

Doxycycline Decreases Production of Interleukin-8 in A549 Human Lung Epithelial Cells*

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Abstract

Doxycycline is an antibiotic that possess anti-inflammatory properties. These anti-inflammatory properties make doxycycline an attractive candidate for possible treatments for a variety of common chronic obstructive airway diseases. Interleukin-8 (IL-8) is a major inflammatory chemokine and a powerful chemo-attractant for both neutrophils and monocytes. We hypothesized that doxycycline might exert its anti-inflammatory effects, at least in part, by modulating IL-8 production. To test this hypothesis, A549 human lung epithelial cells were stimulated with cytomix (IL-1beta, TNF-alpha and gamma-IFN) in the presence or absence of varying concentrations of doxycycline. Doxycycline decreased IL-8 protein production in a concentration- and time-dependent manner. In the presence of 30 microg/ml doxycycline IL-8 protein production was decreased by 63% through out a 30 hr time course. In chemotaxis assays monocyte and neutrophil migration was decreased by 55% and 57% respectively. Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments suggest that doxycycline does not decrease expression of IL-8 mRNA and that use of the RNA polymerase II inhibitor DRB indicates that doxycycline does not effect stability of this mRNA. In the presence of doxycycline p38-alpha mitogen-activated protein kinase (MAPK) expression is decreased by 36% in cytomix-stimulated cells. These data demonstrate that doxycycline can modulate IL-8 release and suggest that it has potential as an anti-inflammatory in those disorders where IL-8 is an important inflammatory mediator.

Introduction

Chemokines are small, excreted, induced proteins that are involved the initiation and regulation of the inflammatory and immunological responses to the presence of foreign antigens in the body. Interleukin-8 (IL-8) or CXCL8 belongs to the CXC subfamily and is a major pro-inflammatory chemokine produced during chronic inflammatory lung diseases (1-3). IL-8 performs a variety of functions including activation of degranulation and the respiratory burst in and chemo-attraction of neutrophils (4-6), chemo-attraction and activation of monocytes (7), chemotaxis of T-cells (8), chemotaxis and adhesion of basophils (9,10),

activation of 5-lipoxygenase with release of leukotriene B₄ (11) and adherence of neutrophils (12) and monocytes (13) to various cell layers.

The common obstructive airway diseases such as bronchitis, asthma, cystic fibrosis, bronchiectasis, chronic obstructive pulmonary disease (COPD) and diffuse panbronchiolitis are all invariably associated with chronic airway inflammation (14-16). These disorders are often progressive even with current therapies, including steroid therapy, suggesting that new, more effective therapies are needed.

The tetracyclines and erythromycin are well known, potent antibacterial agents. In addition both compounds also possess a separate anti-inflammatory mode of action and have been known to reduce inflammation associated with several inflammatory diseases since the 1970's (17,18). These compounds include erythromycin A, and many of its derivatives such as azithromycin and clarithromycin, as well as tetracycline and several related compounds such as doxycycline. These compounds have exhibited anti-inflammatory activity in a variety of inflammatory disorders (18).

We hypothesized that doxycycline possesses potent anti-inflammatory properties, and that treatment with doxycycline leads to a reduction in IL-8 levels in A549 human lung epithelial cells. Such a reduction would lead to an attenuation of many pro-inflammatory responses and possibly limiting tissue damage at the site of inflammation. Our results show that doxycycline decreases the production of IL-8 in cytomix (a combination of IL-1 beta, TNF-alpha and gamma-IFN) stimulated cells resulting in decreased monocyte and neutrophil chemotaxis. These results suggest that doxycycline may be beneficial in the control of the inflammatory response in a variety of chronic airway disorders.

Materials and Methods

Culture of A549 Cells. The human lung epithelial cell line, A549, was purchased from the American Type Culture Collection (Manassas, VA) (19). A549 cells were grown in 25 cm² tissue culture flasks (Corning Costar, Cambridge, MA) in Ham's F-12 containing 10% fetal calf serum, L-glutamine (2 mM) and penicillin-streptomycin (100 U/ml-100 mg/ml) until confluent. After washing with serum-free media doxycycline hydrochloride, dissolved in distilled water, was added to confluent A549 cultures for 3 hr and the cells were cultured 18 hr in serum-free Ham's F-12 with or without cytomix. Cytomix (CM) is a combination of recombinant human tumor necrosis factor-alpha (TNF-alpha), human interleukin-1 beta (IL-1 beta), and human interferon-gamma (IFN-gamma) each at a 5 ng/ml concentration (all cytokines from R&D Systems, Minneapolis, MN) that have been previously used to stimulate the expression of IL-8 (20,21). Concentrations of doxycycline ranged from 3 to 30 microg/ml and are based on reports of clinically relevant serum and tissue levels (22). All control cultures contained the appropriate amount of carrier solvent alone.

Cell Viability Assays. Cell viability was determined by assay for release of lactic acid dehydrogenase into cell culture supernatant fluid using a commercially available assay kit (Sigma, St. Louis, MO) according to the manufacturer's directions.

Determination of IL-8 Protein Levels. IL-8 protein levels were determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. Doxycycline concentrations, cytomix stimulation and culture supernatant collection times were performed as indicated in the figure legends.

Neutrophil and Monocyte Chemotaxis. To perform the neutrophil chemotaxis assay, polymorphonuclear leukocytes were purified from heparinized normal human blood by the method of Böyum (23). The resulting cell pellet routinely consists of > 96% neutrophils and > 98% viable cells as determined by trypan blue and erythrosin exclusion. The cells were suspended in Hanks' balanced salt solution (HBSS) containing 2% bovine serum albumin (BSA, Sigma) at pH 7.4 to give a final concentration of 3.0×10^6 cells/ml. This suspension was used for the neutrophil chemotaxis assay.

Mononuclear cells for the chemotaxis assay were obtained by Ficoll-Hypaque density centrifugation to separate red blood cells and neutrophils from mononuclear cells. This preparation routinely consisted of 30% large monocytes and 70% small lymphocytes determined by morphology and alpha-naphthyl acetate esterase staining with >98% viability as assessed by trypan blue and erythrosin exclusion. The cells were resuspended in HBSS containing 2% bovine serum albumin at pH 7.4 to give a final cell concentration of 5×10^6 cells/ml. The chemotaxis assay was performed in a 48-well micro-chemotaxis chamber (NeuroProbe Inc., Cabin John, MD). Briefly, 25 μ l of the harvested cell culture supernatant fluids were placed into the lower wells and a 10- μ m thick polyvinylpyrrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA), with a pore size of 3 μ m for neutrophil chemotaxis or 5 μ m for monocyte chemotaxis, was placed over the bottom wells. The silicon gasket and upper pieces of the chamber were applied and 50 μ l of the appropriate cell suspension added to the upper wells above the filter. The chambers were incubated in humidified air in 5% CO₂ at 37°C for 30 min for neutrophil chemotaxis or 90 min for monocyte chemotaxis. After incubation, the chamber was disassembled and non-migrated cells were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik (American Scientific Product, McGraw Park, IL), and mounted on a glass slide. Cells that completely migrated through the filter were counted by using light microscopy in ten random high power fields (HPF) per well.

RNA Isolation and RT-PCR. IL-8 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was

extracted from adherent cells using a modification of the methods of Chomczynski and Sacchi (24). The RNA was reverse transcribed using a commercially available RT-PCR kit (Promega, Madison, WI) and commercially available IL-8 primers (R&D Systems, Minneapolis, MN) added at 0.2 μ M final concentration. Using a Perkin Elmer model 480-thermal cycler, reverse transcription was performed on 250 ng of total cellular RNA at 48°C for 45 min. PCR for IL-8 was performed in the same sample tubes at 94°C for 2 minutes followed by 22 cycles consisting of 94°C for 45 sec, 55°C for 45 sec, 72°C for 2 min, followed by 72°C for an additional 7 min. Beta-actin was used as a "housekeeping gene" and RT-PCR was performed in a similar manner as for IL-8 except for an annealing temperature of 55°C. The beta-actin primer sequences that were used are: sense primer 5'-TGACCCAGATCATGTTTGAG-3' and antisense primer 5'-TCATGAGGTAGTCAGTCAGG-3'. The DNA fragments were separated by agarose gel electrophoresis on a 2% gel in TAE buffer and then identified, analyzed and quantified by densitometry. Increasing PCR cycles gave increasing amounts of DNA through a fairly broad range with linearity obtained from about cycles 22-28 beginning with 250 ng of DNA ($r=0.99$ for IL-8 and beta-actin).

Stability of IL-8 mRNA. A549 cells were treated with cytomix or cytomix plus doxycycline for 18 hr as described above and then treated with the RNA polymerase II inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) (Calbiochem, La Jolla, CA), 10 μ g/ml, to inhibit transcription. RNA was isolated from cultures at various times and RT-PCR, using conditions described previously, was used to determine the amount of IL-8 mRNA present at indicated times.

Protein Determination. Protein concentrations were determined spectrophotometrically using the Bradford assay (25).

Western Blotting. Cell extracts were prepared for Western blotting using previously described methods (26). Cell lysates were clarified by centrifugation prior to electrophoresis. SDS-PAGE was performed using a 4% T acrylamide stacking gel and a 10% T separating gel. Twelve micrograms of protein were loaded per lane. After electrophoresis was complete the separated proteins were transferred to a PVDF membrane for further analysis. Transfer was performed using a Tris-HCl (25 mM), glycine (192 mM) and methanol (20% v/v) buffer system. The transfer was performed at 4°C for 16 hr at 30V and then for 30 min at 60V. Antibody for p38-alpha MAPK (Santa Cruz Biotechnology, Santa Cruz, CA) was used to immunochemically detect p38-alpha MAPK protein on the transfer membranes using goat anti-rabbit IgG alkaline phosphatase as the secondary antibody-enzyme conjugate and NBT/BCIP alkaline phosphatase substrate tablets (Sigma, St. Louis, MO) in 0.1 M Tris-HCl pH 9.1 as the substrate system. Five microg of antibody were used at each step during the development procedures. The transfer membranes were blocked with BLOTTO (20 mM Tris base, 180 mM NaCl, 4% nonfat dry milk, 0.02% NaN_3) to minimize nonspecific binding.

Statistical analysis. In cases where multiple experiments were made, differences between groups were tested for significance using one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (Fisher's PLSD). In cases where single measurement was made, the differences between groups were tested for significance using Student's paired t-test. In all cases, a p value of <0.05 was considered significant. The data are expressed as mean \pm SD.

Results

A549 cell culture supernatants from all treatments were assayed for lactic acid dehydrogenase release as a measure of cell viability. There were no significant differences in the lactic acid dehydrogenase activity in the culture supernatants from all treatment types (data not shown).

Cytomix stimulation of A549 cells results in an increase in IL-8 production from 1100 pg/ml in unstimulated control samples to > 230,000 pg/ml (Fig. 1).

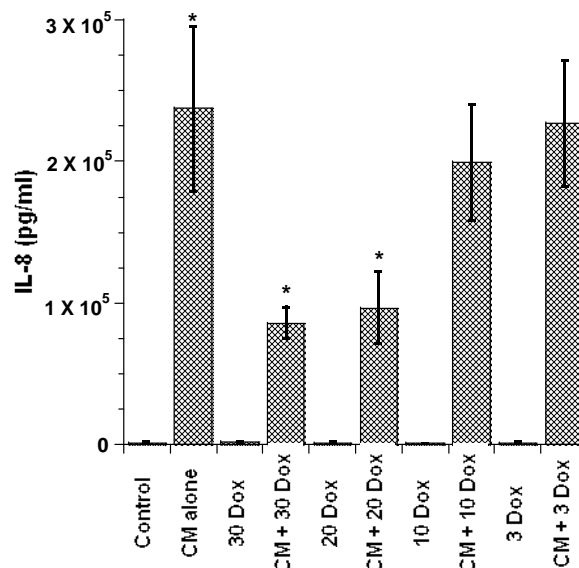


Figure 1. Effect of doxycycline concentration on production of IL-8. Cytomix (CM) with or without 3-30 microg/ml doxycycline (Dox). A549 cells were treated, samples collected and IL-8 ELISA assays were performed as described in Materials and Methods section. Results are from at least three experiments with each condition repeated in triplicate. * $p < 0.01$.

Doxycycline alone at all concentrations tested did not significantly increase IL-8 production over control samples. Increase in doxycycline concentration in cytomix-stimulated cells results in a concentration-dependent decrease in IL-8 production. At the highest doxycycline concentration used (30 microg/ml) IL-8 production was decreased 60% over cytomix treatment alone ($p < 0.01$) (Fig. 1).

In a time course study 30 microg/ml doxycycline treatment of cytomix-stimulated cells suppressed IL-8 production starting at 2 hr and continuing to the end of the

study at 30 hr ($p < 0.01$ at all times). Suppression ranged from 70% at 2 hr to 80% at 30 hr ($p < 0.01$) (Fig. 2).

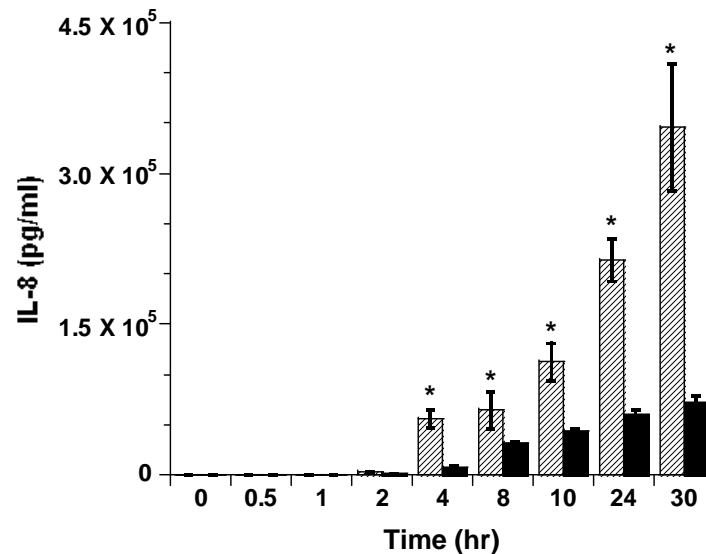


Figure 2. Time course for IL-8 production in the presence of cytomix (CM) and 30 microg/ml doxycycline (Dox). A549 cells were treated and IL-8 ELISA assays were performed as described in Materials and Methods section. Results are from at least three experiments with each condition repeated in triplicate. Hatched columns are CM alone. Black columns are CM + Dox. *In all cases $p < 0.01$.

Doxycycline treatment decreased neutrophil chemotaxis by 57% in cytomix-stimulated cells relative to cytomix alone ($p < 0.01$) (Fig 3A). In companion monocyte chemotaxis experiments doxycycline decreased chemotaxis by 55% relative to cytomix alone ($p < 0.01$) (Fig. 3B).

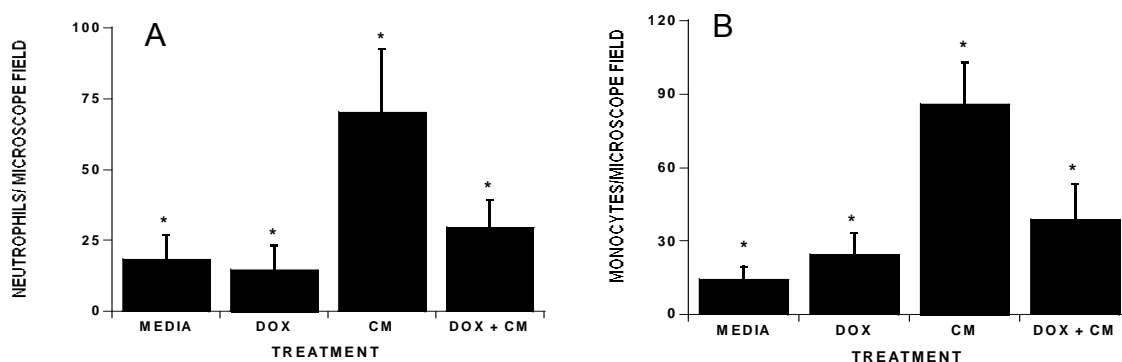


Figure 3. Effect of doxycycline on: (A) neutrophil chemotaxis and (B) monocyte chemotaxis. Results show the number of cells that migrate into the chemotaxis membrane in each microscope field of view examined. At least five high power microscope fields were counted for each treatment. * In all cases $p < 0.01$.

Expression of IL-8 mRNA was not affected by the presence of 30 microg/ml doxycycline. At this doxycycline concentration, cytomix-induced IL-8 mRNA

expression is not altered relative to samples treated with cytomix alone (Fig. 4). Doxycycline alone did not increase IL-8 mRNA expression over untreated control samples (Fig. 4).

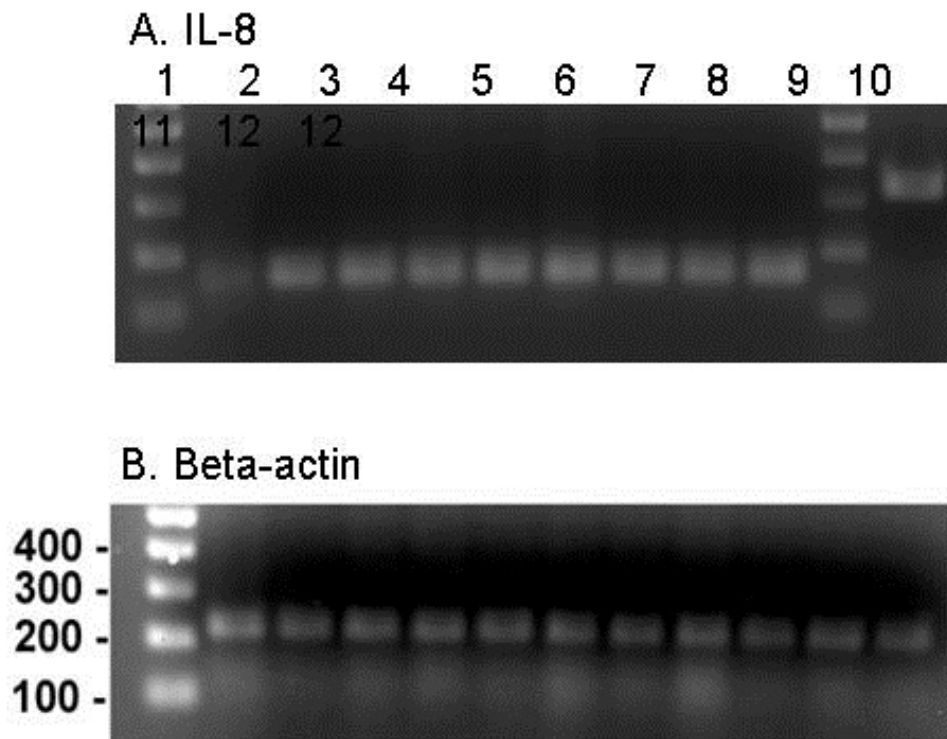


Figure 4. Effect of doxycycline on IL-8 and beta-actin mRNA production. Total cellular RNA was isolated and RT-PCR was performed as described. IL-8 RTPCR Panel A: Lane 1 base pair markers. Lane 2: 30 µg/ml doxycycline alone. Lane 3: 4 hr cytomix (CM). Lane 4: 4 hr CM + 30 µg/ml doxycycline. Lane 5: 12 hr CM. Lane 6: 12 hr CM + 30 µg/ml doxycycline. Lane 7: 18 hr CM. Lane 8: 18 hr CM + 30 µg/ml doxycycline. Lane 9: 30 hr CM. Lane 10: 30 hr CM + 30 µg/ml doxycycline. Lane 11: base pair markers. Lane 12: IL-8 positive control. Beta-actin RTPCR panel B: Lane 1: base pair markers. Lane 2: 30 µg/ml doxycycline alone. Lane 3: 4 hr cytomix (CM). Lane 4: 4 hr CM + 30 µg/ml doxycycline. Lane 5: 12 hr CM. Lane 6: 12 hr CM + 30 µg/ml doxycycline. Lane 7: 18 hr CM. Lane 8: 18 hr CM + 30 µg/ml doxycycline. Lane 9: 30 hr CM. Lane 10: 30 hr CM + 30 µg/ml doxycycline. Results are from duplicate experiments with each condition assayed in triplicate.

Stability of IL-8 mRNA in cytomix-stimulated cells does not appear to be affected by doxycycline (Fig. 5). IL-8 mRNA appears to decrease at the same rate, as indicated by the nearly identical slopes of the decay lines, in the presence or absence of doxycycline over an 8 hr time course.

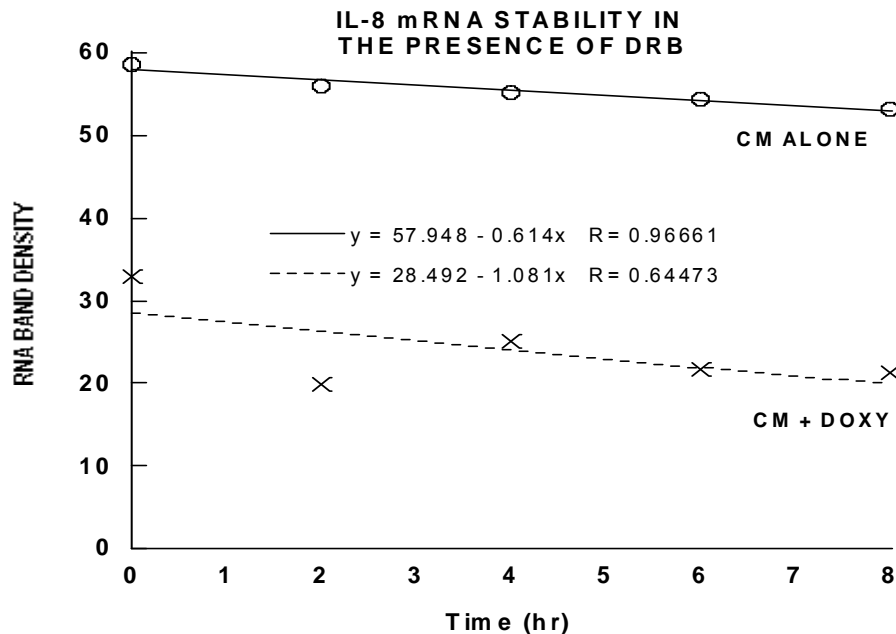


Figure 5. IL-8 mRNA stability. Total cellular RNA was isolated in the presence of 10 microg/ml DRB at the indicated times and RT-PCR performed as described. Solid line: cytomix alone ($r = 0.967$). Dashed line: CM + 30 microg/ml doxycycline ($r = 0.645$). Results are from triplicate experiments.

The effect of doxycycline p38-alpha MAPK was examined (Fig. 6). Treatment of cytomix-stimulated cells with 30 microg/ml doxycycline decreased p38-alpha protein production by 36% over cytomix-stimulation alone.

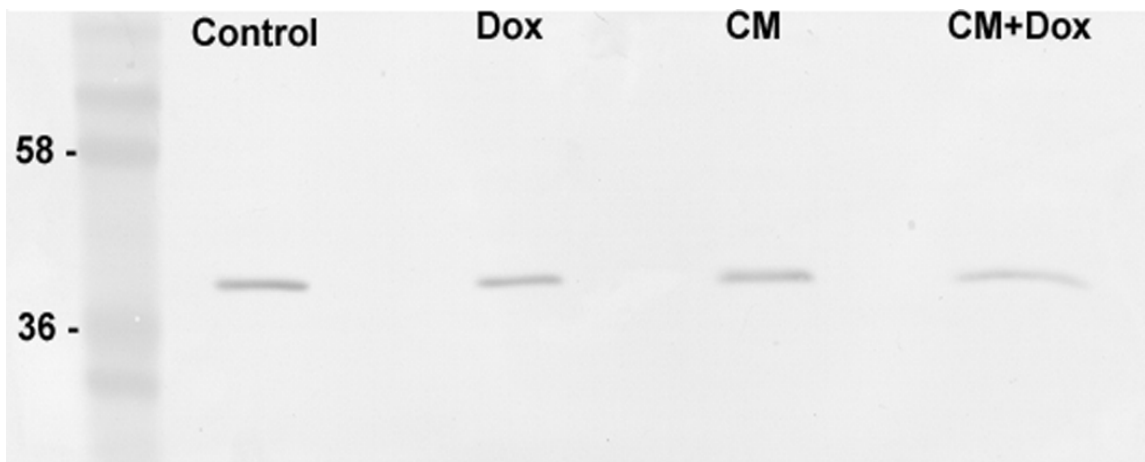


Figure 6. Effect of doxycycline on p38 alpha MAPK. Cells were lysed and electrophoresis and blotting were performed as described. Lane: (1) Molecular weight markers (kDa), (2) Control, (3) Doxycycline (30 microg/ml) alone, (4) CM alone, (5) CM plus doxycycline (30 microg/ml). 20 microg of protein loaded per lane.

Discussion

IL-8 is a major component of the many protein-based chemoattractant molecules that occur in chronic, inflammatory airway diseases (27). As a chemoattractant it recruits both monocytes and neutrophils to inflamed areas of the body. We have shown that doxycycline-treatment of cytomix-stimulated A549 human lung epithelial cells results in a concentration-dependent decrease in IL-8 production (Fig. 1) as well as in a time-dependent decrease in IL-8 production (Fig. 2). The observed decrease in IL-8 production affects neutrophil (Fig. 3A) and monocyte (Fig. 3B) chemotaxis. Chemotaxis of both neutrophils and monocytes are decreased after doxycycline treatment of stimulated cells indicating a possible reduction in the inflammatory response. Expression (Fig. 4) and stability (Fig 5) of IL-8 mRNA appear to be unaffected in cytomix-stimulated cells after doxycycline treatment. The p38 MAPK protein production is also decreased in the presence of doxycycline (Fig. 6).

The lack of effect of doxycycline of IL-8 mRNA expression and stability suggests that the observed decrease in IL-8 protein production may be due to post-translational events and not directly related to regulation of gene activity through involvement of activator protein-1 (AP-1), nuclear factor-kappa beta or other transcriptional regulatory systems. These events could possibly be a variety of post-translational modifications, such as glycosylation of specific amino acid residues in the IL-8 protein, incorrect or lack of removal of the N-terminal hydrophobic signal peptide, or by increased proteolytic degradation of IL-8 by the proteasome, matrix metalloproteases or other proteolytic activity. Another intriguing possibility is the report by Li *et al.* (28) that decreased p38 MAPK activity has no effect of IL-8 gene transcription but that active p38 MAPK plays a critical role in IL-8 protein production in a post-transcriptional manner. This observation appears to describe the decreased IL-8 and p38 MAPK protein levels and the apparent lack of effect on IL-8 mRNA that we have described. IL-8 is an important piece of the inflammatory response to invasion of the body by various foreign objects. However, if the inflammatory response is allowed to progress unchecked substantial cell and tissue damage occurs.

IL-8 production occurs in a wide variety of inflammatory lung diseases that are characterized by an influx of neutrophils (1). Asthma is often thought of as a disease that is dependent on T-lymphocytes and eosinophils (1,29). However there is mounting evidence that neutrophils and IL-8, a powerful chemoattractant for neutrophils, are elevated in severe asthma cases (29). Diffuse panbronchiolitis (DPB) is a progressive, chronic, inflammatory airway disease that is largely restricted to Japan (30). Neutrophils, T-lymphocytes and the chemoattractant molecules IL-8 and MCP-1 are thought to play significant roles in the progression of DPB (30). Cystic fibrosis is characterized by a massive influx of neutrophils into the airways (31). In addition neutrophil chemoattractants such as IL-8 are found in very high concentration (31). In COPD IL-8 is a prominent attractant for neutrophils (32-Barnes) and IL-8 levels in induced

sputum correlate with disease severity (32). A common result for these chronic, inflammatory lung diseases is severe, often life threatening, tissue destruction.

Continuous treatment with some antibiotics, particularly macrolides, reduces exacerbations. A randomized controlled trial with erythromycin reduced exacerbations by 35% compared to placebo (33). In a more recent study, treatment with azithromycin for one year lowered exacerbations by 27% (34). Although the mechanism(s) accounting for the reduction in exacerbations is unknown, current concepts suggest the reduction is likely secondary to the macrolides' anti-inflammatory properties. However, concern has been raised about a very small, but significant, increase in QT prolongation and cardiovascular deaths with azithromycin (35). In addition, the recent trial with azithromycin raised the concern of hearing loss which occurred in 25% of patients treated with azithromycin compared to 20% of control (34).

Tetracycline and derivatives such as minocycline and doxycycline have been extensively used to treat a wide variety of chronic, inflammatory diseases (36) including acne, rosacea, rheumatoid arthritis and periodontitis. Nieman and Zerler (37) propose that tetracycline and some of its derivatives may be beneficial in limiting lung damage during adult respiratory syndrome (ARDS). Although macrolide antibiotics such as erythromycin A have been shown to be effective treatments for cystic fibrosis (38) and diffuse panbronchiolitis (39) development of resistant pathogenic bacteria during the required prolonged therapy is a major concern. Doxycycline is not the antibiotic of choice for many respiratory bacterial infections so development of resistance to this drug is of less importance than it is for the macrolides.

Systemic inflammation has been correlated with poorer outcomes in COPD (40). Doxycycline has anti-inflammatory properties in addition to suppressing release of IL-8 including reduction of or inducible nitric oxide production and MCP-1 release from lung epithelial cells (41,42). It seems likely that doxycycline might reduce other inflammatory cytokines as well. These results suggest that doxycycline may provide a unique, effective, low cost therapy for management of a wide range of chronic, inflammatory diseases.

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