LARYNGOLOGY

Differential chemokine expression patterns in tonsillar disease

Differenti pattern di espressione delle chemochine nella patologia tonsillare

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SUMMARY

Expression profiles of CXC- and CC-chemokines in various forms of tonsillar disease were studied to evaluate whether certain chemokines play a predominant role in a specific subset of tonsillar disease. Total RNA was isolated from 89 biopsies (21 hyperplastic palatine tonsils, 25 adenoids, 16 chronic inflammatory palatine tonsils and 27 chronic inflammatory palatine tonsils with histological prove of acute inflammation), reverse transcribed and subjected to PCR amplifying IL-8, Gro-alpha, eotaxin-1, eotaxin-2, MCP-3, MCP-4 and RANTES. 2% agarose gel electrophoresis revealed a predominance of IL-8 in the chronic inflammatory palatine tonsil group compared to tonsillar hyperplasia. Furthermore, eotaxin-2 was strongly overexpressed in adenoid samples compared to chronic inflammatory specimens. Our data suggest that the majority of diseases related to adenoid formation are mediated via an eotaxin-2 expression, whereas chronic inflammatory tonsillitis is associated with IL-8 upregulation. These data imply that adenoids are related to a Th-2, and chronic inflammatory tonsillitis to a Th-1 based immune response.

KEY WORDS: Chemokines • Tonsillar disease • Eotaxin-2 • Interleukin-8

Introduction

In 1884, Waldeyer first described an annular arrangement of lymphatic tissue at the entrance of the aerodigestive tract, which he suggested plays an important part in the host immune system, including the palatine tonsils, pharyngeal tonsil, lingual tonsils and tubal tonsils. The palatine as well as pharyngeal tonsils are known to play a key role in the development of the human host defense due to their location, histologic anatomy and functional capacities. These structures harbour numerous different types of tissues, including epithelium, lymphoid follicles, blood vessels and connective tissues. The combination...
of these different tissues as well as their anatomic arrangement in crypts facilitates optimised exposure and processing of antigens, which aid in evoking effective immune responses. The palatine as well as the pharyngeal tonsils are secondary lymphoid organs and an integral part of the mucosa associated lymphatic tissue complex. Multiplication, differentiation and stimulation of B-lymphocytes represent one of their major functions and are essential in processing the specific immune response. B-lymphocytes constitute more than 50% of the tonsillar lymphocyte population, in contrast to the blood and other lymphoid organs. The induction of the tonsillar-specific immune responses involves several distinct processes where chemokines play an integral role. Chemokines are chemotactic cytokines, which can be classified into four subgroups depending on their arrangement of amino acids, namely CXC-, CC-, C- and CX3C-chemokines. CXC-chemokines, like interleukin (IL)-8 and growth-regulated peptide–alpha (GRO-alpha), are responsible for chemotaxis of neutrophils, whereas CC-chemokines, like eotaxin-1, eotaxin-2, monocyte chemotactic protein (MCP)-3, MCP-4, and RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), promote the attraction of lymphocytes, monocytes, eosinophils and basophils.

The presented work investigated the importance of different chemokines, i.e. IL-8, GRO-alpha, eotaxin-1, eotaxin-2, MCP-3, MCP-4 and RANTES in tonsillar inflammatory disease and tonsil hyperplasia.

**Materials and methods**

**Tissue samples**

This study included 89 patients who underwent surgery at the Department of Otorhinolaryngology, Head and Neck Surgery at the University of Schleswig-Holstein, Campus Kiel, Germany, for hyperplasia of either the palatine or pharyngeal tonsil, or chronic inflammation of the palatine tonsil, defined as recurrent acute tonsillitis over a period of >6 months. Samples were retrieved during surgery after written patient consent was obtained, in accordance with the ethical commission of the Christian-Albrechts-University of Kiel, subjected to the 1975 Helsinki Declaration. Tissue samples were divided into four subgroups, based on origin, patient past medical history, clinical presentation and histologic diagnosis established by certified pathologists of the University of Schleswig-Holstein, Campus Kiel. The subgroups included 21 hyperplastic palatine tonsils, 25 adenoids, 16 chronic inflammatory palatine tonsils and 27 chronic inflammatory palatine tonsils with histologic signs of acute inflammation as well as presence of cryptal ulcerations and leukocyte infiltration. At the time of procedure, the median of age for each subgroup was 6, 5, 26, and 7, respectively. Immediately after collection, samples were frozen in liquid nitrogen, and stored at -80°C for further processing.

**RNA isolation**

Frozen tissue samples were ground by mortar, and 1 ml of TRIzol™ reagent (Gibco, Ingolstadt, Germany) was applied upon 200 mg of tissue. Total RNA was isolated following the manufacturer’s instructions. After determination of the RNA content using the UVIKon-931 UV-spectralphotometer (Kontron, Hamburg, Germany), samples of total RNA were adjusted to 1.0 µg for first strand cDNA synthesis. Quality assessment of the RNA was conducted using a 1% agarose ethidium-bromide stained gel electrophoresis.

**Reverse transcription**

1.0 µg RNA was heat-denatured (65°C, 10 min), chilled on ice, and subjected to random hexadeoxynucleotide primed reverse transcription using the first strand cDNA synthesis kit (Pharmacia, Freiburg, Germany). Reverse transcription (final volume 15 µl) was conducted at 37°C for 60 min in the presence of 0.2 µM of random hexanucleotide primer and 40 U RNase inhibitor (RNAsin, Gibco, Germany). Following synthesis of the completed first strand cDNA the resulting RNA-cDNA double-stranded helix was heat-denatured (95°C, 5 min) to provide cDNA as a template for polymerisation.

**Primers**

We used the following oligonucleotides for high-stringency PCR reaction as listed below. Glutaraldehyde-3-phosphate-dehydrogenase (G3PDH) was used to compare expression of the genes mentioned below:

- **RANTES** sense: 5’-CAT CCT CATT GCT ACT GCC CTC TG-3’, **RANTES** antisense: 5’-TAA CTG CTG GTC GTC GTC-3’;
- **Eotaxin-1** sense: 5’-CAT CCT TGC CTC CTG CTC TG-3’, **Eotaxin-1** antisense: 5’-CGG GTT CAC GCC ATT CTC CT-3’;
- **Eotaxin-2** sense: 5’-CAC ATC ATC CCT ATC GCC GGC TCT-3’, **Eotaxin-2** antisense: 5’-GTT TGC GAG ATC ACT GAT CAC CAG GGC CC-3’;
- **MCP-3** sense: 5’-GAG CTA CAG AAG GAC CAC CAG TGC-3’, **MCP-3** antisense: 5’-AAG TCC TGG ACC CAC TCT TG-3’;
- **MCP-4** sense: 5’-TCA TCT TCC TAC AAT AAC ATA-3’, **MCP-4** antisense: 5’-GTG CGT TAT TTG AGT ATT GCT GAT CTT T-3’;
- **IL-8** sense: 5’-CTT GCA GAG ACA GCA GAG CAC-3’, **IL-8** antisense: 5’-ACT GTG AGG TAA GAT GTG GGC-3’;
- **GRO-alpha** sense: 5’-TGA ACT GCG CTG CCA GTG C-3’, **GRO-alpha** antisense: 5’-ATGAGCCCGAGCCTTCTCAT-3’.
Polymerase chain reaction (PCR)
Reverse transcribed cDNA products (0.2 5µl) were incubated in 50 µl reaction mixture containing 0.2 µM 5'-3' sequence specific sense oligonucleotide primers, 0.2 µM of 3'-5' corresponding antisense oligonucleotide primers, 200 µM dNTP's, 1.5 mM MgCl₂, 5.0 µl 10x PCR-buffer, and 2.5 U Taq-polymerase (Gibco, Ingolstadt, Germany). The reaction mixture was covered with a mineral oil layer (Applied Biosystem, Weiterstadt, Germany) to prevent evaporation. The PCR was conducted in a Biometra T3 thermocycler. Following initial denaturation (3 min at 95°C), high stringency PCR was run for 34 cycles (94°C for 75 sec, 60°C for 30 sec, and 72°C for 2 min) with an increased annealing temperature of 67°C in the first two cycles, to amplify the RANTES, Eotaxin-1, Eotaxin-2 and G3PDH cDNA. In case of the other chemokines, the PCR parameters were modified to a 40 cycles of 95°C for 60 sec, 60°C for 30 sec, and 72°C for 2 min, with an increased annealing temperature of 68° to 60° over the first 8 cycles. After PCR, all samples were subjected to ethidium-bromide stained 1.5% agarose gel electrophoresis.

Densitometry
The amplicons were evaluated in quantity using Herolab E.A.S.Y. Win32 software (Herolab, Wiesloch, Germany). At first, G3PDH bands were compared among each other, in order to assess relative sample signal strength. Subsequently, all other signals of the chemokine bands were adjusted to the relative strength by division through the G3PDH band signal.

Statistical analysis
All densitometric data obtained from the SQRT-PCR were analysed using SPSS 9.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). All data assembled in this study were tested for normal distribution using the Kolmogoroff-Smirnov test. Expression profiles of each chemokine were analysed among each group using simple block variance analysis and Kruskal-Wallis test for normally and non-normally distributed data, respectively. A p-value < 0.05 was considered significant, and a p-value < 0.01 was considered highly significant.

Results
Quality assessment of the isolated RNA was made by agarose gel electrophoresis exhibiting non-fragmented RNA with sufficient quantity for reverse transcription and subsequent processing. After RT-PCR procedures, samples were processed by agarose gel electrophoresis, and the amplicons of the chemokines were measured and adjusted to the relative G3PDH signal strength, comparative analysis was initiated. All data followed normal distribution and are displayed as mean ± SD in the following.

Four cohorts were examined: patients with tonsillar hyperplasia (n = 21), adenoids (n = 25), chronic tonsillitis (n = 16) and chronic tonsillitis with histological proof of acute inflammation (further in the text referred to as “acute tonsillitis”) (n = 27).

RANTES and Eotaxin
Relative RANTES total-mRNA expression showed a median of 1.37 for tonsil hyperplasia, 1.58 for adenoids, 1.32 for chronic tonsillitis and 1.56 for acute tonsillitis (Table I, Fig. 1A). As shown in Table II and Fig. 1B, relative mRNA expression for eotaxin-2 was 1.02 in tonsillar hyperplasia (median value), 1.34 for adenoids, 1.16 for chronic tonsillitis and 1.16 for acute tonsillitis. A significant overexpression of eotaxin-2 was observed in adenoids compared to patients with chronic tonsillitis (p < 0.05).

IL-8 and GRO-alpha
Relative expression of IL-8 are shown in Table III and Figure 2A. A relative IL-8-mRNA expression was found to be a median of 1.12 in tonsillar hyperplasia, 1.97 for adenoids, 1.82 for chronic tonsillitis and 1.41 for acute tonsillitis. IL-8 was significantly overexpressed in patients with chronic inflammatory tonsillar disease (with and without acute inflammation) compared to tonsil hyperplasia. Relative GRO-alpha mRNA expression levels are shown in Table IV and Figure 2B. Median values were 0.84 for tonsillar hyperplasia, 1.07 for adenoids, 0.60 for chronic tonsillitis and 0.55 for acute tonsillitis. All other examined cytokines were not significantly expressed in the groups analysed.

Discussion
Waldeyer’s tonsillar ring acts as the first line of immune defence against microbes, entering the body nasally or orally. Especially in children, the immunogenic properties of the palatine tonsils are of particular importance. Chemokines are small signalling proteins, whose expression in various tissues is variably regulated during immune responses as well as acute and chronic infection. Previous studies have suggested a functional role for chemokines in hepatitis, colitis, pancreatitis, asthma and various malignancies as well as acute and chronic infections of the upper aerodigestive tract 18-23. Most chemokines are only produced and secreted upon appropriate stimulation of cells by bacterial or viral products 24. In cases of acute and chronically infected tonsils, accumulation of certain subsets of chemokines and neutrophilic dynamics has been observed 25.
Table I. Relative expression of total RNA for RANTES. A total of 89 tissue samples were analysed.

|                      | Tonsillar hyperplasia (n = 21) | Adenoids (n = 25) | Chronic tonsillitis (n = 16) | Acute tonsillitis (n = 27) |
|----------------------|-------------------------------|-------------------|-----------------------------|---------------------------|
| Minimum              | 0.41                          | 0.00              | 0.54                        | 0.00                      |
| 1. Quartile          | 1.07                          | 1.34              | 0.97                        | 1.15                      |
| Median               | 1.37                          | 1.58              | 1.32                        | 1.56                      |
| 3. Quartile          | 1.79                          | 2.11              | 1.45                        | 1.97                      |
| Maximum              | 5.57                          | 2.46              | 1.95                        | 2.46                      |

Table II. Relative expression of total RNA for Eotaxin-2. A total of 89 tissue samples were analysed.

|                      | Tonsillar hyperplasia (n = 21) | Adenoids (n = 25) | Chronic tonsillitis (n = 16) | Acute tonsillitis (n = 27) |
|----------------------|-------------------------------|-------------------|-----------------------------|---------------------------|
| Minimum              | 0.55                          | 0.40              | 0.63                        | 0.30                      |
| 1. Quartile          | 0.89                          | 1.16              | 0.84                        | 0.80                      |
| Median               | 1.02                          | 1.34              | 0.95                        | 1.16                      |
| 3. Quartile          | 1.31                          | 1.60              | 1.21                        | 2.14                      |
| Maximum              | 3.83                          | 2.50              | 1.82                        | 2.86                      |

Fig. 1. (A) Relative expression of total RNA for RANTES. (B) Relative expression of total RNA for eotaxin-2. Expression was correlated to the expression of the housekeeping gene G3PDH in each sample. The plots represent 89 tissue samples. * p < 0.05, ** p < 0.01.

Fig. 2. (A) Relative expression of total RNA for IL-8. (B) Relative expression of total RNA for gro-alpha. Expression was correlated to the expression of the housekeeping gene G3PDH in each sample. The plots represent 89 tissue samples. * p < 0.05, ** p < 0.01.
IL-8 has been described to be very potent in neutrophil activation and migration 26–27. Our data shows significant overexpression of IL-8 in chronic inflammatory tonsillar disease. Since IL-8 expression has been reported to be effectively stimulated by TNF-α and IL-1β for neutrophil chemotaxis as well as activity, our data suggests that a Th1 response is predominantly involved in the pathogenesis of chronic tonsillitis.

Both IL-8 and GRO-α are known to be synthesised by neutrophils and fibroblasts in response to various stimuli 28–29. In vitro, IL-1β and TNF-α seem to be potent stimulators of chemokine production, whereas IFN-γ inhibits their production 10. GRO-alpha is further produced by endothelial cells, fibroblasts and monocytes after stimulation with lipopolysaccharide, IL-1 or TNF-α in vitro. In addition, it induces neutrophil accumulation and chemotaxis 29. In our data however, GRO-alpha was not significantly overexpressed in chronic tonsillar diseases.

Tonsillar hyperplasia appears to be a result of increased proliferation of lymphoid tissue predominantly triggered by bacterial infections. Previously, tonsil size has been shown to be directly proportional to the mean bacterial load 30. The kind of bacteria found in hyperplastic tonsils does not seem to greatly differ from those in recurrently active infected tonsils. However, Haemophilus infection, besides Staphylococcus aureus and Streptococcus pyogenes appears to be more common in tonsillar hyperplasia 30. Our investigations demonstrate that hyperplastic tonsillitis is characterised by an acute inflammatory chemokine pattern as IL-8 expression on mRNA levels correlates with the presence of actively infected tissue. IL-8 expression was significantly elevated in acutely infected tissue compared to hyperplastic tonsils (p < 0.01), and in adenoids and chronic tonsillitis compared to hyperplastic tonsils (p < 0.05). Therefore, as IL-8 is an acute phase chemokine expressed in chronic tonsillitis, this suggests an inflammatory process in the pathogenesis of chronic tonsillitis. An elevation of IL-8 in acute infections has been described previously 31–32, and therefore anticipated for tonsillar disease. However there have been studies showing an equal expression of IL-8 in hyperplastic tonsils and chronic tonsillitis 33. Another reason for a high expression of this chemokine in chronic and hyperplastic disease could be its additional extensive effect on cell proliferation 34.

A similar result would have been expected for the expression of GRO-alpha, since this chemokine also plays an important role in host immune defence by conveying chemotaxis and activation of neutrophils, similar to IL-8. However, in our data, mRNA expression levels of GRO-alpha were not significantly elevated in acute nor chronic tonsillitis compared to tonsillar hyperplasia.

MCP 1-4, RANTES, eotaxin, eotaxin-2 und eotaxin-3 are CC-chemokines. RANTES is a selective attractant for T-cell and monocytes migration 35. Proinflammatory cytokines, such as TNF-α or IL-1β, have been described to be some of the most potent stimulators of RANTES expression. Furthermore, a combination of TNF-α and INF-γ strongly stimulate production of RANTES 36. Another important function of RANTES is its ability to enhance the mucosal as well as systemic humoral production of antibodies, via an elevation of the production of IFN-γ, IL-2-, IL-5-and IL-6, and further an induction of co-stimulatory molecules as well as expression of cytokine receptors for CD 4+T cells 37.

In our data, RANTES expression was evident in all subgroups analysed with no significant differences in relative mRNA expression levels within these groups.

Eotaxin-2 is also described to be a potent chemoattractor for eosinophils in vitro and in vivo 38–39. In eosinophils, eotaxin-2 causes a dose-dependent increase of the production of free radicals, mobilisation of intracellular calcium and upregulation of CD11b 40. Eotaxin-2 expression was evident in all analysed subgroups with a significant upregulation in adenoids, suggesting an involvement of Th2- immune responses as eotaxin-2 which is known to play a crucial role in the pathogenesis of atopic diseases involving Th 2- cell activation 41. Significant upregulation of eotaxin-2 has been

| Table III. Relative expression of total RNA for IL-8. A total of 89 tissue samples were analysed. | Table IV. Relative expression of total RNA for Gro-alpha. A total of 89 tissue samples were analysed. |
|----------------------------------|----------------------------------|
| **Tonsillar hyperplasia**         | **Adenoids**                      |
| **(n = 21)**                     | **(n = 25)**                     |
| **Chronic tonsillitis**          | **(n = 16)**                     |
| **Acute tonsillitis**            | **(n = 27)**                     |
| Minimum                          | 0.13                             | 0.00                             |
| 1. Quartile                      | 0.78                             | 0.43                             |
| Median                           | 1.12                             | 0.84                             |
| 3. Quartile                      | 2.55                             | 1.43                             |
| Maximum                          | 2.90                             | 1.90                             |
| **Table III. Relative expression of total RNA for IL-8. A total of 89 tissue samples were analysed.** | **Table IV. Relative expression of total RNA for Gro-alpha. A total of 89 tissue samples were analysed.** |
| **Minimum**                      | 0.27                             | 0.61                             |
| **Adenoids (n = 25)**            | 1.20                             | 0.59                             |
| **Chronic tonsillitis (n = 16)** | 1.82                             | 0.60                             |
| **Acute tonsillitis (n = 27)**   | 1.41                             | 0.91                             |
| **Maximum**                      | 4.98                             | 2.14                             |
| **Median**                       | 2.61                             | 2.14                             |
| **1. Quartile**                  | 1.07                             | 1.12                             |
| **3. Quartile**                  | 2.06                             | 1.28                             |
| **Maximum**                      | 2.86                             | 2.97                             |
observed in bronchial asthma 42 and allergic rhinitis 43. However, the significance of eotaxin-2 expression in lymphatic tissue of the Waldeyer’s tonsillar ring still largely remains uncertain, like their role in the maturation of the adaptive immune system in the mucosa of the upper aerodigestive tract. However, it is remarkable that eotaxin-2 as well as RANTES are constitutively expressed in the mucosa of the gastrointestinal tract, an organ with distinctive antigen contact, such as Waldeyer’s tonsillar ring 44-45.

To further evaluate the role of the chemokines analysed in the function of the lymphatic tissue of the Waldeyer’s tonsillar ring, it is essential to evaluate their expression on protein level to examine alterations through potential post-transcriptional splicing. Also, immunohistochemical studies would be of interest to evaluate their expression in relation to certain cell populations. These studies would greatly aid in understanding the pathophysiology of tonsillar disease, from which patients could benefit in the future.

Conclusions

The presented data suggest that the majority of diseases related to adenoid formation are mediated via an eotaxin-2 expression, whereas chronic inflammatory tonsillitis is associated with IL-8 upregulation. Thus, these data imply that adenoids are related to a Th-2 response, and chronic inflammatory tonsillitis to a Th-1 based immune response.

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