Abstract. In chronic rhinosinusitis (CRS) an important feature is the infiltration of eosinophils, triggered by T-helper type 2 cells (TH2). Binding of the CpG oligodeoxynucleotide (CpG-ODN) ligand to toll-like receptor 9 (TLR9) induces a shift from a TH2- to a TH1-type response. We evaluated the hypothesis that CpG-ODN could reduce the predominantly TH2-driven response in our cultures. Materials and Methods: Twenty samples from CRS patients with (CRSwNP) and without nasal polyposis (CRSsNP) were cultivated. The expression of interleukin 5 (IL-5), eotaxin 3 and matrix metalloprotease 9 (MMP-9) were evaluated with and without CpG-ODN. Results: Addition of CpG did not influence the expression of IL-5 and eotaxin-3 DNA. Elevated MMP-9 expression in cultures from CRSwNP and CRSsNP patients could be established. Conclusion: CpG does not reduce the attraction of eosinophils since no reduced IL-5 expression was measured in our cultures. Yet, MMP-9 - an important factor in tissue remodelling - was elevated in cultures from CRS patients.

Chronic rhinosinusitis (CRS) is one of the most common chronic diseases in the United States and Europe. Up to 16% of the Western population suffer from CRS (1). Currently, CRS is defined by symptoms and endoscopic findings such as inflammation of the nasal and paranasal sinus mucosa, that presents for durations of longer than three months (2). Furthermore, it is associated with mucosal alterations ranging from inflammatory thickening to nasal polyps (3). Patients complain of thick mucus production and subsequent nasal congestion, reduced olfaction and secondary acute exacerbations with an increased bacterial infection of the nasal sinuses. These symptoms have a serious impact on the patient’s quality of life. One study has even suggested that their quality of life is more severely impaired than patients with congestive heart failure (4). Due to the unknown pathophysiology, CRS treatment is still a challenging process. Furthermore, we have to distinguish CRS with nasal polyps (CRSwNP) from CRS without nasal polyps (CRSsNP). A predominant histological feature of CRS is a persistent underlying infiltration by eosinophils (5). This accumulation of eosinophils might be caused by an influx of T-helper type 2 (TH2) cells with a subsequent dominant TH2 cytokine profile (2). The TH2 lymphocytes and eosinophils release interleukin (IL)-3, IL-5, IL-13 and the pro-inflammatory cytokine profile (2). The TH2 lymphocytes and eosinophils release interleukin (IL)-3, IL-5, IL-13 and the pro-inflammatory cytokine profile (2). These cytokines promote the production, recruitment and activation of eosinophils. IL-5 expression was observed in nasal polyps of patients suffering from CRSwNP (10). In CRSsNP, IL-13 is also significantly elevated in sinus lavage from patients with CRS compared to non-CRS controls. IL-13 seems to affect the function of matrix metalloproteases, which remodel the extracellular matrix (11). For example, addition of IL-13 could induce a significant increase in β-catenin expression in eosinophilic CRS cultures compared to non-eosinophilic cultures (7). Eotaxin, especially eotaxin-3, is supposed to play a pivotal role in the regulation of eosinophilic inflammation and extracellular matrix breakdown in CRSwNP. Due to the fact that MMP-9’s exact role in polyposis still remains unclear (12) and MMP-9 is involved in tissue remodelling (11) this study was also set-up to determine the expression of MMP-9. Additionally, Lim et al. found elevated expression of MMP-9 in CpG stimulated murine macrophages (13). Compared to bacterial DNA, vertebrate DNA shows an under-representation of the cytosine phosphate-guanosine (CpG) complex. In vertebrate DNA, the cytosine in the CpG motif is mostly methylated compared to bacterial DNA, that is not methylated (14). The unmethylated CpG dinucleotides cause an activation of the innate immune system, via toll-like...
receptor 9 (TLR9). The activation of the immune system may be reproduced by the application of synthetic CpG-oligodeoxynucleotides (ODN) (15). TLR9 is expressed by leukocytes and able to detect CpG motifs in DNA (16). Binding of the CpG-ODN ligand to TLR9 induces a shift from a TH2- to the TH1-type response in the target tissue (17). It has been shown that the TH2 dominant inflammatory responses in the lungs could be prevented by the application of CpG-ODN (18). Furthermore, eosinophilic inflammation was abrogated in a murine model of asthma by CpG-ODN (19).

In the present study, we tested the hypothesis that CpG-ODN would reduce the predominantly TH2-driven chronic inflammation in our cultures.

Materials and Methods

Patients, tissue collection and cultures. Tissue collection and experiments were carried out in the same manner as already outlined in a previous study (20). Cultures were obtained from patients suffering from CRSwNP or CRSsNP and undergoing functional endoscopic sinus surgery (FESS) at the Department of Otorhinolaryngology at the University Hospital Mannheim, Germany. Each group consisted of cultures from five different patients. The specimens from the control group were obtained from patients suffering from inverted papilloma during FESS. Written consent was obtained from all patients and the study was approved by the Ethics Committee of the Faculty of Medicine, Mannheim, University of Heidelberg, Germany.

Test agent. The test agents were oligodeoxynucleotides (Eurofins MWG Operon, Ebersberg, Germany) containing the following sequence: CpGas: TCC ATG AGC TTC CTG AGT CT and CpGsc: TCC ATG AGC TTC CTG AGT CT (Scramble siRNA).

Incubation with CpG-ODN. ODNs were added in a concentration of 20 μg/ml when the cultures showed at least 70% confluence (day 0) to all samples, except for the "negative control". IL-5, eotaxin-3 or MMP-9 concentrations in 100 μl of supernatants was determined (20).

Storage of culture supernatants was performed in sterile test tubes at −20°C until an ELISA technique was evaluated after 24, 48, 72 and 96 h.

Cytokine immunoassay. Storage of culture supernatants was performed in sterile test tubes at −20°C until an ELISA technique was used to examine the expression of the interleukin, cytokine and proteinase. The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against the objectives. The exact products used included the following: DuoSet Human Total MMP9 Elisa/Order No. DY 911/R+D Systems/Minneapolis/USA; BD OptEIA Human IL-5 Elisa Set/Order No. 555202/BD Biosciences, Franklin Lakes, New Jersey, USA; BD OptEIA Human Eotaxin Elisa Set/Order No. 555175/BD Biosciences, Franklin Lakes, New Jersey, USA.

According to the manufacturer’s instructions, each ELISA assay determined IL-5, eotaxin 3 or MMP-9 concentrations in 100 μl of supernatant. The cells were grown in 96-well plates with 12 strips of 8 walls coated with an antibody against either of the proteins of interest. After 24-96 h of incubation, the expression of markers of interest in the supernatants was determined (20).

Statistical analysis. Statistical analysis was performed with SAS (SAS/STAT; Version 8, SAS Institute Inc., Cary, NC, USA). p-Values were calculated by using the Student’s t-test and Dunnett’s procedure against the untreated control group (21). The level of statistical significance was defined to be p<0.05, as no Bonferroni adjustments were necessary. Plotting was done using “R”, an open source environment for statistical computing and graphics (22).

Results

Regarding the incubation duration, there was no significant difference in expression of IL-5, eotaxin-3 or MMP-9 after 24, 36, 72 and 96 h.

IL-5. Treatment with CpG did not have any significant effect on the expression of IL-5. But, IL-5 concentration seemed to be altered when comparing the control group (0.095 pg/ml ±0.027 pg/ml; p=0.097) with the CRSwNP group (0.142 pg/ml ±0.357 pg/ml), and when comparing the CRSsNP group (0.073 pg/ml ±0.134 pg/ml; p=0.05) with the CRSwNP group. No significant effect on the expression of IL-5 was found when using CpGas or CpGsc as an addendum. For details see Figure 1.

Eotaxin-3. No significant effect on the expression of eotaxin-3 in the different groups could be shown by adding CpG to the cultures, regardless of whether it was CpGas or CpGsc (control group: 4.72 pg/ml±2.343 pg/ml; CRSwNP group: 4.262 pg/ml±1.577 pg/ml; CRSsNP group: 4.405 pg/ml±2.293 pg/ml). For details see Figure 2.

MMP-9. During treatment with CpG, a significant alteration in the MMP-9 expression was observed. These alterations were observed between the control group (6.43 pg/ml±1.78 pg/ml) and the CRSwNP group (47.186 pg/ml±48.914 pg/ml; p<0.001), as well as between the control group and the CRSsNP group (33.037 pg/ml±53.244 pg/ml; p<0.001). However, between the two CRS groups, no significant effect was observed (p=0.418). As an addendum, no significant effect on the expression of MMP-9 was shown when using CpGas or CpGsc. Figure 3 shows graphs of data.

Discussion

The innate immune system includes the so-called toll-like-receptors (TLRs). The family of TLRs consists of 13 members (TLR1 to TLR13). They recognize various structures such as lipoproteins, flagellin, lipopolysaccharide and nucleic acids (23). TLR9 mediated recognition of viral and bacterial DNA (in terms of CpG) activates the innate immune system and plays a critical role in adaptive immunity (24). It has already been shown that TLR9 expression is the same in patients with CRSwNP or CRSsNP and in patients without CRS. Due to the fact that TLR9 stimulation results
in a strong TH1 response and induces a TH2/TH1 shift (25), we expected that the CpG-ODN addition to our cultures would reduce the predominantly TH2-driven chronic inflammatory state in our cultures. In a murine allergic model it has already been shown that CpG-ODN prevents the development of TH2-mediated eosinophilic inflammation and symptoms of allergic rhinosinusitis (19).

**IL-5.** The factor concentration did not have a significant effect on the expression of IL-5. Only a trend to an alteration of IL-5 expression could be seen when comparing the control group with the CRSswNP group and the CRSswNP with the CRSsNP group. The cytokine IL-5, which is also called eosinophil differentiation factor, is a regulation factor of eosinophil growth, activation and expression (26). Eosinophil-mediated diseases are linked to increased levels of IL-5 expression (e.g., asthma, rhinitis and eosinophilic esophagitis) (27). It also plays a critical role in CRS. In CRSswNP, significantly elevated IL-5 levels were reported (28), in accordance to our results, which displayed an elevation tendency. Additionally, high mRNA and protein levels of IL-5 have been detected in nasal polyposis (29). Hussain et al. showed that treatment with CpG oligodeoxynucleotides could prevent the development of TH2-mediated eosinophilic inflammation and symptoms in a murine model of allergic rhinosinusitis (19). This has also been shown in other diseases with eosinophilic inflammation. In murine and primate models of allergen-induced airway hyper-responsiveness, CpG inhibits IL-5, eosinophilic inflammation, and airway hyper-responsiveness (30, 31). In our cultures, we could not confirm these findings, a fact that could be attributed to the different culture models used (30, 31). Our results showing a tendency of altered IL-5 levels in
CRSwNP compared to both the control and CRSsNP groups, which supports the well-established theory that IL-5 plays a role only in CRSwNP (32). Another explanation for our results might be that the limited surface area of cell cultures—compared to the sinus mucosa—does not allow for revelation of alterations in eosinophilopoiesis.

Eotaxin 3. Eotaxin, also called chemokine (C-C motif) ligand 26 (CCL26), macrophage inflammatory protein 4-alpha (MIP-4-alpha), or thymic stroma chemokine-1 (TSC-1), is an eosinophil specific β-chemokine. Shinkai et al. demonstrated that eotaxin 3 is involved in the accumulation of leukocytes, especially eosinophils. The hypothesis states that eosinophils are activated by eotaxin 3 expressed on vascular sites of endothelial cells, extravasate from the bloodstream, and then migrate to the center of the inflamed tissue through the action of eotaxin (33). In eosinophilic inflammatory diseases such as atopic dermatitis, allergic rhinitis, asthma and parasitic infections, it is assumed that eotaxin is a key protein in their pathophysiology (34). In our cultures, the addition of CPG did not influence eotaxin 3 DNA concentration at all. This is partly inconsistent with the results from Yao et al., where eotaxin 1, 2 and 3 levels were all elevated in eosinophilic nasal polyposis (6). Furthermore, Olze et al. found a correlation between eotaxins and tissue eosinophilia in CRSwNP (35). A consistent explanation why eotaxins, as key chemotactic factors for eosinophils in eosinophilic nasal polyposis, were not altered by CPG treatment in our experiments, cannot be given by the authors.

MMP-9. During the treatment with CpG, significant alterations in the MMP-9 expression could be evaluated when comparing the control group with both the CRSwNP group and the CRSsNP group. Metalloproteinases are proteolytic enzymes mainly responsible for the remodelling of the extracellular matrix (ECM). Since they play an important role in tissue remodelling, they are of particular interest in the research of airway diseases. During tissue remodelling, tissue structures and interstitial stroma of the affected mucosa are broken-down and repaired. Many different metalloproteinase sub-groups, which share important common properties (e.g., they require an active Zn$^{2+}$ site for their catalytic function), have been described (36). MMPs are active in the respiratory mucosa. Aggrecan, elastin, collagens and gelatine are mainly degraded by MMP-9. Elevated concentrations of MMP-9 can be found in diseases like lung cancer, asthma, allergic reactions or lung trauma (37-41). In nasal polyposis and chronic sinusitis, differences in MMP regulation and tissue inhibitors of metalloproteinase (TIMP) regulation could contribute to the differences in tissue remodelling observed with histomorphological examination (36, 42). However, MMP 9’s exact role in polyp and pseudocyst formation still remains unclear (12). Elevated expression of MMP-9 has been described in murine macrophages by TLR9 stimulation with CpG (13). Therefore, our results confirm the findings of Lechapt-Zalcman et al. who reported an elevated concentration of MMP-9 in inflammatory endothelial cells in nasal polyps (43). No significant difference between patients with CRSwNP and CRSsNP in MMP-9 expression could be found, that is in line with a study by Watelet et al. (36). We assume that this is associated with the lack of difference in expression of TLR9 between controls and patients with CRSwNP or CRSsNP (44).

Conclusion

In conclusion, addition of CpG did not have any influence on the expression of IL-5 or the concentration of eotaxin 3 DNA. However, it affected MMP-9 expression in terms of an elevation in cultures from CRS patients with or without nasal polypsis no matter if Cpgas or CpGsc was used. Due to these results, we assume that CpG does not reduce the attraction of eosinophils. Elevated levels of MMP-9 expression in CRS could not be reduced by the addition of CpG to the cultures. Due to the fact that CpG stimulation increased the percentage of epithelial and polyp cells expressing TLR9, we expected more differences in MMP-9 expression, but could not confirm that. In allergic rhinosinusitis in BALB/c mice, the addition of CpG during ovalbumin sensitization prevents the development of TH2-mediated eosinophilic inflammation and symptoms result in a model of allergic rhinosinusitis (19). Our data could not confirm these reduced IL-5 levels in our cultures. As some results only show trends towards a change, more studies are necessary to elevate these effects.

Conflicts of Interest

There exist no conflicts of interest.

References


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