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Activation of MMP-9 by human lung epithelial cells in response to the cystic fibrosis-associated pathogen *Burkholderia cenocepacia* reduced wound healing in vitro

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Wright C, Pilkington R, Callaghan M, McClean S. Activation of MMP-9 by human lung epithelial cells in response to the cystic fibrosis-associated pathogen *Burkholderia cenocepacia* reduced wound healing in vitro. *Am J Physiol Lung Cell Mol Physiol* 301: L575–L586, 2011. First published July 8, 2011; doi:10.1152/ajplung.00226.2010.—*Burkholderia cepacia* complex is a group of bacterial pathogens that cause opportunistic infections in cystic fibrosis (CF). The most virulent of these is *Burkholderia cenocepacia*. Matrix metalloproteinases (MMPs) are upregulated in CF patients. The aim of this work was to examine the role of MMPs in the pathogenesis of *B. cepacia* complex, which has not been explored to date. Real-time PCR analysis showed that *B. cenocepacia* infection upregulated MMP-2 and MMP-9 genes in the CF lung cell line CFBE41o– within 1 h, whereas MMP-2, -7, and -9 genes were upregulated in the non-CF lung cell line 16HBE14o–. Conditioned media from both cell lines showed increased MMP-9 activation following *B. cenocepacia* infection. Conditioned media from *B. cenocepacia*-infected cells significantly reduced the rate of wound healing in confluent lung epithelia ($P < 0.05$), in contrast to conditioned media from *Pseudomonas aeruginosa*-infected cells, which showed predominant MMP-2 activation. Treatment of control conditioned media from both cell lines with the MMP activator 4-aminophenylmercuric acetate (APMA) also resulted in clear activation of MMP-9 and to a much lesser extent MMP-2. APMA treatment of control media also delayed the repair of wound healing in confluent epithelial cells. Furthermore, specific inhibition of MMP-9 in medium from cells exposed to *B. cenocepacia* completely reversed the delay in wound repair. These data suggest that MMP-9 plays a role in the reduced epithelial repair observed in response to *B. cenocepacia* infection and that its activation following *B. cenocepacia* infection contributes to the pathogenesis of this virulent pathogen.

microbial pathogenesis; *Burkholderia cepacia* complex; matrix metalloproteinases; epithelial wound repair

BURKHOLDERIA CEPACIA COMPLEX is an opportunistic bacterial pathogen that chronically colonizes the lungs of patients with either cystic fibrosis (CF) or chronic granulomatous disease (CGD) and causes life-threatening infections in both populations (31, 32, 44). It is a group of 17 related species that are inherently resistant to antibiotic treatment (48, 49). CF is a genetically inherited disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR), a chloride channel. A common feature of CF is chronic opportunistic respiratory infections that patients acquire throughout their lives, resulting in a continuous cycle of infection and inflammation that gradually damages the lungs, thereby reducing lung function. Although *Pseudomonas aeruginosa* is the most commonly ac-

quired pathogen in CF patients, other pathogens, including *B. cepacia* complex, can also colonize and contribute to lung inflammation and damage (31). Although strict segregation measures have reduced the incidence of *B. cepacia* complex infection, two species, *B. cenocepacia* and *B. multivorans*, can be acquired from the environment, and patient-to-patient transmissibility is still an issue (3, 4, 31, 37). Of these, *B. cenocepacia* is particularly virulent. Jones et al. (22) have demonstrated that CF patients with *B. cenocepacia* infection had a shorter survival time than those with *P. aeruginosa* or *B. multivorans* infection.

Matrix metalloproteinases (MMPs) are a group of related but distinct enzymes that regulate various inflammatory and repair processes. They are either secreted or anchored to the cell surface and therefore their activity is directed against membrane proteins or extracellular proteins, including inflammatory mediators (40). Many MMPs, such as collagenase-1 and -3 (MMP-1 and MMP-13, respectively), stromelysins-1, -2, -3 (MMPs-3, -10, and -11), and gelatinase-B (MMP-9), are not expressed in normal healthy resting tissues or are only barely detectable (39). Matrilysin (MMP-7) is expressed on the wounded edge or within injured epithelial cells of the lung and is required for reepithelialization, but it is also expressed in the noninjured and noninflamed epithelium in adult tissues (40). In vitro and ex vivo studies have shown that MMP-9 also plays a role in reepithelialization in the lung (29). Dysregulation of metalloproteinases has also been associated with inflammation and tissue damage (11, 39, 40). MMPs are also upregulated in the CF lung (23, 30).

The induction and activation of MMP-7 in mucosal epithelia is highly sensitive to the presence of virulent bacteria, such as *P. aeruginosa*, but not *Staphylococcus aureus*, *Haemophilus influenzae*, or *Klebsiella pneumoniae*. The induced expression of MMP-7 in Calu-3 cells was attributed to *P. aeruginosa* flagellin (30). *P. aeruginosa* virulence factors, most likely elastase, have also been shown to overactivate host MMP-2 and prevent the formation of tight junctions during repair (7). MMP-7 has been shown to degrade E-cadherin (35), whereas MMP-9 plays a role in claudin turnover (20, 50), suggesting that host MMPs could play a role in disruption of tight junction integrity following infection because they are regulated by cytokines, including IL-13 (28). MMPs have been shown to be responsible for early dissemination of *Mycobacterium tuberculosis* in mice, and MMP-9 has been shown to play a causative role in granuloma formation during *M. tuberculosis* infection (45). The role of MMPs in the pathogenesis of the CF pathogen *B. cepacia* complex has not been explored to date, and therefore we investigated whether MMP expression plays

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a role in *B. cepacia* complex host-cell response in lung epithelia.

MATERIALS AND METHODS

Materials. All materials were purchased from Sigma, with the following exceptions: TRIzol, Superscript III, *Taq* polymerase and Ultrosor G (Invitrogen), Vitrogen (Nutacon); MMP-9 inhibitor I (Merck Chemicals), SYBR green PCR Mastermix (Roche), and Fluorokine E human MMP-9 fluorescent assay kit (R&D Systems).

Cell culture. Both human lung epithelial cell lines, 16HBE14o– and CFBE41o–, were a generous gift from Dr. Dieter Gruenert, University of California, San Francisco. The 16HBE14o– cells (passage 2.85 to ~2.105) were maintained on Vitrogen/fibronectin-coated flasks in minimum essential medium (MEM) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, and 10% FBS. (6) CFBE41o– cells (passage 4.85 to 4.105) were derived from a CF patient that was homozygous negative for the most common CFTR mutation, $\Delta F508$, and were also maintained on coated flasks in the same medium, supplemented with 1% nonessential amino acids (15). Confluent monolayers of 16HBE14o– cells were obtained by seeding at 2×10^5 cells/ml in 1 ml in 24-well plates for 48 h prior to zymography. CFBE41o– cells were seeded at 1×10^5 cells/ml in 1 ml. Medium was changed at 24 h and replaced with complete medium without antibiotics. A confluent monolayer was present at 48 h. Polarized monolayers were also prepared by seeding 16HBE 14o– or CFBE41o– cells at 7×10^5 /cm on coated Transwell polycarbonate filters. The cells were incubated overnight before removal of the apical medium, grown for 6 days with an air-liquid interface, and fed basolaterally on alternate days, with medium in which the FBS was replaced with 2% Ultrosor G (27).

Bacterial infection of lung epithelial cells. The two *B. cepacia* complex strains used in this study, *Burkholderia cenocepacia* strain (BC7) and *Burkholderia multivorans* strain (LMG13010), were both obtained from the Belgian Co-ordinated Collections of Micro-organisms, University of Ghent, Belgium and routinely grown on *B. cepacia*-specific agar (18). *P. aeruginosa* (PAO1) was used as a reference strain and was obtained from American Type Culture Collection. Overnight bacterial cultures were grown in Luria-Bertani broth to an OD_{600nm} of 0.6 and then diluted to a given concentration. A suspension of 2×10^7 CFU/ml was made up in MEM and added to the confluent cultures of lung epithelial cells at a multiplicity of infection of 50:1. The plate was centrifuged to settle bacteria for 5 min at 700 g and incubated at 37°C and 5% CO₂ for the appropriate time.

Isolation of RNA and cDNA biosynthesis. Following bacterial infections, medium was removed, cells were directly lysed in TRIzol, and total RNA was isolated according to manufacturer's instructions. An aliquot of 1 μ g of RNA (A260/280 >1.7) was used to synthesize by cDNA using Superscript III according to the manufacturer's instructions.

Expression of MMP genes. Initial studies to explore the effects of bacteria on transcription of metalloproteinase genes in CFBE41o– and 16HBE14o– cells were carried out semiquantitatively over a 24-h period. A selection of MMP genes, MMP-2, 3, 7, and 9 cDNAs (1 μ l), was amplified in a 50- μ l reaction by reverse transcriptase PCR (RT-PCR). The primers used were as follows: GAPDH forward 5'-CCCCTTCATACCCTCACGTA-3', reverse 5'-ACAAGCTCCCGTTCTCAG-3'; MMP-2 forward 5'-AGGGCACATCCATGACAGC-3', reverse 5'-CCTTCTGAGTTCACCAAC-3'; MMP-3 forward 5'-TCCGCCTGTCTCAAGATGAT-3', reverse 5'-CCA-AAAGTGCCTGTCTTTA-3'; MMP-7 forward 5'-TGTATGGG-GAACTGCTGACA-3', reverse 5'-AGACTGCTACCATCCGTCCA-3'; MMP-9 forward 5'-CAGTCCACCCTTGCTCTT-3', reverse 5'-AGGGACAGTTGCTTCTGGAG-3'.

PCR was carried out for 35 cycles of denaturation (30 s, 95°C), annealing (30 s, 58°C), and elongation (45 s, 72°C).

Real-time PCR was carried out by using the same primers as those for regular PCR together with FastStart Universal SYBR Mastermix (Roche) according to manufacturer's instructions at concentrations of 380 nM for MMP primers and 190 nM for GAPDH. The samples were amplified in triplicate on a 7300 Real-Time PCR System (Applied Biosystems) as follows: 40 cycles of denaturation (15 s, 95°C) and annealing/elongation (1 min, 66°C) were carried out. The threshold cycles (Ct) for each primer set were obtained from triplicate samples and averaged. The delta Ct was the calculated difference between the average Ct for the target gene and the average Ct for GAPDH as a control for total starting RNA quantity. The delta-delta Ct method of calculation was then used to assess fold change in gene expression relative to the GAPDH gene.

Gelatin zymography. Gelatin zymography experiments were performed on conditioned media harvested from control untreated cells, from infected lung epithelial cells as described above, or from 4-aminophenylmercuric acetate (APMA)-treated supernatants to assess the level of MMP-2 and MMP-9 enzyme secreted. Conditioned media were harvested from cells at appropriate time points posttreatment and stored at –80°C until use. A volume of 20 μ l of cell-conditioned medium was mixed with 5 $\times$ nonreducing Laemlli buffer (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) and loaded onto a 10% SDS-PAGE gel containing 0.1% gelatin. Samples were electrophoresed at 140 V for 90 min and then the gels were rinsed in distilled water and washed in two changes of 2.5% Triton-X 100 to remove SDS for 1 h. Gels were then incubated for 24 h in digestion buffer (50 mM Tris pH 8, 0.5 mM CaCl₂, 5 μ M ZnCl₂) at 37°C. Bands were visualized by Coomassie blue staining for 1 h followed by destaining in methanol-acetic acid-water (1:1:8) for 10 min. Gelatinase activity corresponded to areas of clearance of the gelatin from the gels. The images were captured on a SynGene G:box system (SynGene, Cambridge, UK) and the clear bands were analyzed by SynGene Gene Tools software. The activity of all samples were normalized by setting the activity of the relevant control to one, and the data were presented as the fold change in activity.

Determination of active and total MMP-9 secretion. The amount of total and active MMP-9 secreted was determined by using a Fluorokine E human MMP-9 fluorescent assay kit according to the manufacturer's instructions. Conditioned cell medium (diluted 1:10) or MMP-9 standard (200 μ l per well) were added to individual wells precoated with MMP-9-specific capture antibody, incubated for 2 h, and washed four times. Total MMP-9 was determined in the presence of APMA, whereas no activator was added to wells to determine active MMP-9. The fluorogenic substrate was added for a further 18 h at 37°C and fluorescence generated was measured on a Thermo Fisher Varioskan Flash spectrophotometer. The MMP-9 activity was determined by comparison with a standard curve of the log of the active MMP-9 concentration against the log of the relative fluorescence units, determined by regression analysis.

Transepithelial resistance. For tight membrane integrity experiments, 16HBE14o– or CFBE41o– cells were seeded at 7×10^5 cells/ml on Transwell filters that were precoated with Vitrogen. The integrity of the polarized monolayer was determined by measuring transepithelial electrical resistance (TER) using an epithelial volt-ohmmeter (World Precision Instruments) and corrected for the resistance value of the cell-free filter. Only polarized monolayers with a TER of >200 Ω /cm² over that of cell-free filters were used. TER values were 510 ± 100 and 484 ± 56 Ω /cm² for CFBE41o– cells and 16HBE14o– cells, respectively. Cells were pretreated with GM6001 (10 μ M) for 30 min prior to, and during, the period of infection with bacteria; the TER was measured at regular intervals and compared with parallel wells exposed to bacterial alone or inhibitor alone.

Wound repair assays. Confluent monolayers of either 16HBE14o– or CFBE41o– were cultured as stated above with and without bacterial infection. Medium was replaced with MEM alone for 24 h. Conditioned media from uninfected cells were harvested and treated with 1 mM APMA (Sigma) or DMSO (solvent control) for 16 h at

37°C with gentle agitation to activate the MMPs released. Conditioned media either from infected cells or following APMA treatment were either used immediately or stored at -80°C for use in zymography. To confirm the role of MMP-9, conditioned media from *B. cenocepacia* strain BC7-infected cells were also preincubated with 20 nM MMP-9 inhibitor 1 for 30 min prior to application. Confluent monolayers were also cultured for wounding. A wound was inflicted by scraping with a sterile pipette tip across the entire monolayer as described by Olsen et al. (38). Images of the wounds were captured via a microscope video eyepiece camera USB at time intervals, and the monolayers were replaced in the incubator at 37°C and 5% CO₂ between imaging. The distance between the cell migration fronts was measured by using an ocular measurement graticule at five predetermined points along the length of the scrape. The values are presented as repair of the wound in micrometers.

Statistical analysis. The data changes in transepithelial resistance and wound healing experiments for individual strains were compared with one-way ANOVA with Tukey's posttest for pairwise comparisons (Minitab). Two-tailed Student's *t*-test (assuming unequal variances) was used to compare the real-time PCR data of individual strains relative to the untreated negative control, densitometry analysis of zymograms, and fluorescence analysis of MMP-9 expression or activation relative to control cells. A *P* value of <0.05 was deemed to be significant in each case.

RESULTS

Semiquantitative analysis of MMP gene expression indicated upregulation of MMP-2 and MMP-9 mRNA. To enable us to focus on the MMP genes of interest, the expression of MMP-2, 3, 7, and 9 genes was investigated in two independent cell lines in response to *B. cepacia* complex infection or *P. aeruginosa* strain PAO1 infection over a 24-h period by semiquantitative PCR, initially. Both the 16HBE14o- and CFBE41o- cell lines expressed all of the examined genes, albeit MMP-3 mRNA was expressed at very low levels and was barely detectable in CFBE41o- cells (Fig. 1, A and B). Semiquantitative reverse transcriptase PCR suggested an upregulation of MMP-2 and MMP-9 mRNA at earlier time points in both of these cell lines, in response to infection with *B. cenocepacia* strain BC7, *B. multivorans* strain LMG13010, and *P. aeruginosa* strain PAO1. To confirm the upregulation of these genes as an early response to infection, the expression of these two genes was therefore further examined by real-time PCR at the earliest time point of 1 h only. Although no clear upregulation of MMP-7 gene expression was observed by PCR when normalized with GAPDH, previous studies on Calu-3 cells demonstrated MMP-7 upregulation following exposure to *P. aeruginosa* strain PAO1 (24); thus this was also included in further studies.

MMP-2 and MMP-9 mRNA upregulated in lung epithelial cells in response to *B. cepacia* complex infection. The apparent upregulation of MMP-2 and MMP-9 genes in the 16HBE14o- and CFBE41o- cell lines in response to exposure to *B. cenocepacia* strain BC7, *B. multivorans* strain LMG13010, or *P. aeruginosa* strain PAO1 was quantitatively examined by real-time PCR at the early time point of 1 h (Fig. 2). MMP-2 gene expression was increased within 1 h in both cell lines in response to *B. cepacia* complex infection. The *B. cenocepacia* strain BC7 upregulated MMP-2 gene expression in both 16HBE14o- cells (3-fold, *P* < 0.05) and CFBE41o- cells (4-fold, *P* < 0.01), thus having a somewhat greater effect on CFBE41o- cells than on 16HBE14o- cells. Although *B. multivorans*

Fig. 1. Upregulation of matrix metalloproteinase (MMP)-2 and MMP-9 genes in response to *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* infection by semiquantitative RT-PCR. Confluent monolayers of 16HBE14o- cells (A) and CFBE41o- cells (B) were exposed to bacteria (multiplicity of infection 50:1) for 1, 2, 4, or 24 h and the expression of MMP-2, MMP-3, MMP-7, and MMP-9 mRNA was determined by semiquantitative RT-PCR. Upregulation of both MMP-2 and MMP-9 genes is apparent at 2 h in response to *B. cepacia* complex. No clear upregulation of the MMP-7 gene was observed. Images are representative gels from 2 independent experiments. neg, Negative; pos, positive; CTRL, control cells treated with bacteria-free medium; BC7, *B. cenocepacia* strain; LMG13010, *B. multivorans* strain; PAO1, *P. aeruginosa* strain.

strain LMG13010 also upregulated the MMP-2 gene in 16HBE14o- cells by 4.6-fold (*P* < 0.01), there was no statistically significant upregulation in CFBE41o- cells, relative to controls. There was a 2- to 3-fold increase in MMP-7 gene expression in response to both *B. cepacia* complex strains and to PAO1, in 16HBE14o- cells only. In contrast, there was no MMP-7 response in CFBE41o- cells within 1 h of infection with any of the three strains examined. *B. cenocepacia* BC7 infection also resulted in an upregulation of the MMP-9 gene in both cell lines; however, no alteration in MMP-9 gene expression was observed in response to *B. multivorans* strain LMG13010 or to *P. aeruginosa* in either cell line. Interestingly, there was no upregulation of any of the three genes examined in response to *P. aeruginosa* (PAO1) in CFBE41o- cells was observed at the early 1 h time point, but increased expression of both MMP-2 mRNA (6-fold) and MMP-7 mRNA (2.8-fold) was observed in 16HBE14o- cells (*P* < 0.01).

MMP-9 is activated following *B. cenocepacia* infection. The secreted levels of MMP-2 and MMP-9 were examined by gelatin zymography of conditioned media at 24 and 48 h (Fig. 3). There was a clear activation of MMP-9 in the presence of *B. cenocepacia* strain BC7 over 48 h, in the conditioned medium from both the 16HBE14o- cells (3-fold) and CFBE41o- cells (2-fold) (Fig. 3A, *P* < 0.05). This activation was confirmed by MMP-9-specific fluorometric assay (Fig. 3B), which indicated that a 4-fold and 3.8-fold increase in MMP-9 activity was

Fig. 2. Expression of MMP genes in response to *B. cepacia* complex and *P. aeruginosa* infection by real-time PCR. 16HBE14o– cells (A) or CFBE41o– cells (B) were infected with *B. cenocepacia* strain BC7, *B. multivorans* strain LMG13010, or *P. aeruginosa* strain PAO1 for 1 h. MMP-2, MMP-7, and MMP-9 mRNA expression was determined by real-time quantitative PCR relative to GAPDH mRNA. A: upregulation of MMP-2 and MMP-7 mRNA was observed in response to infection with all 3 species, whereas MMP-9 upregulation was only observed in response to *B. cenocepacia*. B: MMP-2 and MMP-9 were both upregulated in response to *B. cenocepacia* infection in CFBE41o– cells. Bars represent the average of 3 separate experiments + SE. *Significant difference in expression from uninfected cells ($P < 0.05$). **Significant difference in expression from uninfected cells ($P < 0.01$).

achieved in 16HBE14o– and CFBE41o– cells, respectively ($P < 0.05$). Zymography showed an upregulation of MMP-9 by 1.7-fold 48 h following infection of 16HBE14o– cells with *B. multivorans* strain LMG13010 (Fig. 3A), but which was not apparent in CFBE41o– cells. Despite the lack of apparent activation of MMP-9 in CFBE41o– cells by zymography, quantitation of MMP-9 activity demonstrated a 2.0-fold and 2.4-fold increase in MMP-9 activity in 16HBE14o– and CFBE41o– cells ($P < 0.05$) following 48-h exposure to *B. multivorans* strain LMG13010. The difference in zymography and fluorometric assay may be due to the fact that zymography underestimated the relatively weak MMP-9 activation by *B. multivorans* LMG13010 in CFBE cells. No activation of pro-MMP-2 was observed for either *B. cepacia* complex strain. In comparison, much more MMP activation was evident following infection of 16HBE41o– cells with PAO1 (Fig. 3C). When the conditioned medium from PAO1 was applied without dilution to zymography gels, extensive gelatinase activation was observed as seen by a large smear of gelatin degradation (Fig. 3C), and it was difficult to determine which gelatinase was activated. However, dilution of the conditioned medium to 1:100 and below showed that the predominant MMP activated following PAO1 infection was MMP-2 in 16HBE14o– cells. Comparable studies indicated that CFBE41o– cells also secreted high levels of MMP-2 in response to PAO1 (Fig. 3D).

Secretion of active MMP-9 is observed basolaterally and not apically in polarized lung epithelial cells. To explore the activation in polarized cells, 16HBE14o– or CFBE41o– cells were cultured on semipermeable Transwell filters. These were infected apically either with *B. cenocepacia* strain BC7 or with *B. multivorans* strain LMG13010, and the filtered conditioned medium from the apical and basolateral compartments was analyzed by zymography. No alteration in MMP expression or activation was observed in the apical supernatants of polarized 16HBE14o– cells or CFBE41o– cells (data not shown). In contrast, basolateral levels of activated MMP-9 were increased following apical infection with *B. cenocepacia* strain BC7, which was evident up in the basolateral chamber to 72 h postinfection in both cell lines (Fig. 4, A and B). Exposure to *B. multivorans* strain LMG13010 did not have any apparent effect on MMP secretion of the lung cell lines, either basolaterally or apically on zymograms.

MMPs are not involved in *B. cepacia* complex-related tight junction disruption. We and others have previously shown that coinfection of lung epithelial cells with a series of *B. cepacia* complex isolates resulted in disruption of epithelial integrity, as evidenced by a drop in transepithelial resistance and a reduction in zonula occludens-1 (ZO-1) expression (9, 25). To explore whether activation of MMPs were responsible for this effect, we preexposed polarized CFBE41o– cells to the MMP broad-spectrum inhibitor, GM6001, prior to, and during, infection with either *B. multivorans* strain LMG13010, or the *B. cenocepacia* strain BC7. The concentration used (10 μ M) is recommended for inhibition of a range of MMPs. Exposure of 16HBE14o– or CFBE41o– cells to these strains resulted in a drop in TER within 4 h (Fig. 4C), as we have previously demonstrated for Calu-3 cells. However, inhibition of MMP activity by incubation with GM6001 prior to and during infection with *B. cepacia* complex strains, did not prevent the tight junction disruption. The drop in TER was comparable with that of the individual strains without inhibitor in both cell lines. This clearly indicates that MMPs are unlikely to have any role in the observed tight junction disruption of *B. cepacia* complex-infected polarized lung epithelial cells.

MMP-9 slows down wound repair in confluent lung epithelia. To explore what effect *B. cepacia* complex activation of MMP-9 has on pathogenesis, the effect of activated MMPs on the confluent lung epithelial cells was investigated by use of a wound healing model. Both 16HBE14o– (Fig. 5, A and C) and CFBE41o– (Fig. 5, B and D) cells rapidly migrated over the wound in untreated control conditioned media, albeit the rate of migration in CFBE41o– cells was slower than that of 16HBE14o– cells ($P < 0.05$ at later time points 4 to 8 h). Conditioned medium from *B. cenocepacia* strain BC7-infected 16HBE14o– or CFBE41o– cells reduced the rate of wound repair in both 16HBE14o– ($P < 0.001$) and CFBE41o– cells ($P < 0.05$), respectively (Fig. 5). In addition, conditioned medium from 16HBE14o– cells infected with *B. multivorans* strain LMG13010 also significantly reduced the rate of wound repair in 16HBE14o– cells ($P < 0.05$), but not in CFBE41o– cells. The apparent difference in the effect of *B. multivorans*, which has a weaker effect on MMP-9 activity, on wound repair may be linked to the slower rate of repair in CFBE41o– cells. In contrast, application of conditioned medium from PAO1-infected cells (diluted 1:500 to normalize for active gelatinase)

Fig. 3. *B. cenocepacia* infection increases MMP-9 activity in both cystic fibrosis (CF) and non-CF lung epithelial cells. 16HBE14o- or CFBE41o- cells were treated with bacteria-free medium (CTRL), *B. cenocepacia* strain BC7, *B. multivorans* strain LMG13010, or *P. aeruginosa* strain PAO1, for 24 and 48 h. A: representative gelatin zymograms of conditioned media from *B. cepacia* complex-infected cells and corresponding MMP-9 densitometry. Activation of MMP-9 but not MMP-2 was observed compared with control. Densitometry data (solid bars, 16HBE14o-; shaded bars, CFBE 41o-) represent the average of 3 separate experiments and are graphed $\pm$ SE. Act, activated. *Significant difference in expression from uninfected cells ($P < 0.05$). B: MMP-9 activity in 16HBE14o- (solid bars) and CFBE41o- (shaded bars) 48 h after exposure to *B. cepacia* complex or *P. aeruginosa* as determined by fluorometric assay (* $P < 0.05$, ** $P < 0.005$). Data represent means of 3 independent experiments $\pm$ SD. C and D: representative zymogram showing that 16HBE14o- cells and CFBE41o- cells treated with *P. aeruginosa* strain PAO1 secreted high levels of MMP activity, which required dilution to below 1:100 to observe that the predominant activated MMP was MMP-2. Representative zymograms of 3 independent experiments are shown.

did not show any effect on the rate of wound repair in either cell line under the conditions used (Fig. 5).

Specific inhibition of MMP-9 activity was achieved by incubating conditioned cell supernatants with MMP-9 specific inhibitor (20 nM), which is four times the reported IC_{50} value for MMP-9 inhibition but considerably less than the IC_{50} values for its two secondary targets MMP-13 or MMP-1 (IC_{50} values of 113 nM and 1.05 μ M, respectively). This concentration was chosen to ensure complete inhibition of MMP-9 activity. Incubation of conditioned medium from *B. cenocepacia*-treated cells with this inhibitor reduced MMP-9 activity from 4.5 ng/ml (16HBE14o- treated) and 3.3 ng/ml (CFBE41o- treated) to undetectable levels in both cases as determined by MMP-9 fluorometric assay. When MMP-9 activity was specifically suppressed in conditioned me-

dium from *B. cenocepacia*-infected cells, the delay in wound repair that had been observed was reversed and the wound repair was comparable with that of controls for both cell lines (Fig. 6, $P < 0.05$). For further confirmation of the role of MMP-9, conditioned media from control monolayers were also treated with 1 mM APMA, a potent activator of MMPs. Zymography verified that there was clear activation of MMP-9 and to a lesser extent MMP-2 in these conditioned media following APMA treatment (Fig. 7A). APMA-treated conditioned media applied to wounded cells also inhibited cell migration and wound repair in both cell lines (Fig. 7, B and C; $P < 0.01$, in both cases), again indicating that the presence of activated MMP-9 prevented epithelial wound repair, rather than an alternative bacterial compo-

Fig. 4. Basolateral secretion of active MMP-9 from polarized lung epithelial cells and lack of effect of MMPs on tight junction integrity. **A**: representative gelatin zymograms of conditioned media from basolateral chamber of polarized 16HBE14o- and CFBE41o- cells 48 and 72 h after infection with *B. multivorans* strain LMG13010 or *B. cenocepacia* strain BC7. **B**: corresponding densitometry. *Significant change in MMP-9 activity ($P < 0.05$). **C**: inhibition of MMP activity does not impair *B. cepacia* complex-induced opening of tight junctions. Polarized 16HBE14o- and CFBE41o- cells were treated with GM6001 before and during infection with *B. multivorans* LMG13010 or *B. cenocepacia* BC7 and tight junction integrity was monitored by transepithelial electrical resistance (TER), demonstrating that MMP inhibition does not alter tight junction integrity. Data are presented as % change in TER relative to medium treated control $\pm$ SE from 3 independent experiments.

DISCUSSION

B. cenocepacia has been established as the most virulent of all the *B. cepacia* complex species identified (31, 32), being more frequently associated with cepacia syndrome and patient deaths relative to other species in the complex (22). *B. cenocepacia* also elicits a potent proinflammatory response. Studies have shown that *B. cepacia* complex LPS can elicit up to 10 times more TNF- α and promote more neutrophil burst compared with LPS from other CF pathogens including *P. aeruginosa* (19, 36, 52). MMPs are widely implicated in airway damage in chronic pulmonary diseases including CF; however, the role of MMPs in the pathogenesis of *B. cepacia* complex infection has not been examined to date.

The potential for *B. cepacia* complex infection to affect MMP expression was examined by semiquantitative PCR initially to screen for the upregulation over a wider time frame and include four MMPs. Upregulation of MMP-2 and MMP-9

genes was evident following infection with both *B. cepacia* complex strains and with *P. aeruginosa*; therefore quantitative analysis was carried out on both of these genes together with MMP-7, which has previously been shown to be upregulated in response to *P. aeruginosa*. To focus on the early effects of infection, real-time PCR analysis was carried out on cells at 1 h postexposure to bacteria. This confirmed the upregulation of the MMP-2, -7 and -9 genes in response to BC7 infection in 16HBE14o- cells. MMP-2 and MMP-9 were also upregulated in the CFBE41o- cells within 1 h. Interestingly, the CFTR-expressing 16HBE14o- cells were generally more responsive to infection from all three species than the CFBE41o- cells. In particular, MMP-7 gene upregulation was not observed in response to infection with any of the bacteria examined in the CFBE41o- cells within 1 h. MMP-7 is widely reported to be upregulated in response to *P. aeruginosa* expression; however, all the studies have been carried out in CFTR-expressing cells

Fig. 5. Effects of bacterial infection on wound repair in lung epithelial cells. Confluent lung epithelial cells were grown for 24 h and subjected to scrape wounding and allowed to recover over time at 37°C and 5% CO₂ in the presence of conditioned media from control cells or from cells infected with *B. cenocepacia* (BC7), *B. multivorans* (LMG13010), or *P. aeruginosa* (PAO1). A and B: representative phase-contrast images of 16HBE14o- (A) and CFBE41o- (B) cells were captured at 0, 2, 4, 6, and 8 h postwounding to illustrate recovery. A delay in recovery was evident in cultures exposed to conditioned media from *B. cenocepacia* BC7-infected cells. C: migration of 16HBE14o- cells over the wound was measured at hourly intervals and plotted against time. D: migration of CFBE41o- cells over the wound was measured at hourly intervals and plotted against time. Data in C and D are graphed $\pm$ SE and are representative of 3 independent experiments in each case. Significant difference wound closure following exposure to conditioned medium from infected cells relative to uninfected cells: ** $P < 0.01$, * $P < 0.05$.

Fig. 6. Inhibition of MMP-9 activity in conditioned medium from *B. cenocepacia*-infected cells reverses the delay in wound repair. Confluent lung epithelial cells were grown for 24 h and subjected to scrape wounding and allowed to recover over time at 37°C and 5% CO₂ in the presence of conditioned media from control cells or from cells infected with *B. cenocepacia* (BC7) in the absence or presence of MMP-9 inhibitor I. **A** and **B**: zymography illustrating the inhibition of MMP-9 activation in 16HBE14o- cells (**A**) and CFBE41o- cells (**B**) in the presence of MMP-9 inhibitor I. **C** and **D**: representative phase-contrast images of 16HBE14o- (**A**) and CFBE41o- (**B**) cells were captured at 0, 4, and 8 h postwounding to illustrate recovery. The delay in recovery evident in cultures exposed to conditioned media from *B. cenocepacia* strain BC7-infected cells was reversed in cultures exposed to conditioned media from *B. cenocepacia* strain BC7-infected cells in the presence of MMP-9 inhibitor I. **E**: migration of 16HBE14o- cells over the wound was measured and plotted against time. **F**: migration of CFBE41o- cells over the wound was measured and plotted against time. Data in **E** and **F** are graphed $\pm$ SE and are representative of 3 independent experiments in each case. Significant difference wound closure following exposure to conditioned medium from infected cells relative to uninfected cells: * $P < 0.05$.

or tissues (24, 30). To the best of our knowledge, no study has been carried out to examine the effect of *P. aeruginosa* on MMP-7 expression in cells or tissues with a CF phenotype. It is possible that the MMP-7 upregulation in response to *P. aeruginosa* is CFTR dependent or is slower in cells with a CF phenotype and so should be examined further.

Because of the consistent early upregulation of MMP-2 and MMP-9 genes in both cell lines, with and without a CF phenotype, we focused on the gelatinases for further study. Clear activation of MMP-9 but not MMP-2 in response to *B. cenocepacia* exposure was evident in gelatinase zymography. In addition, we have established that the activation of MMP-9 is polarized and is exclusive to the basolateral side of CFBE41o- monolayers. This is significant because basolateral activation will have most impact on lung tissue following infection, having greater access to the basement membrane components and immune cells. Although the gelatinase activity following *B. cenocepacia* infection was much less than that following *P. aeruginosa* infection, the predominant gelatinase activated following *P. aeruginosa* infection was MMP-2. The activation of MMP-2 in response to *P. aeruginosa* strain PAO1 exposure has previously been shown, although exposure of human airway epithelial cells to supernatants from *P. aeruginosa* strain PAO1 (1% dilution) did not result in any change in the expression of MMP-9 or MMP-2 genes (7). Our observed

upregulation of MMP-2 gene expression is probably due to the fact that we used whole cells, rather than supernatants.

We have previously shown that when *B. cenocepacia* strain BC7 or *B. multivorans* strain LMG13010 was applied to polarized epithelial cells, the tight junctions were disrupted with concomitant degradation of the tight junction protein ZO-1 (9). Given previous reports relating MMP-9 having activity on the epithelial tight junction protein claudin 1 and subsequent effects on tight junction integrity, we therefore wanted to examine whether MMPs were involved in this previously observed disruption of TER following *B. cepacia* complex infection. TER is a widely used marker of tight junction integrity, one of several indicators of epithelial barrier disruption. Exposure of polarized 16HBE14o- or CFBE41o- cells to either *B. cenocepacia* strain BC7 or *B. multivorans* strain LMG13010, in the presence of GM6001, a broad-spectrum inhibitor of MMPs-1, -2, -3, -7 and -9 (1, 14), did not have any effect on tight junction integrity. This suggests that the observed MMP-9 activation following *B. cepacia* complex infection, or any other inhibited MMPs, did not play a role in this response. The ability for MMPs including MMP-7 and MMP-9 to degrade tight junction and adherens junction proteins has been shown previously. Asahi et al. (2) have suggested that the tight junction protein ZO-1 is a substrate for MMP-9 in a blood-brain barrier model. They showed that

Fig. 7. 4-Aminophenylmercuric acetate (APMA) activation of conditioned medium from uninfected cells results in MMP-9 activation and consequent delay in wound repair. Confluent lung epithelial cells were grown for 24 h and subjected to scrape wounding and allowed to recover over time at 37°C and 5% CO₂ in the presence of conditioned media from control cells with or without APMA pretreatment. **A**: zymogram of conditioned media from confluent epithelial cells exposed to control medium only (MEM), following exposure to the vehicle only (DMSO) or exposed to APMA. **B**: representative phase-contrast images of 16HBE14o- and CFBE41o- cells were captured at 0, 2, 4, 6, and 8 h postwounding to illustrate recovery. A delay in recovery was evident in both 16HBE14o- and CFBE41o- cultures exposed to conditioned media treated with APMA. **C**: migration of 16HBE14o- cells over the wound was measured at hourly intervals and plotted against time. **D**: migration of CFBE41o- cells over the wound was measured at hourly intervals and plotted against time for control cells and those exposed to APMA (or DMSO only)-treated conditioned medium. Data in **C** and **D** are graphed $\pm$ SE and are representative of 3 independent experiments in each case. *Significant difference wound closure following exposure to conditioned medium following APMA treatment relative to control conditioned media ($P < 0.01$).

ZO-1 degradation, but not occludin degradation, during cerebral ischemia was reduced in MMP-9^{-/-} mice relative to wild type. However, this is a complex model, and many other explanations for this exist. Harkness et al. (17) used confocal microscopy to demonstrate that MMP-9 caused subtle changes in the expression of ZO-1 in the rat endothelium of the central nervous system. In contrast, Ichiyasu et al. (20) have demonstrated that the absence of MMP-9 in knockout mice increased levels of the tight junction regulatory protein, claudin-1, with a concomitant drop in transepithelial migration of dendritic cells. The tight junctions are very tightly regulated, and the degradation of one tight junction protein in isolation cannot be conclusive regarding the integrity of the tight junction complex

as a whole. An important feature of tight junctional regulation is that other junctional proteins can compensate for the degradation or lack of a single protein (33). It is clear from our data that MMPs do not contribute to the disruption of tight junctions associated with *B. cepacia* complex infection.

A delay in wound repair was observed in cells treated with conditioned medium from both cell lines infected with *B. cenocepacia* and with conditioned medium from *B. multivorans*-infected 16HBE41o- cells. The finding that specific inhibition of MMP-9 completely reversed this delay in wound repair in both cell lines following *B. cenocepacia* infection strongly suggests that MMP-9 is responsible for the delay in wound repair following *B. cenocepacia* infection. The lack of

a delay in wound repair in CFBE41o– cells exposed to conditioned medium from *B. multivorans* exposed cells, despite a twofold increase in MMP-9 activity, was unexpected but may relate to the overall slower repair in this particular cell line. A delay in wound repair may have become apparent over a longer period of time. MMPs have been associated both with tissue destruction and with tissue repair (38–40). Olsen et al. (38) have recently demonstrated that exposure of 16HBE14o– cells to arsenic upregulated both MMP-9 expression and activity. No alteration in MMP-2 activity was observed. Consistent with our data, the activation of MMP-9 correlated with an impairment of wound repair in confluent monolayers of lung epithelial cells. In addition to delayed wound repair, it is likely that MMP-9 activation may further exacerbate the host response to *B. cepacia* complex infection. It has been shown that high levels of MMP-9 promote infiltration of inflammatory cells and exacerbate the symptoms of asthma (26). In addition, MMP-9 upregulation and activation is expressed during various stages of tuberculosis infection including the tuberculous granuloma, where it is associated with tissue destruction (41). MMP-9 expression was also induced in vivo in mice infected with another respiratory pathogen, *Francisella tularensis*, and was demonstrated to play a clear role in pathogenesis of this infection, in terms of histopathology, neutrophil recruitment, and proinflammatory response (34). Exposure of 16HBE14o– cells to supernatants or mucopurulent material from CF airways upregulated a number of MMP genes, including MMP-9 but not MMP-2 (42). This effect was downregulated in response to azithromycin. Further roles in proinflammatory activity of MMP-9 have been demonstrated: MMP-9 processing of the proinflammatory cytokine IL-8 has been shown to increase its chemotactic activity (46, 47) and MMP-9 has been shown to activate IL-1 β precursor, another mediator of the inflammatory response (21). Gaggari et al. (12) have shown that MMP-9 is involved in a proteolytic cascade that generates the neutrophil chemoattractant proline-glycine-proline (PGP) from collagen, and this peptide is substantially upregulated in CF sputum samples. The further upregulation of MMP-9 in *B. cenocepacia*-colonized patients is likely to further contribute to elevated PGP levels and the subsequent chronic inflammatory response.

MMP-9 levels have also been implicated with poor survival in *P. aeruginosa* pneumonia non-CF patients (10). Bronchoalveolar lavage (BAL) fluid taken from surviving ventilator-associated pneumonia patients had a lower ratio of MMP-9 to TIMP relative to those that died. In addition, MMP-9 levels progressively declined in patients' BAL in response to antibiotic therapy (10). MMP-9 activity was also elevated in sputa from lung transplant patients, and those with the highest levels of MMP-9 activity were associated with patients with chronic lung transplant rejection (5). Transplanted CF patients that have been colonized with *B. cenocepacia* have poorer prognosis compared with patients colonized with other members of the complex (8). The activation of MMP-9 in response to *B. cenocepacia* infection together with its likely role in lung damage, shown here, may provide one mechanism whereby *B. cenocepacia*-colonized patients have poorer outcomes on transplant than patients colonized by *B. cepacia* complex species other than *B. cenocepacia* and is worthy of further study.

The activation of MMP-9, together with the observed delay in wound repair in CFTR-expressing cells following infection with *B. multivorans* strain LMG13010, may have relevance for patients with CGD. *B. multivorans* is the most frequently isolated species among CGD patients (13). Furthermore, it was recently shown that *B. multivorans* associated more with cells from CGD patients than from healthy donors, and infection was associated greater levels of proinflammatory cytokines (51). Our data show that MMP-9 activation may also play a role in delayed the repair of lung damage in addition to a role in the inflammatory response in these patients.

In summary, previous studies have shown that the expression of MMP-9 is enhanced in human airway CF epithelium relative to non-CF epithelia and elevated in serum of CF patients and correlated with pulmonary exacerbations (16, 43). The further activation of MMP-9 in response to *B. cenocepacia* infection and its effect on delaying epithelial repair, as demonstrated here, would contribute to chronic lung damage that is a key feature of CF lung disease and it is likely to further exacerbate inflammation in *B. cenocepacia*-colonized patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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