12-mediated collagen gel contraction was restricted to FNE and its homologue FNZN among a group of S. equi proteins with affinity for eukaryotic ECM proteins (Table 1). Addition of PDGF-BB or human plasma FN stimulated contraction to a degree similar to FNE (Fig. 5A). FNE acted synergistically with PDGF-BB and plasma FN, with the highest rate of contraction achieved for the combination FNE and plasma FN (Fig. 5B).

Because FNE stimulated binding between collagen and FN and because added plasma FN acted synergistically with FNE (Fig. 5B), we hypothesized that FN could be involved in the mechanism by which FNE induced C2C12 cell-mediated collagen gel contraction. In a first series of experiments we took advantage of the 4D cells. These cells, similarly to C2C12 cells, were unable to contract collagen gels because they lack collagen-binding $\beta_1$ integrins (Fig. 5C). FNE did not induce significant 4D cell-mediated collagen gel contraction, nor did PDGF-BB or added plasma FN when compared with control (Fig. 5C). The combination of FNE and plasma FN, but not the combination of FNE and PDGF-BB, however, effectively induced collagen gel contraction (Fig. 5D). These results suggest that FNE-induced collagen gel contraction depended on cell-produced FN.

Based on the above data it seemed reasonable to suggest that FNE-induced C2C12 cell-mediated contraction involved FN produced by the cells. To further investigate this possibility we analyzed expression and production of FN. FN mRNA could be detected in C2C12 but not in 4D cells (Fig. 6A). Production of FN by C2C12 cells could be demonstrated in experiments using metabolic labeling and immunoprecipitation of FN. C2C12 cells cultured in collagen gels synthesized detectable FN after 2 and 4 h and PDGF-BB up-regulated this synthesis 2-fold (Fig. 6B). These results are compatible with that PDGF-BB induced C2C12 cell-mediated collagen gel contraction by stimulating FN synthesis. These findings provide a plausible explanation for the synergism between PDGF-BB and FNE (Fig. 5B), namely that PDGF-BB increased cell surface expression of FN and thereby enhanced the FNE effect on contraction. To further test the hypothesis of a dependence on FN for contraction induced by FNE, we used siRNA directed against murine FN. Transfection with FN-siRNA effectively inhibited C2C12 cell FN synthesis by $\sim$85% compared with control siRNA (Fig. 6C). The ability of FNE to induce C2C12 cell-mediated collagen gel contraction, either alone or in combination with PDGF-BB, was completely abolished after transfection with siRNA. Adding exogenous FN restored the synergistic effect of the combination of FNE and FN (Fig. 6D).

FNE-induced Cell-mediated Collagen Gel Contraction Required the Integrin $\alphaV\beta3$—C2C12 cells do not express collagen binding $\beta1$ integrins but express the FN binding integrins $\alpha5\beta1$ and $\alphaV\beta3$. To investigate whether FNE-induced collagen gel contraction is mediated by $\alphaV\beta3$, we investigated the effects of anti-murine $\beta3$ integrin IgG or a specific cyclic Arg-Gly-Asp-based inhibitor. Both abolished FNE-induced C2C12-mediated collagen gel contraction (Fig. 7), suggesting that $\alphaV\beta3$ and not $\alpha5\beta1$ was used by the cells.

FNE Restored Dermal Interstitial Fluid Pressure Lowered as a Result of Anaphylaxis—Several components that inhibit fibroblast-mediated collagen gel contraction in vitro lower dermal IFP, whereas components stimulating contraction are able to normalize dermal IFP that has been lowered as a result of inflammatory processes (11, 13, 15–17). Because FNE could induce C2C12 cell-mediated collagen gel contraction, we tested whether it could restore dermal IFP lowered as a result of anaphylaxis. IFP was lowered by the mast cell degranulator C48/80

### Table 1

| No addition | Bacterial protein |
|-------------|------------------|
| FNZN        | 91.70 ± 1.53     |
| FNE         | 5.04 ± 1.59      |
| FNE         | 85.04 ± 1.59     |
| PDGF-BB     | 5.04 ± 1.59      |
| PDGF-BB + FN| 69.30 ± 2.64     |
| PDGF-BB + FNE| 72.30 ± 2.64   |
| FNE + FN    | 69.30 ± 2.64     |

$^{a}p < 0.05$ when compared to no addition (Student’s $t$-test).

*Fig. 5. FNE-stimulated collagen gel contraction is dependent on FN. Collagen gels were made with additions of FN (50 μg/ml) and/or FNE (100 nM). Gels were floated with or without PDGF-BB (final concentration 20 ng/ml). PDGF-BB, FNE, and FN stimulated collagen gel contraction mediated by C2C12 cells compared with control (A). Combination of PDGF-BB and FN or PDGF-BB and FNE increased the contraction compared with addition of any of the factors alone. Combination of FNE and FN had a synergistic effect, most pronounced in early time points (B). In 4D cells (fibronectin null-mutation), none of the added factors (PDGF-BB, FNE, and FN) had a significant effect on collagen gel contraction compared with control (C). Combination of PDGF-BB and FNE did not induce any significant contraction whereas combination of FN and FNE, similar as in C2C12 cells, induced a synergistic effect on contraction compared with control (D and E). In all panels, data are means from a minimum of three independent experiments, and error bars are S.E.*
and after induction of cardiac arrest local injections of FNE 30 min after induction of anaphylaxis restored IFP, whereas injections of vehicle control had no effect (Fig. 8).

Discussion

We show that the soluble ECM-binding protein FNE that is secreted by S. equi, subspecies equi induces C2C12 cell-mediated collagen gel contraction by linking cell-surface bound FN to collagen type I fibers. Integrins αVβ3 and α5β1 bind FN and would both be candidate receptors for the FN-FNE-collagen fiber complexes and mediators of the intracellular force required for contraction. The reason for the cellular preference of αVβ3 usage in FNE-induced contraction is not clear. It is possible that cryptic sites are opened in the complex of FN-FNE-collagen fibers that enhance binding by αVβ3 rather than by α5β1.

The detailed structure of the single collagen binding site for FNE in collagen type I is not known but it was dependent on the triple-helix confirmation. Furthermore, because FNE bound collagen type II with high affinity, it is likely that this site is present also in collagen type II. Rotary shadowing experiments have identified three collagen type I binding sites for recombinant α-subunit A domains of the collagen binding integrins α1β1 and α2β1 (33). One of these sites is also recognized by integrin α11β1 (34). These integrin binding sites were separate from the FNE binding site identified herein and α2β1- or α11β1-mediated cell adhesion to collagen type I was not inhibited by FNE. FNE bound collagen independent of cations or
collagen carbohydrates. Together our data show that collagen type I contains one single high affinity FNE binding site, which is distinct from known integrin binding sites and dependent on proper triple helical folding.

The affinity of FNE for FN was higher than for collagen type I. Native dimeric plasma FN harbors at least two binding sites for FNE (7) and furthermore this molecule is more flexible in solution than is collagen type I, which appears as a stiff rod. These differences most likely explain why a lower concentration of FNE was needed to achieve maximal binding of soluble collagen type I to immobilized FN than that needed for maximal binding of soluble FN to immobilized collagen type I and also why maximal amount in picomoles of bound FN was around six times higher than maximal amount of bound collagen type I.

Collagen gel contraction mediated by C2C12 myoblasts and FN-deficient 4D cells, both lacking collagen binding β1 integrins but expressing αVβ3 (data not shown), can be used as an in vitro model of IFP regulation during inflammation. The reason for this is that available data suggest that in such tissue the collagen binding β1 integrins become inactive (10). In such tissues PDGF-BB normalizes IFP in a process dependent on the αVβ3 integrins (13). PDGF-BB stimulated FN synthesis by C2C12 cells cultured in collagen gels. This finding offers an explanation for the synergy between PDGF-BB and FNE in collagen gel contraction. An increase in cellular FN would facilitate the formation of an FNE-stimulated complex between FN and the collagen fibers. Interestingly, the rate of initial contraction was higher for the combination of plasma FN and FNE compared with PDGF-BB and FNE. This is in accordance with an increased FN synthesis induced by PDGF-BB as a prerequisite for contraction, a process that would require more time. The combination of PDGF-BB and FNE was unable to induce contraction by the FN-deficient 4D cells but plasma FN together with FNE induced a rapid contraction by these cells. Moreover, knock-down of FN synthesis in C2C12 cells rendered these cells unable to mediate collagen gel contraction, also in the presence of FNE or PDGF-BB. These findings suggest that the effects of PDGF-BB on cell-mediated contraction not only depend on αVβ3 (13, 19, 20) but also on FN. The FNE-induced contraction may demonstrate a more general mechanism for cell-mediated collagen gel modulation, particularly in situations where collagen-binding β1 integrins are absent or perturbed, i.e. inflammation. Extracellular matrix proteins produced by cells after stimulation by e.g. TGF-β and/or PDGF that bind both FN and collagen could potentially promote contraction by a mechanism similar to that by which FNE induced contraction.

Generally, pathogens provoke a series of innate immune responses in the host including edema formation. The significance of inflammatory edema resulting from infection is presumably to enhance clearance of infectious organisms. Mechanistically edema results from increased transcapillary filtration pressure, induced by an increased capillary hydrostatic pressure, lowered colloid osmotic or IFP and/or an increase in vascular permeability for plasma proteins; changes that accompany inflammation. Previous data show that PDGF-BB, which stimulates collagen gel contraction in vitro, can normalize lowered IFP as result of anaphylaxis by an αVβ3-dependent mechanism (8, 11, 12). Here we show that FNE, similarly to PDGF-BB, normalizes IFP that had been lowered by the mast cell degranulator C48/80. The results suggest that during S. equi subspecies equi infection FNE can “compact” the interstitium thereby making it possible to encapsulate the infection. This will have the following consequences for the interstitium (9): First, lowered diffusivity of macromolecules such as IgG. The diffusion coefficient for proteins this size in the ECM is normally less than 5% of their free diffusion coefficient in water and will be further lowered following a compaction of the ECM. Another consequence of the lowered diffusivity is that cytokines attracting inflammatory cells will be likewise hindered in their diffusion thereby lowering the attraction of such cells. Second, hydraulic conductivity will be lowered. Finally, the movement of inflammatory cells is more restricted in a compacted ECM. FNE is secreted due to a frameshift mutation in the Tfe gene from the virulent S. equi subspecies equi that causes strangles in horses. It is an interesting possibility that the secreted FNE may function as a virulence factor by compacting the interstitium, thereby counteracting a lowering of IFP and the resulting edema formation even at a distance from the bacterial growth. The FNE homologue FNZ that is present on the horse commensal S. equi subspecies zooepidemicus is unlikely to have this activity because it is anchored to the bacterial cell wall.

In summary, we provide support for a mechanism of cell-mediated collagen gel contraction based on the soluble bacterial protein FNE forming a molecular bridge between collagen fibers and cell surface FN, in turn bound by the integrin αVβ3. The integrin αVβ3 is required for PDGF-BB-induced normalization of dermal IFP lowered as a result of anaphylaxis and possibly also for counteracting excessive edema formation during inflammation (13). The finding that FNE induced normalization of IFP is therefore compatible with the presence of a novel virulence mechanism in which bacteria can be protected from innate immune reactions by inducing a compacted connective tissue.

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