in vivo *26*: 369-374 (2012)

In Vitro Effects of Doxycycline on Inflammatory Cytokines and Gelatinases in Chronic Rhinosinusitis

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Abstract. *Background/Aim: The pathophysiology of chronic rhinosinusitis (CRS) is unknown, but the majority of patients suffer from eosinophilic infiltration. We hypothesised that doxycycline might alter the eosinophil-associated expression of interleukin-5 (IL-5) and eotaxin-3 in CRS and also the expression of matrix metalloproteinase 9 (MMP-9), being involved in the tissue-remodelling in CRS. Materials and Methods: After obtaining samples from 10 CRS patients with and without nasal-polyposis undergoing functional endoscopic sinus surgery and two healthy individuals, the expression of IL-5, eotaxin-3 and MMP-9 were evaluated by an ELISA technique. The tested agent was doxycycline at 0.1 or 1 mg/ml. Results: IL-5 levels remained unchanged, but eotaxin-3 levels actually increased under doxycycline treatment. The only marker showing a slight drop was MMP-9, albeit not significant. Conclusion: As first clinical trials with doxycycline in the treatment of CRS produced reasonable results we could demonstrate that the underlying pathology is more complex, and doxycycline affects only a part of the factors believed to support the chronic infection of the respiratory mucosa.*

Chronic rhinosinusitis (CRS) has been defined as a disease of the nasal and paranasal sinus mucosa persisting for more than three months. CRS is characterized by a chronic inflammation of these mucosa with mucosal changes ranging from inflammatory thickening to the development of nasal polyps (1). The presence of polyps then divides CRS in two subgroups: CRS with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP). The pathophysiological cause of nasal polyposis is still unknown, but the majority of patients suffer from eosinophilic inflammation with a dominant T-helper type 2 (Th2) cytokine profile (2). Furthermore, the accumulation of activated eosinophils within tissues are a persistent feature of this disease (3). Activated eosinophils contribute to nasal polyp pathology by the production of inflammatory mediators and cytokines (4). Not only is CRS frequently observed in patients with asthma, but the two diseases also have a number of common pathological features: the activation of Th2-like lymphocytes and eosinophils secreting interleukin (IL)-3, IL-5, IL-13 and eotaxin (5-8). Thus some authors even state CRS as being the "asthma of the upper airways" (9). IL-5 is considered to play a pivotal role in the accumulation of eosinophils and activation in nasal polyps (10). For example, IL-5 expression was observed in nasal polyps and inferior turbinates, whereas no detectable IL-5 was found in serum of patients suffering from CRSwNP (11). Furthermore, eotaxin, especially eotaxin-3, is believed to be another crucial player in the regulation of eosinophilic inflammation and extracellular matrix breakdown in CRSwNP. However, for example in CRSsNP, IL-13 is also significantly elevated in sinus lavage from patients compared to controls. Airway remodeling is also influenced by IL-13. IL-13 alters the function of matrix metalloproteases, which remodels the extracellular matrix (12). The application of IL-13 induced a significant increase in β-catenin expression in eosinophilic (CRSwNP and CRSsNP) CRS cell culture compared to non-eosinophilic culture (7). The increased expression of β-catenin might be a marker for airway remodeling in CRS. But a study indicated that there may be a significant difference between CRSwNP and CRSsNP in the expression of matrix metalloproteinases and, in consequence in the nasal tissue remodeling (13). Tetracyclines seem to be effective in treatment of various chronic inflammatory airway diseases (14). One possible reason for this might be the effects of tetracyclines on the matrix metalloproteinases. Another reason might be its

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Key Words: Chronic rhinosinusitis, doxycycline, CRS, IL-5, MMP-9, eotaxin-3, inflammatory cytokines, gelatinases.

antibacterial activity against Staphylococcus aureus and thus a reduction in *S. aureus* enterotoxin. A first study has shown the positive effects of doxycycline and steroids in patients with CRSwNP, with a reduction in the size of the nasal polyps being described (15). We hypothesized that doxycycline administration might reduce the expression of IL-5 and eotaxin-3 in CRSwNP; compared these results to those from cultures from patients suffering from CRSsNP and individuals without CRS at all. Furthermore, this study was designed to analyze the expression of MMP-9, it being involved in the tissue remodeling in CRS, and the possible effects of doxycycline in the alteration of tissue in chronic inflammatory airway disease.

Materials and Methods

Patients, tissue collection and cultures. Five CRSwNP and five CRSsNP cultures were obtained from ten patients undergoing functional endoscopic sinus surgery at the Department of Otorhinolaryngology at the University Hospital Mannheim, Germany in 2006-2010. Written consent was obtained from all patients for the use of tissue samples of the resected paranasal mucosa. The cultures of the control group were obtained from nasal turbinates from two healthy individuals. A fibroblast culture for each sample of paranasal sinus mucosa was set up. After removal of adjacent connective tissue, the tissue specimens were cut into pieces. This was followed by their incubation in trypsin solution (0.25% trypsin in phosphate buffered saline, PBS, Sigma-Aldrich, Munich, Germany) overnight at 4˚C. The suspension was then added to a human fibroblast monolayer and subsequently cultured in FAD2-medium (Dulbecco's modified Eagle's medium and Ham's F12 in a 3:1 ratio supplemented with fetal calf serum, insulin, triiodothyronine hydrocortisone, epidermal growth factor, cholera toxin) at 37˚C in an atmosphere with 10% CO_2. After reaching subconfluency, the feeder layer was detached by incubation with 0.02% ethylenediamine tetraacetic acid (EDTA) in PBS for 4 min at room temperature and the cells were further cultured overnight in serum free fibroblast growth medium (KGM; Clonetics, San Diego, CA, USA; Fisher Scientific Co., Pittsburgh, PA, USA). Cells were passaged by trypsinization (0.1% trypsin and 0.02% EDTA dissolved in PBS for 5 min at 37˚C).

Treatment with doxycycline. After 24, 48, 72 and 98 hours of incubation with 0.1 or 1 mg/ml doxycycline the expression of IL-5, eotaxin-3 and MMP-9 in the culture supernatants were evaluated (13).

Cytokine immunoassay. The supernatants of the cultures were stored in sterile test tubes and stored at –20˚C until use. The expression of the examined IL-5, eotaxin-3 and MMP-9 were evaluated by an enzyme-linked immunosorbent assay (ELISA) test (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against the target proteins. The exact products used were the following: DuoSet Human Total MMP9 Elisa: R+D Systems, MN, USA. BD OptEIA Human IL-5 Elisa: BD Biosciences, Franklin Lakes, NJ, USA. BD OptEIA Human Eotaxin Elisa Set: BD Biosciences, Franklin Lakes, NJ, USA. According to the manufacturers instruction, each ELISA assay determined its target proteins in 100 μl of supernatant. The cells were grown in 96-well plates with 12 strips of 8 walls coated with an antibody against the proteins of interest. After 24-96 hours of incubation, the expression of the markers of interest in the supernatants was determined. According to information provided by the manufactures, the human MMP-9 assay measures the 92 kDa Pro-MMP-9 and the 82 kDa active MMP-9. It does not measure the 65 kDa form. The MMP-9 assay is able to detect a range of 31.2-2,000 pg/mL. The IL-5 assay has a detection range of 7.8-500 pg/ml and the eotaxin assay has a range of 6.3-400 pg/ml. Like all ELISA assays, interference of drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference therefore cannot be excluded.

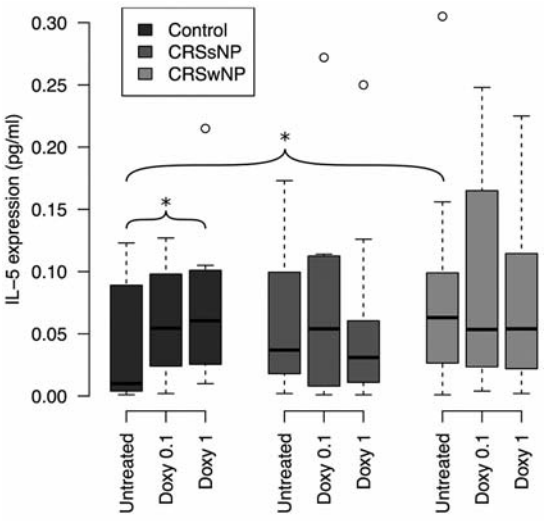

Figure 1. *Box-whisker plot of the expression in correlation with the concentration of doxycycline used. The boxes represent the interquartile range with the whiskers extending up to 1.5 times this range. Outliers are marked as circles and the median is marked with a solid line. Control: healthy tissue; CRSsNP: Chronic rhinosinusitis without nasal polyps; CRSwNP: Chronic rhinosinusitis with nasal polyps. *indicates a p-value <0.05.*

Statistical analysis. Statistical analysis was performed with SAS (SAS/STAT, Version 8, SAS Institute Inc., Cary, NC, USA). An analysis of variance (ANOVA) with student's *t*-test and Dunnet's procedure against the untreated control group (1) was used to calculate *p*-values. As no Bonferroni adjustments were necessary, the level of statistical significance was set to $p<0.05$. Further statistical analysis and plotting was carried out using R, an open-source environment for statistical computing and graphics (2).

Results

Incubation duration. Analyzing the effect of incubation duration (24, 48, 72 and 96 h), there was no statistically significant difference in expression of IL-5, eotaxin-3 or MMP-9 in any of the tested situations.

IL-5. The concentration (0.1 or 1 mg/ml) of doxycycline used did not have a significant effect on the expression of IL-5 in

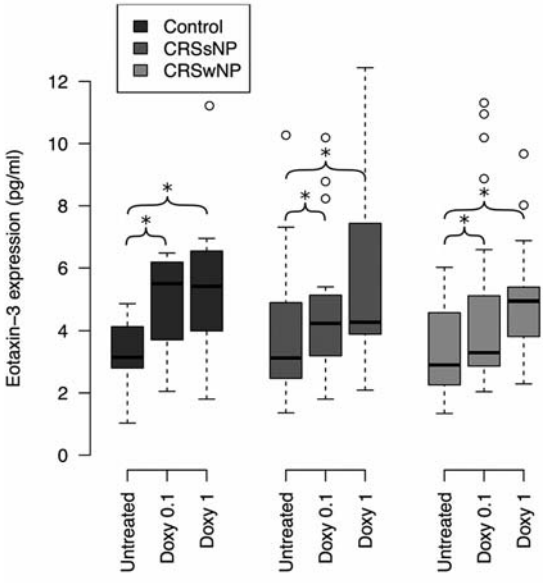

Figure 2. *Box-whisker plot of the expression in correlation with the concentration of doxycycline used. The boxes represent the interquartile range with the whiskers extending up to 1.5 times this range. Outliers are marked as circles and the median is marked with a solid line. Control: healthy tissue; CRSsNP: Chronic rhinosinusitis without nasal polyps; CRSwNP: Chronic rhinosinusitis with nasal polyps. *indicates a p-value <0.05.*

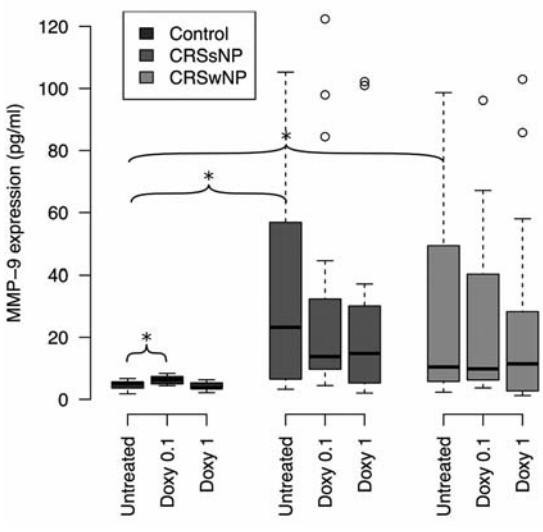

Figure 3. *Box-whisker plot of the expression in correlation with the concentration of doxycycline used. The boxes represent the interquartile range with the whiskers extending up to 1.5 times this range. Outliers are marked as circles and the median is marked with a solid line. Control: healthy tissue; CRSsNP: Chronic rhinosinusitis without nasal polyps; CRSwNP: Chronic rhinosinusitis with nasal polyps. *indicates a p-value <0.05.*

general *i.e.* effects were not dose dependent. The untreated CRSsNP cultures (0.053 pg/ml ±0.049 pg/ml) tended to have a higher expression of IL-5 compared to the untreated control cultures (0.033 pg/ml ±0.048 pg/ml) (p=0.069). The first number in brackets always represents the mean value whereas the number behind the "±" sign represents the standard deviation. Expression of IL-5 in untreated CRSwNP (0.069 pg/ml ±0.067 pg/ml) was significantly higher than in the untreated control cultures (0.033 pg/ml ±0.048 pg/ml) (p=0.032). Only in the control group, doxycycline generated a significant increase in expression of IL-5 (doxycycline 1mg: 0.075 pg/ml ±0.067 pg/ml) compared to the untreated group (p=0.013). In the CRSsNP and in the CRSwNP group, the doxycycline effects were not statistically significant (Figure 1).

Eotaxin-3. Doxycycline dose had a significant effect on the expression of eotaxin-3. No group effect was adherent. The higher the doxycycline concentration, the greater the increase in eotaxin-3 expression in all cultures. Figure 2 gives a graphical interpretation of the data. The p-values were far below p=0.001 for all cultures and all of the treatments when compared to the corresponding untreated groups.

MMP-9. MMP-9 was only significantly elevated through treatment with doxycycline in the cultures from healthy tissue. Doxycycline treatment at 1 mg concentration nevertheless reduced the MMP-9 levels of CRSwNP cultures by 11.4 pg/ml (34%) and 4.6 pg/ml (12%) in CRSsNP, although not significantly. CRS cell cultures exhibited a significantly increased expression of MMP-9 compared to the control group (3.27 pg/ml ±2.09 pg/ml) with p<0.001. The mean value of MMP-9 concentration in CRSsNP was 34.23 pg/ml ±39.06 pg/ml and the mean value of MMP-9 concentration in CRSwNP was 27.33 pg/ml ±31.58 pg/ml. The box-whisker plots in Figure 3 depict the considerable increase in MMP-9 expression in the CRS groups.

Discussion

The etiopathogenesis of CRS with and without nasal polyps remains unknown. Consequently, the current treatment is symptomatic and involves topical corticosteroids and functional endoscopic sinus surgery (3). There are strong indications of a role for eosinophils in the pathology and a connection to eosinophil diseases such as asthma (4-5). We investigated whether doxycycline is able to reduce the levels of IL-5, eotaxin-3 and MMP-9 as markers for eosinophilic activation and tissue remodelling in CRS (6-8) in cell cultures of patients with CRSsNP and CRSwNP. Our control group were cell cultures of nasal turbinates from healthy individuals.

IL-5. IL-5 is also called eosinophil differentiation factor and is a cytokine regulating eosinophil growth and activation (9). Authors agree that many eosinophil mediated diseases, such

as asthma, rhinitis, and eosinophilic esophagitis are related to increased levels of IL-5 expression (10). Due to the higher concentration of IL-5 in nasal polyps both at the mRNA and at protein levels, CRS seems to have a linkage with this cytokine (11, 12). The significantly elevated IL-5 levels in patients with CRSwNP is confirmed by our data. IL-5 is also of great interest regarding nasal polyps since selectively antagonizing IL-5 with a monoclonal antibody caused a reduction in nasal polyp size maybe through reduction of E-cadherin expression in cell cultures (13, 14). Nevertheless, doxycycline did not meet our expectations regarding lowering of IL-5 expression levels. Although Van Cauwenberge *et al*. described a sustained clinically relevant reduction of polyp size for more than 3 months when administering low-dose doxycycline (14), we saw no difference in IL-5 expression levels. Moreover no reduction of IL-5 expression through systemic application of antibiotics was seen (15), which partly supports our findings. Danielsen *et al*. states that an unchanged IL-5 level in nasal polyps compared to the surrounding tissue is not uncommon as it may describe an equilibrium between the nasal polyps and the surrounding tissue. Interpreting this data, an increased expression of IL-5 in the polyp, therefore, only indicates a potential for polyp growth and so not necessarily an apparent polyp growth (16).

Eotaxin-3. Eotaxin-3 also known as macrophage inflammatory protein 4-alpha, thymic stroma chemokine-1, and chemokine (C-C motif) ligand 26, is an eosinophil specific β-chemokine involved in the accumulation of eosinophils. Shinkai *et al*. discovered its potential to attract eosinophils in vascular endothelial cells to sites of inflammation (17). Many eosinophilic inflammatory diseases, such as atopic dermatitis, allergic rhinitis, asthma and parasitic infections, are therefore believed to be related to increased levels of eotaxin-3 (18). In contrast to other authors, we did not confirm differential eotaxin-3 expression between CRS with and without nasal polyps and the control group (8, 19). Studies directly reporting on eotaxin-3 and the use of doxycycline are lacking. Nevertheless, eosinophilic cationic protein, a protein selectively synthetized in eosinophil granulocytes and being in directly involved in inflammatory reactions as well as in the nasal polyp size, seems to be lowered under the use of doxycycline (15, 20). Therefore a plausible explanation for the significant increase of eotaxin-3 under the use of doxycycline cannot be given. More experiments targeting more eosinophilic markers should clarify the significance of doxycycline in the treatment of CRS.

MMP-9. MMP-9 levels were much greater in the CRS cell cultures compared to the untreated control, yet surprisingly a significant increased expression of MMP-9 in the control group was detected when applying 0.1mg doxycycline. The elevated level of MMP-9, which preferentially degrades gelatin, elastin, aggrecan, and collagens, suggests that this enzyme may be associated with tissue remodeling in nasal polyps, although its precise role in polyp and pseudocyst formation remains unclear (7, 21-25). MMPs are furthermore involved in multiple airway diseases, such as lung cancer, asthma (26), allergic reactions (27, 28) and lung trauma (29, 30) as one of the main sites MMPs are active is the respiratory mucosa (22). Similar expression of MMP-9 in CRSwNP and CRSsNP matches data published before (7), although CRSsNP and CRSwNP are believed to be two different diseases originating from two different pathological mechanisms (31). Doxycycline has shown its potential to ameliorate oxidative stress in tissues with its antioxidant property (32). Oxidative stress is furthermore strongly related to tissue remodelling processes and therefore to MMP-9. Zeydanli *et al*. stated tetracycline class antibiotics and, in particular, a low-dose doxycycline treatment of diabetic rats for four weeks improved endothelial vascular dysfunction of thoracic aortas. Doxycycline treatment furthermore normalized oxidative stress markers, such as of MMP-2 and MMP-9, in these rats (33). As a slight decrease of MMP-9 levels was also detected for CRS in our experiments, these results seem to be in line with their findings. A study of Wang *et al*. with primary human epithelial cells found increased Mucin 5AC levels associated with increased MMP-9 transcripts, protein and activity. However, the increase of Mucin 5AC transcripts and protein were diminished after cells had been treated with doxycycline or MMP-9 siRNA. Doxycycline inhibited MMP-9 transcription, protein production and activity (34). The slight increase of MMP-9 in our control group nevertheless cannot be explained in this way.

Conclusion

Doxycycline seems to have a varied effect on the expression of eosinophilic and tissue remodelling markers important for the growth and perpetuation of nasal polyps and for recalcitrant infection in CRSwNP.

Interleukin-5 levels remain unchanged, eotaxin-3 levels actually increased under doxycycline treatment. The only marker showing a slight drop was MMP-9, albeit not significant.

Although the first clinical trials with doxycycline in the treatment of CRS produced reasonable results, we demonstrated that the underlying pathology is complex, and doxycycline only partly affects the factors believed to support the chronic infection of the respiratory mucosa. More basic research is needed in order to understand the therapeutic mechanisms underlying doxycycline in the treatment of CRS.

Conflict of interest

There is no conflict of interest.

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Received January 20, 2012
Revised February 21, 2012
Accepted February 23, 2012