Quantification of the mRNA encoding Tumor Necrosis Factor α (TNFα) and its receptors in human nasal polyps

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ABSTRACT

Purpose: The object of the study was to assess the expression of the genes encoding TNFα and its receptors (TNF-R1 and TNF-R2) in patients with nasal polyps (NP).

Material and methods: The number of the mRNA copies was assessed by QRT-PCR in RNA extracts from 16 eosinophilic (ENP) and 5 neutrophilic nasal polyps (NNP), and 9 normal mucosa (NM) samples. The expression of corresponding proteins was demonstrated using immunohistochemistry.

Results: The mean level of mRNA copies for TNFα in ENP (82229c/μg) was not significantly higher when compared with controls (74869c/μg). NNP demonstrated significantly lower mean TNFα gene expression (7021c/μg) than the controls (p<0.05). A statistically higher mRNA TNFα copy number in ENP than in NNP was also revealed (p<0.01). A noticeably lower mRNA expression of TNF-R1 in ENP and NNP was seen as compared to the control group (10198c/μg vs. 30749c/μg, p<0.05 and 3440c/μg vs. 30749c/μg; p<0.05 respectively). In ENP the mean TNF-R2 mRNA copy number was markedly higher than in NNP (185c/μg vs. 7.6c/μg, p<0.05). TNF-R2 mRNA level did not differ significantly between ENP and the control group (185c/μg vs. 469c/μg). TNF-R1 expression was significantly higher than TNF-R2 at the mRNA (p<0.01) and protein (p<0.05) level both in ENP and NNP. No significant correlations in proteins expression were detected between ENP and NNP.

Conclusions: TNF-R1 has been identified to be a prevalent form of the TNFα receptor in nasal polyps which may reflect the apparent dominance of this form in TNFα signalling. The findings raise the possibility that the eosinophils from NP may influence biological responses through TNFα-dependent mechanisms. The differences between ENP and NNP relating to TNFα and the expression of its receptors may reflect the distinct character of those diseases.

Key words: TNFα, TNF-R, QRT-PCR, immunohistochemistry, nasal polyps, polymerase chain reaction

INTRODUCTION

The pathophysiology of nasal polyps (NP) is considered to be the ultimate manifestation of chronic inflammation of the upper respiratory tract of unknown aetiology. Histomorphologically, NP show oedema, inflammatory cell infiltration with numerous, activated eosinophils and various degrees of tissue remodelling in the epithelium, glands, connective tissue and vessels [1]. Tissue eosinophilia is a general characteristic of NP and the oedematous-eosinophilic type of NP is the predominant histological form, with an incidence rate of over 90%. The neutrophilic, fibroinflammatory form of NP is much less common [2].

Due to the complexity of this process, it is not surprising that NP formation is regulated through the release of a wide spectrum of inflammatory mediators and cytokines. Cytokine that acts as a key mediator in the local inflammatory and immune response is a tumor necrosis factor α (TNFα). TNFα is a pleiotropic inflammatory cytokine produced by many cells including monocyte/macrophages, T-cells, NK cells, mast cells, eosinophils and epithelial cells. TNFα exerts an extreme spectrum of bioactivities and appears to affect most body organs. However, its final effect strongly depends on the targeted cells, duration and quantity of TNFα expression. In
patho- and physiological situations, TNFα shows a remarkable functional duality, being strongly engaged both in tissue regeneration/expansion and also destruction. TNFα induces the elaboration of chemokines and up-regulates the expression of cell adhesion molecules (ICAM-1, VCAM-1, P-selectin and E-selectin) in the endothelial cell which promotes leukocyte adhesion to the vessels [3] and transendothelial migration of eosinophils being the most abundant inflammatory cells in NP [4].

The effects of TNFα, like other cytokines, are mediated by its binding to high-affinity receptors (TNFRs). TNFRs are a family of proteins that consist of, to date, at least 27 members. However, TNFα only has the ability to bind two of them called TNF-R1 (p55/60) and TNF-R2 (p75/80) [5]. TNF-R1 is constitutively expressed in most tissues, whereas the expression of TNF-R2 is more limited and typically found in cells of the immune system. The importance of TNF-R2 is likely to be underestimated [6].

The aim of the paper was to analyze the expression of genes coding TNFα and related TNF-R1 and TNF-R2 receptors in nasal polyps by the estimation of mRNA expression. The study was performed in respect to the histological findings which divided NP into eosinophilic (ENP) and neutrophilic (NNP). Simultaneously, TNFα and its receptors protein expression were examined by immunohistochemistry.

MATERIAL AND METHODS

Subjects
Twenty-one patients (14 males and 7 females) with NP treated surgically at the Department of Otolaryngology, Wroclaw Medical University were included in the study. All the subjects met the diagnostic criteria for chronic rhinosinusitis as established by the Task Force on Rhinosinusitis (AAO-HNS) [7]. The patients' ages ranged from 44 to 71 years (mean 52.6 years). The extent of the disease was assessed by CT and endoscopically. Patients had been free of any medication for at least 2 weeks before surgery and had bilateral polyps in the nasal cavities on endoscopic examination. The presence of comorbidity or smoking history was also excluded. The subjects underwent polypectomy for nasal obstruction with subsequent tissue sampling for further RNA isolation.

The control group consisted of 9 healthy persons (7 males and 2 females). The absence of NP was assessed by clinical history, endoscopic examination and imaging. Prick tests were performed to rule out the existence of allergy. The history of other diseases was also excluded. Control tissue samples were taken from unchanged middle concha.

Nasal polyp specimens and control mucosa were immediately placed into a sample tube containing 1 ml phosphate buffered saline (PBS) and frozen directly at -70°C until further investigations. A part of each sample was fixed in 10% buffered neutral formalin, processed routinely, and embedded in paraffin wax for subsequent immuno-histochemical examination. The study was approved by the Local Ethical Committee of Wroclaw Medical University.

Histologic examination
Serial sections of paraffin-embedded samples were stained with hematoxylin-eosin to visualize inflammatory cells and to exclude other pathologies. The diagnosis of eosinophilic nasal polyps (ENP) was determined if the percentage of eosinophils was greater than 80% of all leukocytes or the presence of clusters of eosinophils was seen. When the dominant cells in the tissue were lymphocytes and plasmocytes the diagnosis of NNP was established. According to this 16 (76%) cases were classified as eosinophilic and 5 (24%) patients as a neutrophilic nasal polyps.

Immunohistochemistry
The expression of TNFα, TNF-R1 and TNF-R2 proteins were demonstrated using conventional immunohistochemical avidin-biotin-peroxidase (ABC) complex techniques with 3,3-diaminobenzidine-tetrahydrochloride as a substrate (DAKO). The sections, after being deparaffinized in xylene and rehydrated through graded alcohol were pretreated in a microwave oven in citrate buffer (pH 6.0) for over 8 minutes. Secondly, slides were incubated overnight at 24°C temperature with an anti-human TNFα (MAB610), TNF-R1 (MAB225), and TNF-R2 (MAB226), mouse monoclonal antibody, diluted 1:20. The antibodies were manufactured by R&D Systems. Subsequently, all the sections were treated with hydrogen peroxide to quench endogenous peroxidase activity. The sections were counterstained with Mayre’s haematoxylin, dehydrated, mounted and then examined under a microscope. Controls were performed by replacing the primary antibodies with normal mouse serum. The IgG concentration of these two reagents were equivalent.

The number of positive cells, expressed as labeling index (LI), in a high power field were divided by the total number of cells present in that field, and this was expressed as a percentage. All the sections were verified by a pathologist and evaluated by two investigators (ZP, FM).

QRT-PCR method
Total RNA was extracted from freshly frozen NP tissue samples with the use of TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the producer’s protocol. The concentration of RNA was determined spectrophotometrically by the use of GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, USA).

The DNA Engine Opicon™ systems (MJ Research, USA) were applied to quantify the amount of mRNA TNFα and its receptors TNF-R1, TNF-R2 by the use of real time QRT-PCR technique. The reaction mixture consisted of: 25 µl 2x QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Valencia, CA, USA), 0,5 µl QuantiTect RT Mix and 0,5µM forward starters and reverse, 0,1 µg RNA. Starters used for amplification:
Primer Express™ Version 2.0 (PE Applied Biosystems, USA). The thermal conditions for one-step RT-PCR were as follows: reversed transcription at 50°C for 30 minutes, 95°C for 15 minutes and then 45 cycles of amplification at 94°C for 15 seconds and at 53.3°C for 30 seconds, 72°C for 30 seconds. The transcription activity of β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as endogenous controls were evaluated in each sample. The RT-PCR specificity was assessed on the basis of the melting temperature for each amplimer. The standard curve was drawn for the commercially accessible patterns of β-actin copies using β-actin Control Reagent Kit (Applied Biosystems, USA) to calculate the number of mRNA copies of genes tested. Qualitative results were recalculated per 1 μg of the total RNA (c/μg).

Statistical analysis
The analysis was performed using the Statistica 5.0 package (Statsoft, Poland). All values were expressed as means ± SE. In order to check the normality of the distribution, the Shapiro–Wilk test was performed. In case of a normal distribution the Student t test was performed; otherwise the Mann-Whitney U test was used. Correlations were calculated by using Spearman’s rank order test. The level of confidence was established at p<0.05.

RESULTS

TNFα mRNA expression
The mRNA for TNFα was detected in all cases of both the study and the control group. TNFα mRNA levels ranged from 1676 c/μg to 265928 c/μg in eosinophilic and from 1477 c/μg to 18802 c/μg in neutrophilic NP; in the unchanged mucosa of the control group from 1490 c/μg to 180066 c/μg (Fig. 1).

The mean level of mRNA copies for TNFα in ENP (82229 ± 85379 c/μg) was not significantly higher when compared with controls (74869 ± 71665 c/μg) (p=0.821). NNP patients demonstrated significantly lower mean TNFα gene expression (7021 ± 6949 c/μg) than the control cases (p<0.05). A statistically higher mRNA TNFα copy number in ENP than in NNP was also revealed (p<0.01).

Quantification of mRNA for TNFα receptors in NP samples
Among eosinophilic and neutrophilic polyps mRNA for TNF-R1 was detected in 13 (81%) and 4 (80%) cases respectively. All the cases of the control group expressed TNF-R1. The mean level of copies of mRNA for TNF-R1 in ENP and in NNP was noticeably lower compared to the control group (10198 c/μg vs. 30749 c/μg, p<0.05 and 3440 c/μg vs. 30749 c/μg; p<0.05 respectively). There was no statistical difference in mean TNF-R1 expression levels between ENP and NNP (p=0.098).
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TNF-R2 mRNA expression was observed in 11 (69%) cases of ENP, 3 (60%) NNP and 9 (100%) specimens of the control group. It was also found that in ENP and the control cases the mean TNF-R2 mRNA copy number was significantly higher than in NNP (185 c/μg vs. 7.6 c/μg, p<0.05 and 469 c/μg vs. 7.6 c/μg, p<0.05). TNF-R2 expression did not differ significantly between ENP and the control group (185 c/μg vs. 469 c/μg; p=0.150) (Fig. 1).

**TNFα mRNA and TNFRs mRNA copy number correlation**

Among eosinophilic NP, the mRNA copy number for TNFα correlated positively with both mRNA level for TNF-R1 (r=0.845; p<0.001) and TNF-R2 (r=0.665; p<0.01). In that group significant coexpression of mRNA for both TNFα receptors was noted (r=0.574; p<0.05). No any statistically significant correlations were observed between TNFα and TNF-R1 or TNFα and TNF-R2 expression in neutrophilic polyps. A linear correlation was detected between the mRNA levels for TNFα and TNF-R2 (r=0.673; p=0.047) in the control group. TNF-R1 expression was significantly higher than TNF-R2 at the mRNA level both in ENP and NNP (p<0.01).

**Immunohistochemistry**

In eosinophilic nasal polyps TNFα, TNF-R1 and TNF-R2 proteins were seen in 11/16 (69%), 13/16 (81%), and 11/16 (69%) cases; among NNP in 3/5 (60%), 4/5 (80%), and 3/5 (60%) cases; in healthy mucosa in 0%, 1/9 (11%), and 1/9 (11%) cases respectively. The percentage of the immunoreactive cells for the investigated proteins are presented in Tab. 1.

![Figure 2. Representative photomicrographs of eosinophilic nasal polyps biopsy sections immunohistochemically stained with antibodies against TNFα (a), TNF-R1 (b) and TNF-R2 (c) (magnification x200).](image)

Intense, homogenous labeling for TNFα was seen in ENPs and average to weak among NNP (Fig. 2, 3). In healthy mucosa TNFα protein expression was limited to hardly a few endothelial and epithelial cells with very weak immunostaining. Staining for TNF-R2 protein was exhibited much more intensively than for TNF-R1 in all the subgroups. In ENP clearly more intensive labelling for both TNF-R1 and TNF-R2 were seen also in granulocytes like cells surrounding subepithelial glands and sparsely in endothelial cells. No significant correlations in proteins expression were detected between ENP and NNP. Either in ENP and NNP the TNF-R1 protein level was statistically higher than TNF-R2 (p<0.05).

**DISCUSSION**

Owing to its strong proinflammatory and immunostimulatory activities, TNFα is an important mediator of the progression of many chronic disorders including NP. In our study, the mRNA for TNFα was revealed both in the control subjects and NP with insignificant prevalence in the latter group. The results are in contrast to the study by Finotto et al. [8] in which eosinophils from normal nasal mucosal samples compared to
NP did not show mRNA for TNFα. The relatively high levels of the mRNA copy for TNFα observed in the control group are difficult to explain. However, it is known that the TNFα protein is constitutively synthesized by a number of cells, including airway epithelial cells, even under physiological conditions and plays a significant role in development and cell apoptosis [9]. Additionally, TNFα expression can be activated by many factors including viral and bacterial infections.

TNFα protein expression was markedly higher in both ENP and NNP than in the control group. Recruited eosinophils in NP are considered the primary source of TNFα which confirms their role in the promotion of inflammatory reactions. The absence of TNFα positive cells in the control group contrasts with Bachert et al.’s [10] data which did not show a notable difference in TNFα protein concentration between NP and normal mucosa.

The discrepancy between high copy number of mRNA for TNFα in normal mucosa and the absence of detectable protein may be explained by the observation that in eosinophils under some circumstances including physiological conditions mRNA for TNFα may not undergo translation. It is postulated to be relevant to an adenine and uridine (AU)-rich element (ARE) in the 3’untranslated region (3’UTR) of TNFα transcripts, an important determinant of TNFα mRNA translational blockage [11].

TNFα and its receptors expression is regulated by both transcriptional and posttranscriptional mechanisms. The molecular basis governing this regulation is not however entirely understood. Regulation on the transcriptional level occurs mainly at the stage of initiation. Bacterial lipopolysaccharide and IFNγ have been shown to be major agent up-regulating the concentration of transcription factors of the NF-κB/Rel family, and subsequently can activate the TNFα transcription and release [12]. Besides it has been demonstrated also that 1,25(OH)2D3 and stimulation with IL-1 increases the TNFα synthesis [13,14].

The induction of the cellular responses mediated by TNFα is initiated by its binding to specific cell-surface receptors. The strongest factor inflecting the expression of superficial TNFRs is TNFα itself. It was proven in vitro that TNFα treatment leads to increased mRNA expression for both TNF-R1 and TNF-R2 [15]. At the same time, the most frequent cause of TNFRs decrease is receptor internalization occurring after TNFα binding.

In the control group high level of mRNA for TNF-R1 was noted and was accompanied by weak immunoreaction for TNF-R1. From these data it might be surmised that expression of TNFRs regulation resembles multilevel control of TNFα synthesis mentioned before [16]. Typically, TNF-R1 is constitutively expressed at a low level whereas the level of TNF-R2 expression is significantly less ubiquitous.

Increased mRNA for TNFRs among the control group might also be a part of tissue response to upregulated TNFα expression. In was proven before that dramatically reduced TNFα sensitivity might be achieved by expression of TNFα receptors at higher than optimal levels [17].

Despite intensive research, the individual signalling roles of the two TNFRs are still under considerable debate. The two receptors signal shows distinct and largely non-overlapping sets of activities. Therefore, mutual relations between both TNF-R1 and TNF-R2 may be in part responsible for bidirectional TNFα activity. TNF-R1 is a cellular transducer of major TNFα activities including induction of apoptosis and coordination of the inflammatory process. TNF-R1 deficient mice developed a low incidence of arthritis and mostly in
much attenuated form [18]. TNF-R1 signalling events result also in cell proliferation through Nuclear Factor kappa B (NF-κB) transcription factor activation what has also been reported in fibroblasts [19]. Consequently, excessive TNF-R1-mediated signalling may be responsible in part for distinct fibro-inflammatory character of the neutrophilic nasal polyps.

TNF-R2 efficiently assist TNF-R1 effects and mediates also enhanced proliferation of cells including T, B and NK cells [20]. Thus, the elevated expression of TNF-R2 might be related to the inhibition of apoptosis or upregulated cell divisions which would have a survival or proliferation effect [21].

The results of our study may have important implications for new therapeutic approaches in the treatment of NP. A consequence of the obliteration of TNFR function is restrained inflammatory response which was carried out before in otitis media with effusion [22].

In conclusion, the current study presents for the first time the expression of TNFα receptors in nasal polyps. TNF-R1 was found to be the prevalent form of TNFα receptor in NP which may reflect the apparent dominance of TNF-R1 in TNFα signalling. TNF-R1 expression was significantly higher than TNF-R2 at the mRNA and protein level both in ENP and NNP. A statistically higher mRNA expression for TNFα in ENP than in NNP was also revealed. The findings raise the possibilities that the eosinophils from NP may influence biological responses through TNFα-dependent mechanisms and therefore participate in complex mechanism of NP formation and eosinophil accumulation. Differences were revealed in TNFα and TNFRs levels between eosinophilic and neutrophilic polyps what may arise from/or cause distinct histological character of that diseases. Due to the fact that none of the currently discussed theories seem adequate to account for all the known facts related to nasal polyps further investigations are necessary.

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