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Macrophage activation by gastric fluid suggests MMP involvement in aspiration-induced lung disease

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Abstract

Asthma occurs in more than 5% of the population in industrialized countries and is now characterized as a chronic inflammatory disease. The chronic aspiration of gastric fluid is considered by many investigators to be a primary inflammatory factor exacerbating or predisposing patients to asthma, with more than 50 medical papers per year linking asthma with gastroesophageal reflux disease (GERD), which can lead to aspiration events. However, the mechanisms involved in the inflammatory effects caused by gastric-fluid aspiration are not clear at the present time. The role of macrophages in the pathogenesis of disease seems likely given the involvement of those cells in a variety of chronic inflammatory diseases. To investigate the potential role of gastric fluid and the mechanisms potentially underlying chronic aspiration-associated pathogenesis, we examined the activation of murine macrophages (Raw 264.7 cell line) with gastric fluid. Inflammatory cytokine production and activation of the NF-κB signaling pathway were observed. Toll-like receptor (TLR)-4-dependent activation was observed under some conditions, indicating that bacterial components within the gastric fluid are involved in macrophage activation. Matrix metalloproteinase-9 (MMP-9) expression by macrophages was enhanced by gastric fluid, suggesting a potential mechanism by which remodeling of airways might be induced by gastric-fluid aspiration.

Introduction

Airway inflammation is complex and most likely involves a variety of factors associated with both innate and adaptive immunity. Recent work has pointed toward an important role of matrix metalloproteinases (MMPs) in regulation of airway remodeling associated

with pulmonary inflammation (Parks et al. 2004). MMPs are extracellular matrix degrading proteinases that regulate extracellular matrix composition, and are capable of cleaving non-matrix proteins, such as growth factors, chemoattractants, and cell surface receptors (Black et al. 2003; Chakraborti et al. 2003). There are 23 different MMPs in humans, which can be classified into three main categories based on their structure and choice of substrate: interstitial collagenases (MMPs -1, -8, -13, and -18, which cleave collagen types I, II, and III), gelatinases (MMPs -2 and -9, which degrade some form of collagen type IV), and the stromeolysins (MMPs -3, -10, and -11, which cleave laminin; Webster and Crowe 2006). These proteinases have roles in a variety of processes, including fetal development, normal tissue remodeling, wound healing, and inflammation.

Given their roles in tissue remodeling and inflammation, it is not surprising that a number of observations have implicated MMPs in the pathogenesis of pulmonary disease. For example, increased expression of MMP-2 and MMP-9 was observed in patients with chronic obstructive pulmonary disease (COPD) and in asthmatic patients (Cataldo et al. 2000). MMP-9 was reported to inhibit neutrophil accumulation, and MMP-12 was found to contribute to the accumulation of eosinophils and macrophages in IL-13-induced airway remodeling (Lanone et al. 2002). Differential expressions of MMP-2, 3, and 9 were reported in asthma patients (Boxall et al. 2006). The production of MMP-12 was stimulated by IL-1β and TNF-α, and was found to promote cell migration (Xie et al. 2005). In addition, deficiency in MMP-9 in a mouse model was found to be associated with decreased lung or alveolar injury (Lanone et al. 2002; Proust et al. 2002; Zuo et al. 2002). Further, increased MMP-2 and -14 activity was found to be associated with asthmatic airway smooth muscle (Henderson et al. 2007). These reports are consistent with the idea that MMP activity is an important factor in the progression and remodeling processes that occur during airway injury.

One of the primary cell lines associated with MMP activity is the macrophage/monocyte cell line. Macrophage activation is regulated by four signaling cascades, two of which are initiated by the bacterial endotoxin lipopolysaccharide (LPS; Adams 1994), which is a ligand for Toll-like receptor (TLR)-4 (Huber et al. 2006). The LPS/TLR-4 ligand/receptor combination triggers a signaling cascade, which results in gene transcription mediated by the transcription factor NF-κB (Alberts et al. 2002). The NF-κB family of proteins consists of the p65 (RelA), RelB, c-Rel, p50, and p52 subunits, which come together to form homo- and heterodimers (Ghosh et al. 1998). The p50/p65 heterodimer is the combination prevalent in most cells (Ghosh et al. 1998). Until LPS binds to TLR-4, inhibitor proteins of NF-κB (IκB) prevent NF-κB from entering the cell nucleus to initiate gene transcription by binding

to the dimer (Alberts et al. 2002). Binding of LPS to TLR-4 signals a cascade that frees NF-κB from its inhibitor, thus allowing it to enter the cell nucleus, where it can initiate transcription of genes involved in macrophage activation (Alberts et al. 2002). Once activated, macrophages produce inflammatory proteins such as TNF-α (Adams 1994; Alberts et al. 2002), allowing TNF-α to be used as a measure of macrophage activation. That LPS activation of the NF-κB pathway is important in the production of MMP-9 has been shown (Lu and Wahl 2005; Rhee et al. 2007a).

There is ample evidence that macrophages are associated with the pathogenesis of pulmonary disease. For example, airway inflammation induced by allergen challenge is associated with the recruitment of immature macrophages to the airway (Lensmar et al. 2006). Further, recruitment of macrophages and release of proteolytic enzymes and inflammatory cytokines were found to be associated with airway inflammatory disease (Tetley 2005). As another example, macrophage infiltration, increased MMP-2 and MMP-9 activity, production of monocyte chemoattractant protein-1 (MCP-1), and increased levels of TNF-α and macrophage inflammatory protein-2 (MIP-2) were observed in bronchoalveolar lavage fluid (BALF) in LPS-induced airway inflammation (Delayre-Orthez et al. 2005).

The association of gastroesophageal reflux disease (GERD) with upper and lower respiratory disease had been known for decades (Harding et al. 1996; Harmanci et al. 2001; Mueller et al. 2004; Yuksel et al. 2006). Chronic gastric-fluid aspiration is considered by many investigators to be one of the inflammatory factors that may exacerbate or predispose some patients to asthma (Rodriguez et al. 2003; Sacco et al. 2000). The fact that patients with GERD have a higher prevalence of asthma has been widely reported (Alhabib et al. 2007; Atalay et al. 2005; Debley et al. 2006), and about 50 papers per year are currently published describing the link between asthma and GERD. However, the potential mechanisms by which chronic aspiration of gastric fluid might affect respiratory disease are not clear at present. Given the preponderance of data pointing toward macrophages and MMPs as important in the pathogenesis of lung disease, we set out to investigate the effects of gastric fluid on cultured macrophages as a potential model for evaluating the underlying mechanisms associated with the connection between GERD and lower respiratory disease. In this study, particular attention was paid to the nature of the MMP produced by gastric-fluid-activated macrophages, as well as to the potential pathways involved in activation of the macrophages. Our experimental results confirm that gastric fluid is a potent inflammatory mediator in chronic airway inflammatory disease, and point toward one potential mechanism by which aspiration of gastric fluid may mediate that disease.

Studies from our laboratory have shown that chronic aspiration can have a profound effect on the normal rat lung function (Appel et al. 2007). Further, we have shown that chronic aspiration can exacerbate asthma-like hypersensitivity in mice (Barbas et al. 2008). The wide range of pulmonary pathology that can be influenced by chronic aspiration is further demonstrated by the observations that chronic aspiration can induce either accelerated acute rejection (Hartwig et al. 2006) or the development of obliterative bronchiolitis (Li et al. 2008) in rat lung transplant models. The studies described in this manuscript are intended to provide the first *in vitro* investigations aimed at elucidating the mechanisms associated with chronic aspiration in the pathogenesis of pulmonary disease.

Materials and methods

Mouse gastric-fluid collection and use

All animals were housed in a pathogen free animal facility. Gastric fluid was collected from 8–12-week-old male BALB/cJ mice using the following procedure: a small midline incision was made over the proximal duodenum. The proximal duodenum was then ligated with 2-0 silk suture, and the gastric fluid was collected in a 15 ml sterile conical tube. The gastric fluid was then filtered through a 70 μm strainer (BD Biosciences) to remove relatively large particulate matter. Gastric fluid was pooled and frozen until needed in experiments.

It was expected that exposure of cultured macrophages to undiluted gastric fluid would prove cytotoxic. The buffering capacity of gastric fluid is quite substantial, and the fluid is not readily neutralized. For example, in a test of the buffering capacity of gastric fluid from rats, 6 parts of a pooled rat serum (pH 7.88) were required to raise the pH of 1 part of pooled gastric fluid from a pH of 3.3 to a pH of 7.0, whereas only 0.2 part of rat serum was required to raise the pH of 1 part of an unbuffered solution of hydrochloric acid over the same range. Indeed, exposure of Raw 264.7 cells to 2% murine gastric fluid diluted in culture medium (see below for details) resulted in complete cell death. In contrast, 1% gastric fluid did not prove cytotoxic and was thus utilized as a maximum working concentration in experiments. It was noted that the pH of 1% gastric fluid diluted in culture medium was not substantially different than the pH of culture medium with no gastric fluid.

Cell culture

Raw 264.7 cells were cultured in the Dulbecco's modified medium with 4 mM L-glutamine and 10% fetal bovine serum, with penicillium/streptomycin to retard bacterial growth. Cells were grown in 5% humidified CO_2 chambers maintained at 37 °C.

ELISA assay

Raw 264.7 cells were treated with or without mouse gastric fluid appropriately diluted in tissue culture medium. Following incubation for a specified amount of time, the supernatant was collected and appropriately diluted for evaluation by ELISA. The ELISA assay was carried out using the mouse TNF-α ELISA assay system of R&D laboratories. The lower limit of detection for ELISAs conducted using this format is about 5 pg/ml.

SDS PAGE and Western blot

Cells cultured on 10 mm plates were treated with or without gastric fluid and LPS appropriately diluted in tissue culture medium. Cells were harvested at appropriate time intervals, and the medium was removed. Nuclear proteins were extracted by first lysing the cells using a hypotonic solution containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, a cocktail of protease inhibitors (BD), and PMSF. Next, the nucleus was disrupted with a high-salt solution containing 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.6 M KCl, 0.2 mM EDTA, 0.5 mM DTT, a cocktail of protease inhibitors (BD), and PMSF. The nuclear protein extracts were boiled with SDS sample buffer, and proteins were separated by SDS PAGE on an 8% polyacrylamide gel. Proteins were then transferred by Western blotting, and p-65 and p-50, two proteins associated with the NF-κB signaling pathway, were identified using antibodies specific for p50 (e-Bioscience) and p65 (Santa Cruz). The proteins were detected using an ECL kit (Amersham). The anti-PARP (Santa Cruz) was used as the internal control for total nuclear protein extracted.

Blocking activation with an anti-TLR-4 antibody

Raw 264.7 cells were plated at a density of 5×10^4 cells/well in 96 well dishes. Sixteen to eighteen hours after plating, fresh culture medium was added to cells and experiments were initiated. Anti-mouse TLR4 (blocking antibody; e-Bioscience, clone MTS510) and Isotype control IgG1 (e-Bioscience) were added to the cells at a concentration of 10 ug/ml prior to treatment with gastric fluid or LPS. Cells were incubated at 37 °C for 1 h. Appropriately diluted mouse gastric fluid or LPS (10 ng/ml) was then added. Cells were incubated at 37 °C for 6 h, after which time the concentration of TNF-α in the supernatant was quantified.

Cell migration assay

Raw 264.7 cell migration was assayed by a Boyden chamber method using a 24-well microchemotaxis chamber and polycarbonate filters (Falcon) with 8 μm diameter pores. Eighty percent confluent cells were starved for 24 h, then collected and re-suspended in serum-free DMEM at a concentration of 2×10^6 cells/ml. These cells were then placed in equal concentrations on the top of each filter. Serum-free DMEM or serum-free DMEM with gastric fluid were placed in the lower compartment. The chamber was incubated in a humidified incubator at 37 °C and 5% CO_2 for 24 h. To evaluate migration in response to treatment, cells were removed from the top portion of the filter by scraping with a cotton swab, and the cells that had migrated to the under surface of the filter were fixed with 100% methanol and stained with 0.95% crystal violet.

Gelatin zymography

Metalloproteinase activity was assayed by gelatin zymography. Raw 264.7 cells were plated 16–18 h before treatment in 10 mm dishes. Cells were treated with or without LPS (10 ng/ml) or gastric fluid (1% v/v), and culture medium was collected 24 h after initiation of the treatment. The medium was concentrated using an Amicon Ultra 4 (Millipore). Next, 10 μl of the concentrated medium was fractionated by SDS PAGE on a zymography gel (7.5% acryl amide, 2 mg/ml gelatin, Sigma). Separated protein was renatured by incubating the gel for 1 h with 2.5% Triton-X 100, and then with renaturing buffer (40 mM Tris, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$) overnight at 37 °C. Finally, the gel was incubated for 16–18 h with gentle shaking at room temperature with a developing buffer containing 50 mM Tris–HCl, pH 7.6, 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Brij35. Gels were stained in 0.1% coomassie blue (R250, Sigma) for 1 h and destained until the bands (unstained regions that were not stained because of the absence of gelatin as a result of MMP activity) were evident.

Statistics

Statistical analysis was performed using Prism 4 software (GraphPad Inc., San Diego, CA). A Student's t-test was used for two-group comparisons. Data are expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Results

Gastric fluid stimulates TNF-α expression in Raw 264.7 cells

As assessed by TNF-α production, macrophages (Raw 264.7) are activated for 24 h by gastric fluid

(diluted 1/1000) in a TLR4-dependent manner, since anti-TLR4 (clone MTS510) but not an Isotype control blocked gastric-fluid-mediated activation of those cells (Fig. 1). Anti-TLR2, on the other hand, did not block activation of gastric-fluid-mediated activation of cultured monocytes (data not shown). Gastric-fluid-diluted 1/100 also activated macrophages, but that activation was not inhibited by anti-TLR4 antibody (data not shown.) Gastric-fluid-diluted 1/50 resulted in the killing of the macrophages.

Gastric fluid stimulates migration of Raw 264.7 cells

Raw 264.7 cells were loaded onto the upper chamber of the transwell flask with the medium containing 1% gastric fluid in the bottom chamber. Cells were incubated in humidified 5% CO_2 incubator for 6 h, and the cells on the lower surface of the filter were fixed and stained with crystal violet as described in the Materials and methods. The number of migrated cells in response to gastric-fluid treatment was increased 5–6 fold when compared to non-stimulated cells (Fig. 2). This result indicates that exposure to gastric fluid stimulates macrophage migration, and suggests that recruitment of inflammatory cells as a result of exposure to gastric fluid may be involved in the pathogenesis of airway disease.

Gastric fluid stimulates MMP-2 and MMP-9 expression in Raw 264.7 cells

Since the connections between MMP activation and inflammatory cell migration have been well established,
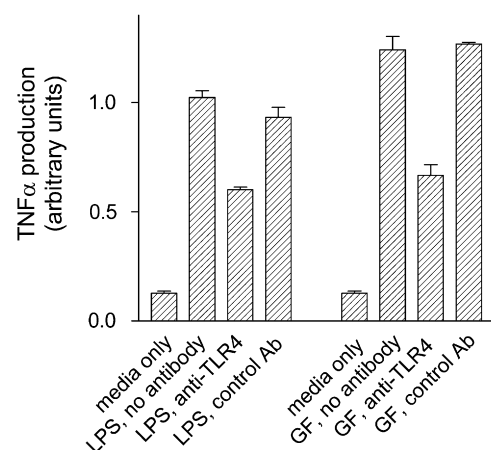

Fig. 1. Anti-TLR-4 antibody blocks stimulation of Raw 264.7 cells by gastric fluid. TNFα production by mouse macrophages in culture was measured following stimulation by 1 ng/ml LPS or by 0.1% gastric fluid for 24 h with and without blocking of the TLR-4 receptor with anti-mouse TLR-4. The experiment was conducted in duplicate, and the standard errors are shown.
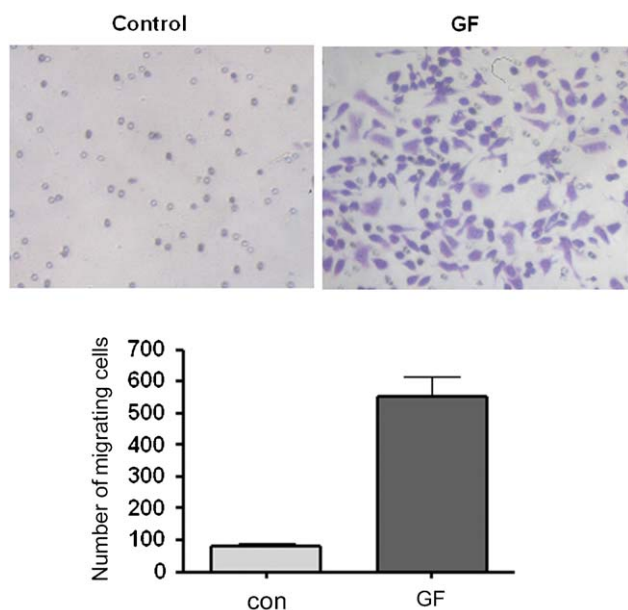
Fig. 2. Gastric fluid stimulates migration in Raw 264.7 cells. Raw 264.7 cells (8×10^4 cells/well) were loaded onto the top wells of the transwell chamber with the medium with or without 1% gastric fluid in the bottom chamber. Cells were incubated in humidified 5% CO_2 for 24 h, and the cells on the lower surface of the filter were fixed and stained with crystal violet. After staining, random fields of migrated cells were photographed in triplicate at 40 × magnification. Cell migration was determined as the average number of cells that had migrated across the filter in the photographed area. Data shown are the average of three independent studies.
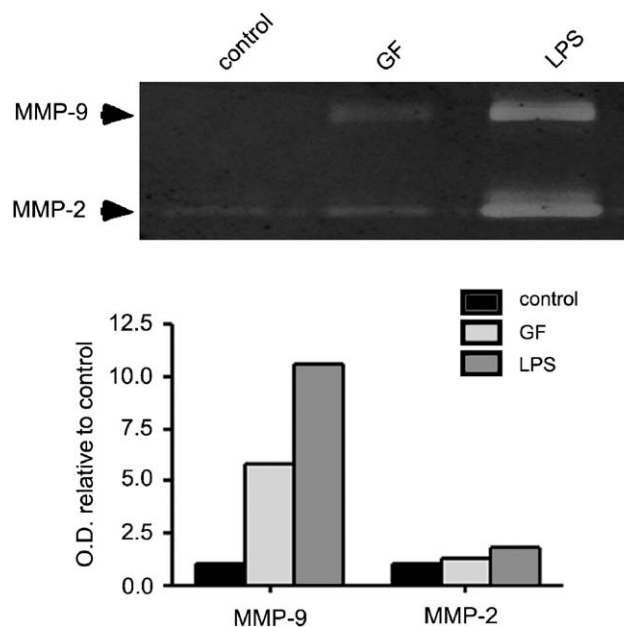

Fig. 3. Gastric fluid stimulates MMP-9 expression and, to a lesser degree, MMP-2 expression in Raw 264.7 cells. Raw 264.7 cells were plated in a 10 mm dish and treated with 100 times of diluted mouse gastric fluid in a serum-free condition. Culture medium was collected and concentrated for the assay of gelatin zymography. MMP-9 and MMP-2 activity was observed after 24 h of gastric-fluid treatment. The optical density, correlating inversely with MMP-2 and MMP-9 activity, was measured by densitometry using software from Labwork[TM]. Data shown are the representative of three independent studies.

we evaluated the expression of metalloproteinase activity after treatment of Raw 264.7 cells with gastric fluid for 24 h. A substantial increase in MMP-9 activity was observed following treatment with either gastric fluid or LPS, although the increase was more substantial with LPS treatment (Fig. 3). A smaller but perhaps biologically significant increase in MMP-2 expression was also observed following treatment with either gastric fluid or LPS. The relative ratio of MMP-9 to MMP-2 activity was similar in gastric-fluid-treated and LPS-treated cells, perhaps indicating that gastric fluid and LPS induced similar types of activation of the MMP family of enzymes. This result indicates that MMPs in macrophages are activated by gastric fluid, and suggests that increased MMP-9 and perhaps MMP-2 activity associated with exposure to gastric fluid may be in involved in the airway remodeling associated with the pathogenesis of airway disease.

Gastric fluid stimulates an NF-κB signaling pathway in Raw 264.7 cells

Regulation of MMP-9 expression by the NF-κB signal transduction pathway has been recently reported (Lu and Wahl 2005; Rhee et al. 2007a, b) With this in mind, we evaluated whether the p50/p65-associated NF-κB pathway might be activated in gastric-fluid-activated Raw 264.7 cells. For this purpose, Raw 264.7 cells were stimulated with appropriately diluted gastric fluid for 3, 6, 12 and 24 h. Nuclear protein was extracted, and the nuclear translocation of p50 and p65 proteins, which is associated with activation of an NF-κB pathway, was examined. Complexes p50 and p65 were observed in the nuclear extract after gastric-fluid treatment. The expression level of p50 and p65 was increased in a time-dependent manner (Fig. 4), indicating that the expression of MMPs and TNF-α induced by gastric fluid may be regulated through NF-κB transcriptional regulation.

Discussion

Airway inflammation may be induced by multiple factors. The role of gastroesophageal reflux disease in pulmonary pathology had been considered for years, and GERD-associated aspiration is a leading suspect as an inflammatory factor in asthma (Rodriguez et al. 2003; Sacco et al. 2000). In the present study, we
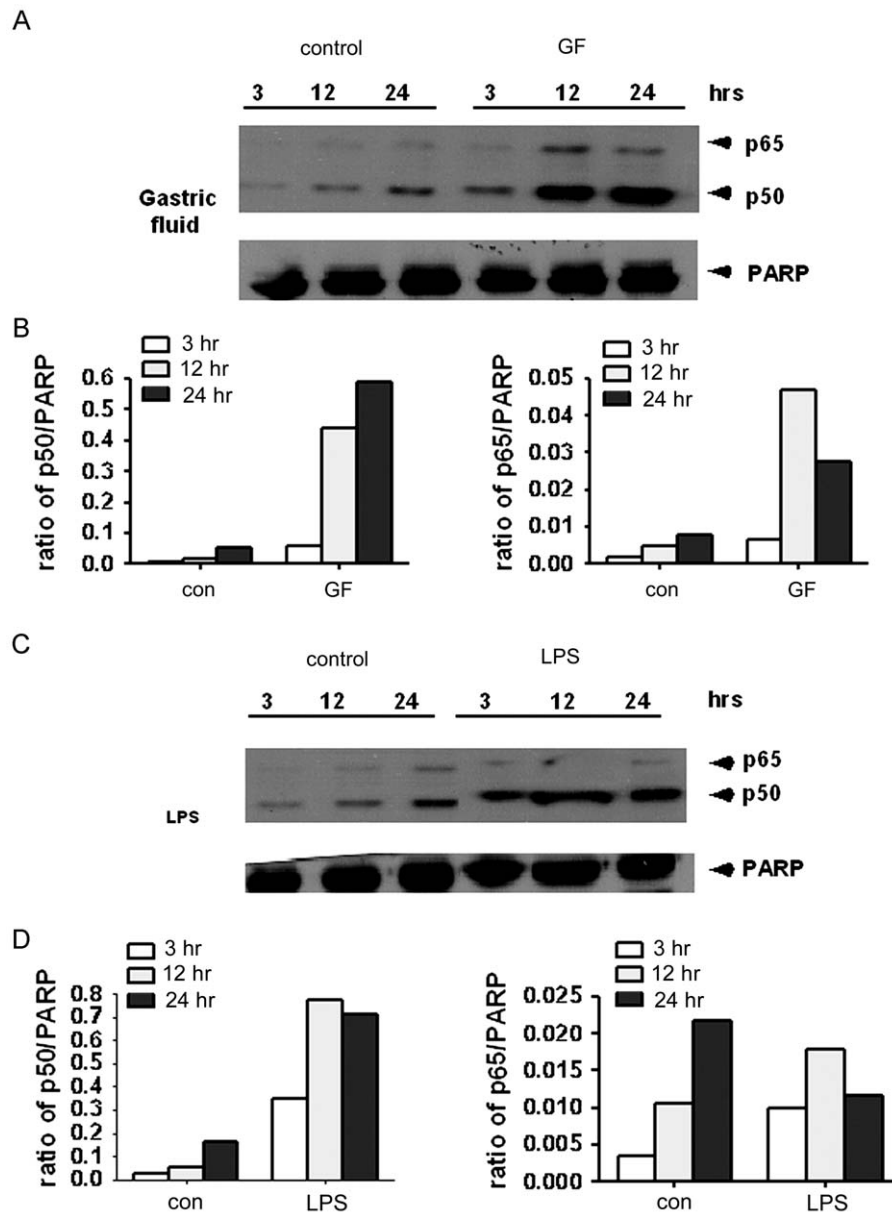
Fig. 4. Activation by gastric fluid of NF-κB signals in Raw 264.7 cells. Raw 264.7 cells were stimulated with (GF) or without (con) 1% gastric fluid for 3, 12, and 24 h. Nuclear protein was extracted for the examination of nuclear translocation of p50 and p65 proteins, which are both associated with an NF-κB signaling pathway. The increased expressions of nuclear p50 and p65 and of the nuclear internal control PARP were measured by densitometry using software from Labwork[TM].

establish an *in vitro* model to assess the potential role of gastric fluid in macrophage-mediated inflammation and tissue remodeling.

Aspiration of undiluted gastric fluid with a pH below 3 or 4 almost certainly leads to cell death and necrosis of lung tissue. However, following dilution of the gastric fluid with fluid present in the lung, it is expected that the concentration of gastric fluid will reach a level at which it is no longer cytotoxic, but at which it is still sufficiently concentrated to be pro-inflammatory. The experiments described in this paper shed light on the potential nature of that pro-inflammatory action.

Using gastric fluid collected from Balb/C mice, we demonstrated that secretion of the inflammatory cytokine TNF-α was increased in Raw 264.7 cells stimulated with as little as 0.1% gastric fluid. This indicates that gastric fluid may be an important mediator in inflammatory process associated with pulmonary injury, and suggests that microaspiration by gastric fluid in patients with GERD may be an important triggering factor for airway chronic inflammation. Further, activation of MMP-9 and to a lesser extent MMP-2, activation of the NF-κB signaling pathway, and stimulation of cell migration were also associated with exposure to gastric

fluid. The nuclear shuttling p50 was more significant than p65 in our model, perhaps indicating that the alternative NF-κB complex, such as c-REL/RELA/p50/IκBα/ε, is probably involved in gastric-fluid activation in Raw 264.7 cells. Further, activation of an NF-κB signaling pathway is likely responsible for the observed stimulation of cell migration since NF-κB is involved in leukocyte production of the macrophage migration mediator Monocyte Chemoattractant Protein (MCP)-1 (Cho et al. 2002). All of these factors probably play a role in asthma and airway remodeling as previously reported (Liang et al. 2007; Lu and Wahl 2005; Rhee et al. 2007a), further implicating GERD-associated aspiration as a potential mediator of pulmonary pathology.

Of particular interest is the observation that gastric fluid at a concentration of 0.1% stimulated macrophages in a TLR-4-dependent manner, suggesting that LPS contained in the gastric fluid is one primary source of stimulus for macrophages exposed to gastric fluid. This is of particular importance given the fact that many patients with GERD are treated with acid blockers, which in turn may inadvertently lead to increased growth of bacteria in the stomach (Zavros et al. 2002a, b). On the other hand, more concentrated gastric fluid (1.0%) stimulated the same cells in a TLR-4-independent manner, suggesting that gastric fluid contains multiple components that might stimulate macrophages. A wide array of microbial components might account for this TLR-4-independent stimulation. In addition, protease activity contained in the gastric fluid may account for stimulation of macrophages through protease-activated receptors (PARs), which are abundant on a variety of immune cells. Interestingly, PAR-1 has been shown to be necessary for leukocyte recruitment via MCP-1 (Chen et al. 2008). This combined with the gastric-fluid-induced macrophage migration observed in this study supports the idea that PARs might play a role in the gastric-fluid-dependent activation of macrophages. Although the pH of the gastric fluid used in this study was sufficiently neutralized by dilution in buffered media such that the primary protease (pepsin) was inactivated, other gastric proteases have activity at neutral pH and may stimulate macrophages. However, a wide range of microbial products in the gastric fluid seem the most obvious candidates as the primary activators of the macrophage population. Future studies should include the effects of gastric fluid on other cells that may be important in the pathogenesis of chronic aspiration-induced lung injury. Such cells might include pulmonary epithelial cells, airway smooth muscle cells, and a variety of leukocytes other than macrophages. For example, Zarbock et al. (2006) demonstrated reversal of acid-induced injury by blocking platelet-neutrophil aggregation.

Independent of which step is involved in TNF-α secretion, the finding that more dilute gastric fluid promotes TNF-α secretion in a TLR-4-dependent manner suggests a potential avenue for treatment that might warrant further investigation. The combination of an agent that neutralizes gastric fluid and a TLR-4 antagonist might be useful in suppressing the airway inflammation caused by reflux of gastric fluid, especially since a TLR-4 antagonist has been shown to have anti-inflammatory effects in mice models of inflammatory bowel disease (Fort et al. 2005).

In summary, the present study demonstrates for the first time that gastric fluid is potentially an important pro-inflammatory mediator. Further, the activation of MMP-9 activity and macrophage migration by gastric fluid provides insight into the mechanisms that may be associated with the processes of inflammation and airway remodeling in patients with GERD-induced pulmonary pathology.

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