doi:10.1111/j.1365-2591.2007.01215.x

Irreversible but not reversible pulpitis is associated with up-regulation of tumour necrosis factor-alpha gene expression in human pulp

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Abstract

Kokkas AB, Goulas A, Varsamidis K, Mirtsou V, Tziafas D. Irreversible but not reversible pulpitis is associated with up-regulation of tumour necrosis factor-alpha gene expression in human pulp. *International Endodontic Journal*, **40**, 198–203, 2007.

Aim To analyse the gene expression of tumour necrosis factor-alpha (TNF-α) in human dental pulps, under normal and inflammatory conditions and to examine the association between any observed alterations in the expression of this cytokine with the severity of the clinical symptoms.

Methodology Eighteen pulpal samples were obtained from single-rooted human teeth. Six of the teeth were normal (group A), six had been diagnosed with reversible pulpitis (group B), and the remaining six were from teeth diagnosed with irreversible pulpitis (group C). TNF-α gene expression was semi-quantitatively analysed in each sample with RT-PCR, and the results from each group of teeth were compared with the Kruskal–Wallis and Mann–Whitney tests.

Results Tumour necrosis factor-alpha was detected in all three groups of dental pulp. Statistical analysis provided evidence of a significant increase of TNF-α gene expression associated with irreversible inflammation compared with healthy controls ($P = 0.002$). No such difference was detected in reversibly inflamed pulp in comparison to healthy teeth ($P = 0.699$).

Conclusion Tumour necrosis factor-alpha gene expression in inflamed human dental pulp tissue is positively associated with the severity of clinical symptoms.

Keywords: human pulps, irreversible pulpitis, reversible pulpitis, tumour necrosis factor-alpha.

Received 17 April 2006; accepted 18 September 2006

Introduction

Tumour necrosis factor-alpha (TNF-α) is a pleiotropic cytokine produced predominantly in response to infection, antigen or injury. This proinflammatory cytokine is synthesized and released by a wide range of cells involved in the immune-inflammatory process and elicits a broad spectrum of biological effects. Amongst other things, TNF-α is known to increase the toxicity of leucocytes, stimulate the synthesis of the acute phase inflammation proteins, and induce the expression of other proinflammatory cytokines (Haynes & Fauci 2001).

Information concerning pulp TNF-α expression *in vivo* is relatively scarce, despite earlier reports about its ability to stimulate, together with other cytokines, human pulp cells to synthesize and secrete proteolytic enzymes that destroy the extracellular matrix (Panagakos *et al.* 1996, O'Boskey & Panagakos 1998, Ueda & Matsushima 2001, Lin *et al.* 2001). In the rat, cells expressing TNF-α were detected immunohistochemically, immediately following surgical

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exposure, in infected pulps as well as in developing periapical lesions (Tani-Ishii *et al.* 1995), whereas TNF-α – together with interleukin (IL)-1α and IL-1β – was detected in pulp interstitial fluid during lipopolysaccharide-induced acute inflammation (Bletsa *et al.* 2006). In a study of normal and inflamed human dental pulps, Pezelj-Ribaric *et al.* (2002) reported high levels of TNF-α protein in samples from teeth clinically diagnosed with irreversible pulpitis, but considerably lower expression of this cytokine in samples from normal teeth. In a different study, Zehnder *et al.* (2003) examined the steady-state mRNA expression of various cytokines (but not TNF-α) in symptomatic and clinically healthy human dental pulps and produced evidence supporting that pulpitis is associated with the up-regulation of IL-6, IL-8 and IL-18. Nevertheless, existing data do not address the issue of correlation of cytokine expression with the extent and severity of the clinical symptoms of pulpitis. The aim of this study was to evaluate whether a correlation between TNF-α gene expression in human dental pulp and the clinically defined reversibility of pulp inflammation, could be detected.

Materials and methods

Sample collection

Eighteen pulpal samples removed from single-rooted human teeth, derived from patients with age ranging from 13.5 to 40 years, were used following their informed consent. All patients had abstained from long-term treatment with anti-inflammatory medication. Clinical and radiographic examination excluded teeth with a diagnosis of pulp necrosis, periapical pathosis, periodontal diseases (periodontal pocket <3 mm) or other injuries, with the exception of crown fractures. The samples were divided into three groups, namely A, B and C (Table 1) according to the following criteria: group A included normal pulp tissue collected from teeth ($n = 6$) with no history of pulpal pain. Clinical and radiographic examination had ensured that these teeth had no caries, crown fractures or any restorations. The samples were collected from teeth extracted for orthodontic reasons or after root-canal treatment for prosthetic reasons. Group B consisted of pulp tissue collected from teeth ($n = 6$) with clinical

Table 1 Individual characteristics of dental pulp source used in this study

Sample	Age (years)	Tooth	Clinical symptoms	Pathology/treatment
Group A				
1	15	35	No history of pulpal pain	No caries or restorations
2	22.5	15		
3	25	25		
4	15	35		
5	14	45		
6	14	35		
Group B				
7	28	15	History of mild instant pain	Caries without pulp exposure
8	30	15	Vitality test on cold produced instant pain	Amalgam or composite restorations
9	32.5	25		
10	19	35		
11	20	35		
12	22	35		
Group C				
13	25	13	History of severe spontaneous pain Vitality test on cold produced immediate pain	Pulp exposure by caries
14	29	22	History of severe prolonged pain to thermal stimuli Vitality test on cold produced immediate pain	Composite restoration, caries near pulp
15	35	15	History of severe spontaneous pain Vitality test on cold produced immediate pain	Large amalgam restoration, caries near pulp
16	36	15	History of severe spontaneous pain Vitality test on cold produced immediate pain	Pulp exposure by caries
17	30	45	History of severe prolonged pain to thermal stimuli Vitality test on cold produced immediate pain	Large amalgam restoration, caries near pulp
18	40	35	History of severe spontaneous pain Vitality test on cold showed immediate pain	Pulp exposure by caries

diagnosis of reversible pulpitis. The teeth in this group had a history of instant pain to thermal stimuli and were designed for pulpectomy and root-canal treatment because of prosthetic reasons. According to the clinical and radiographic examination, these teeth had caries and/or restorations, but showed no evidence of pulp exposure or infection. Pulp sensitivity tests to cold gave instant pain. Group C consisted of pulp tissue collected from teeth ($n = 6$) with a clinical diagnosis of irreversible pulpitis. The teeth in this group had a history of prolonged pain to thermal stimuli or spontaneous pain and were designed for pulpectomy and root-canal treatment. Clinical and radiographic examination revealed the presence of caries and/or restorations with or without pulp exposure. Pulp sensitivity test on cold showed immediate pain reaction.

The pulpal tissue was extirpated by a barbed broach, after the chamber was carefully opened with a round bur. In the cases of tooth extraction the teeth were grooved longitudinally by a fissure bur, without penetrating the root canal. Then the teeth were split in half and the pulpal tissue was gently removed with a spoon excavator. Only pulp samples that were removed in one piece were used; and were immediately transported on ice to the laboratory and stored at −70 °C.

The Ethical Committee of the Dental School of the Aristotle University of Thessaloniki approved all sample collection and experimental procedures. Informed consent was obtained from all participants in this study.

Analysis of TNF-α gene expression

Total RNA was isolated with a commercial kit (Purescript®; Gentra Systems, Minneapolis, MN, USA) following the manufacturer's specifications. Reverse transcription to cDNA was accomplished with M-MulLV reverse transcriptase using random hexamer primers (Invitrogen, Carlsbad, CA, USA). PCR amplification of the TNF-α reverse transcript was performed simultaneously in the same reaction tube, with that of β-actin which served as internal reference so as to compensate for fluctuations in the initial cDNA concentration and sampling/pipetting errors. Reaction conditions were: 30 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, preceded by an initial denaturation step at 94 °C for 5 min and followed by a final extension step of 10 min at 72°C. PCR cycle testing performed prior to our experiments had shown that, in the 30–32 cycle range, cDNA amplification proceeds exponentially and with a practically stable TNF-α/β-actin band intensity ratio within the same sample group. The TNF-α primers were added in a final concentration of 0.2 μM: forward, TTATTACCCCCTCCTTCAGACAC; reverse, AAGTCTGGAAACATCTGGAGAGAG (Zhou *et al.* 2003). The β-actin primers were a generous gift by Dr A. Kritis (Department of Physiology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece) and were added in a final concentration of 0.04 μM: forward, AGGGGCCGGACTCGTCATACT; reverse, ACACTGTGCCCATCTACGAGG (Kritis *et al.* 2002).

The amplified DNA was electrophoretically separated in a 1.5% gel. The light intensity of the TNF-α (347 bp) and β-actin (621 bp) bands following UV-irradiation was analysed through computer-assisted densimetry (Kodak Digital Science™; 1D Image Analysis Software, New Haven, CT, USA). The TNF-α/β-actin band intensity ratio was taken as a measure of TNF-α gene expression in each sample.

Statistical analysis was performed with the Kruskal–Wallis test followed by Mann–Whitney *post hoc* tests (SPSS, version 12.0).

Results

Tumour necrosis factor-alpha gene expression was detected in all three groups (Fig. 1). The distribution of TNF-α/β-actin band intensity ratios is shown in Fig. 2. The Kruskal–Wallis test revealed statistically significant differences among the three groups ($P = 0.008$). A statistically significant increase was observed in group C (irreversible pulpitis; mean ± SD = 1.119 ± 0.137) compared with either group A (healthy pulps; mean ± SD = 0.591 ± 0.088; $P = 0.002$) or group B (reversible pulpitis; mean ± SD = 0.676 ± 0.234; $P = 0.015$). No statistically significant difference in TNF-α gene expression was observed between groups B and A ($P = 0.699$).

Discussion

Pulpitis is a typical inflammatory disease of the pulpal connective tissue, whenever dentine and pulp are affected by caries, operative procedures or trauma.

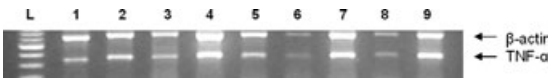

Figure 1 Agarose gel electrophoresis of the simultaneous amplification of tumour necrosis factor-alpha and beta-actin reverse transcripts from representative human pulp samples.

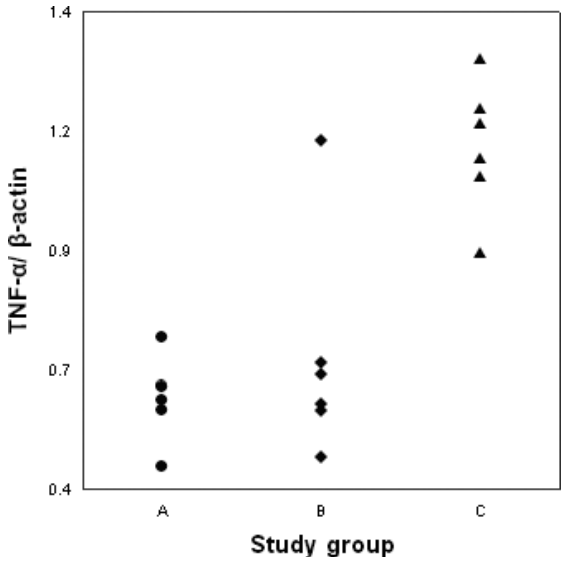

Figure 2 Distribution of tumour necrosis factor-alpha gene expression normalized to that of β-actin in the three study groups. A, healthy pulps (group A); B, pulps with reversible pulpitis (group B); C, pulps with irreversible pulpitis (group C). Dots indicate individual values.

The extent of tissue injury and the host tissue activity interacting within the inflexible environment of the pulp chamber result in a broad spectrum of inflammatory and reparative events. Two discrete stages exist that require different treatment plans, i.e. reversible pulpitis where pulp preservation is indicated or irreversible pulputis where the dentist may perform pulpectomy and root-canal treatment. In general, accurately diagnosing the histopathological condition of the pulp from clinical symptoms or other diagnostic data is not possible. Therefore, although the clinical value of a molecular diagnostic marker may at first appear limited in scope – given the requirement for pulp removal – the emerging correlation of cytokine expression with clinical signs and symptoms of pulpitis will hopefully be of value in terms of better characterizing the development of pulp inflammation, especially since studies of histological findings have largely failed to do that in the past (Seltzer *et al.* 1963). Even though the altered expression of a small number of cytokines, on either the mRNA (IL-6, IL-8, IL-18) (Zehnder *et al.* 2003) or the protein level (IL-2, TNF-α, TGF-β1, macrophage inflammatory protein-3-alpha) (Rauschenberger *et al.* 1997, Pezelj-Ribaric *et al.* 2002, Piattelli *et al.* 2004, Nakanishi *et al.* 2005) has been associated with the presence of inflammation in human pulp samples in the past, no attempt has been made to associate their varied expression with different stages of inflammation, with a single exception (Pezelj-Ribaric *et al.* 2002).

In this study, a semi-quantitative method of RT-PCR was used to compare the gene expression of TNF-α in normal, reversibly inflamed and irreversibly inflamed human dental pulp to demonstrate that: (1) TNF-α gene expression can be detected in healthy and inflamed human pulp, (2) clinically diagnosed irreversible pulpitis is associated with a statistically significant increase in steady-state mRNA levels of TNF-α extracted from inflamed pulp compared with healthy controls and (3) there is no apparent increase of TNF-α gene expression in pulps from teeth with clinical signs and symptoms of reversible pulpitis. The small standard deviations observed in all the three sample groups argue in favour of the reliability of the results, despite the use of a single as opposed to three housekeeping genes as proposed by Vandesompele *et al.* (2002). Besides, β-actin was selected as a housekeeping gene in this study, based on its accepted use as an internal standard of gene expression, be it on the mRNA or the protein level, in a variety of inflamed tissues (Markova *et al.* 2006, Wu *et al.* 2006). In any case, the findings are essentially in agreement with the previously reported observation that protein TNF-α levels are significantly increased in pulps from human teeth with irreversible pulpitis (Pezelj-Ribaric *et al.* 2002) and indicate that this increase may be due, at least in part, to an up-regulation of transcription. On the other hand, the inability to detect a similar increase in pulps from teeth diagnosed with reversible pulpitis constitutes a novel finding. Whilst Pezelj-Ribaric *et al.* (2002) did report significantly increased TNF-α protein levels in pulps associated with what they defined as 'asymptomatic irreversible' pulpitis, the apparent contradiction with the present results is eliminated if that condition is taken to represent an intermediate stage of pulp inflammation between reversible and irreversible pulpitis. Taken together, these results indicate that pulp inflammation proceeds through clearly separate stages based on molecular as well as clinical diagnostic criteria, even though current knowledge does not allow an association between the clinical diagnosis of normal pulp, reversible and irreversible pulpitis with histopathological findings. In fact, this classification, which is difficult in dental practice, is mainly based on the patients' complaints and signs that may not be always reliable. Despite the existence of these difficulties, a classification such as this is necessary for

the clinician to select the treatment plan. In this study, the grouping of collected pulp samples was based on clinical diagnostic criteria described in the literature, namely that reversible pulpitis causes a momentary painful response to thermal change that subsides as soon as the stimulus is removed and does not involve a complaint of spontaneous pain, as opposed to irreversible pulpitis, which causes a more painful response to thermal change that subsides as soon as the stimulus is removed and does involve a complaint of spontaneous pain (Cohen 1998).

The association of TNF-α gene expression in human pulp with the severity of clinical symptoms of pulpitis detected in this study, is in line with recently accumulated evidence suggesting the involvement of cytokine – induced cycloxygenase-2 (COX-2) activity in the pathophysiology of pulp inflammation (Lin *et al.* 2002, Chang *et al.* 2003, Kawashima *et al.* 2005). Indeed, dental pulp levels of prostaglandin E2 whose production can be catalysed by COX-2, have been independently associated with the severity of pulpitis symptoms in the past (Cohen *et al.* 1985).

The fact that TNF-α was recently shown to induce high levels of vascular endothelial growth factor and tissue plasminogen activator expression in human pulp fibroblasts with their probable involvement in the destruction of pulpal tissues through promoting expansion of the vascular network (Chang *et al.* 2003a, Chu *et al.* 2004) argues in favour of TNF-α's possible use as a molecular marker of irreversible processes in pulp inflammation. Regrettably, the limited amounts of tissue available for examination precluded the determination of TNF-α protein levels on the same samples in this study, thus depriving the opportunity of directly comparing the extent of induction in mRNA and protein levels. It is hoped that the development of more sensitive techniques will allow the carrying out of more detailed analyses in the future.

Conclusion

Clinically diagnosed irreversible pulpitis is associated with a statistically significant up-regulation of TNF-α gene expression in the dental pulp. However, steady-state pulpal mRNA levels in reversible pulpitis do not differ significantly from those in healthy teeth, suggesting that, in pulpitis, clinical diagnostic criteria of reversibility can be matched by changes in gene expression.

Acknowledgements

This study was supported by research grants: (1) Research Committee of the Technological Educational Institution of Thessaloniki (RCTEIThe-10/6/31-3-2004) and (2) Ministry of Education in Greece (Pythagoras II – 21/80843). We thank Dr Aristidis Kritis for his gift of the β–actin primers.

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