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ORIGINAL ARTICLE

The concentration of TNF-α correlate with number of inflammatory cells and degree of vascularization in radicular cysts

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OBJECTIVE: To correlate values of tumor necrosis factor-alpha (TNF-α) depending on the count of inflammatory cells with degree of vascularization in cystic fluid of radicular cysts.
MATERIAL AND METHODS: We investigated TNF-α concentration in 43 radicular cysts obtained from patients undergoing surgery, under local anaesthesia, and after aspiration of cystic fluid from non-ruptured cysts by enzyme-linked immunosorbent assay assay in respect of different clinical parameters as well as by histomorphometric analyses.
RESULTS: Significantly higher concentration of TNF-α is associated with smaller radicular cysts, higher protein concentration in cystic fluid as well as with higher presence of inflammatory cells, and increased degree of vascularization in pericystic tissues and cyst wall thickness.
CONCLUSIONS: We believe that determination of TNF-α in cystic fluid simultaneously with other parameters can be an additional parameter for clinical diagnosis of inflammed cysts.
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Keywords: TNF-α; radicular cysts; immunohistochemistry; inflammatory cells; vascularisation; inflammation

Introduction

It is established that the radicular cysts are a result of inflammatory process in the periapical tissues (Stashenko *et al*, 1994). Humoral and cellular immune responses play a role in the pathogenesis of these lesions. Tumor necrosis factor-alpha (TNF-α) is a cytokine initially identified as a cause of hemorrhagic necrosis in certain tumors (Jurisic *et al*, 1999, 2004). It is secreted by macrophages, monocytes and NK cells and it exists in two forms. The biological actions of TNF-α were mediated after binding to its specific cell surface receptors, called TNF receptor superfamily (Jurisic *et al*, 2006). TNF-α mostly triggers apoptosis and necrosis in sensitive tissues, can be linked to osteomyelitis and apical periodontitis, but it can also participate in skeletal homeostasis including osteoclastic formation, and bone resorption in maxillofacial region. TNF-α among other cytokines can regulate fibroblasts activity and collagen formation through modulation of collagenase activity (Bertolini *et al*, 1986; Thomson *et al*, 1987; Silva *et al*, 2004; Bletsa *et al*, 2006).

As the growth of radicular cysts is accompanied by local bone destruction, new vascularization and accumulation of different cells in the cyst wall, significance of investigation of TNF-α in respect of these parameters is very important. During this dynamic and inflammatory process, TNF-α can be accumulated in the cystic fluid. As TNF-α is associated with multiple effects, we correlated its values in respect of different clinical and histological parameters including cyst size, wall thickness and degree of vascularization in 43 radicular cysts.

Materials and methods

Patients

Forty-three radicular cysts were obtained from patients undergoing surgery at the Oral Surgery Department, School of Dentistry, University of Belgrade. The cysts were surgically enucleated under local anesthesia. The mucoperiostal flap was raised, and if necessary, a bur was used to remove the bone under irrigation with sterile saline solution, avoiding injury to the cyst wall. The cystic fluid was aspirated from non-ruptured cysts (any samples contaminated with blood were excluded), then immediately centrifuged (3000 rpm for 20 min) to remove cells, and the supernatant was stored at −70°C until used for assays. After the enucleation, one section of cyst wall was taken for histopathological evaluation. The final diagnosis of the cysts was confirmed on the basis of clinical, radiographic, and histopathological examinations (Table 1). The size of cysts was calculated

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Table 1 Cyst characteristics

The number of cysts	43
Thick wall	77.77%
No thick wall	22.33%
Degree of inflammation	
Slight	7.00%
Moderate	18.60%
Pronounced	74.40%
Degree of vascularization	
Low	8.33%
Intermediate	22.22%
High	69.45%
Degree of epithelial proliferation	
Low	21.10%
Intermediate	37.20%
High	41.70%

as the mean values of the largest dimension (line *a*) on radiography and the dimension perpendicular to it (line *b*) using the formula $(a + b)/2$. Cysts were divided into following groups: with mean size below 2 cm, from 2 to 3 cm, and over 3 cm. The patients had no history of infection in the cystic lesions and they had not taken antibiotics or long-term inflammatory medications during the preceding 2 months. No orocystic communications were clinically detected before surgery. The local human ethics committee approved the study protocol. After receiving information about the character and purpose of the study, the subjects gave written consent for participation.

Determination of proteins
Proteins were determined semiquantitatively and results were expressed as a level of opalescence (low, intermediate, and high).

Determination of TNF-α
The concentrations of TNF-α in the cystic fluids were measured by enzyme-linked immunosorbent assay obtained from Diaclone (Besancon, France) according to the manufacturer's recommended protocol. The concentration of TNF-α from sample were mathematically calculated from the standard curve obtained from different dilutions of known concentration of rh TNF-α in the original assay. The system utilized a highly specific monoclonal antibody in the range from 15.6 to 1000 pg ml^{-1}. Absorbance was measured using a microplate reader at 450 nm (Behringer EL 311, Berlin, Germany).

Histomorphometric study
The material used in this study consisted of pericystic biopsies tissues. The tissues were removed under local anesthesia during routine cysts surgery and immediately fixed in 10% neutral buffer formalin at room temperature. The tissue blocks were sliced into 3-μm-thick sections for routine histological examinations after staining with hematoxylin and eosin (H&E) or immunohistochemistry. A minimum of the three sections in each sample were used to access the presence of the inflammatory cells, degree of vascularization and degree of epithelial proliferation. Briefly, each specimen was graded at 400× magnifications as: grade I (mild) <10 inflammatory cells per field; grade II (moderately inflamed), 10–50 inflammatory cells per field, and grade III (highly inflamed) more than 50 inflammatory cells per field. Grading of each specimen was based on the average inflammatory cell number in three consecutive microscopic fields starting from the epithelial-connective tissue border and proceeding gradually deeper into lamina propria. Degree of vascularization was characterized as low (below 10 blood vessels mm^{-2}), moderate (11–15 blood vessels mm^{-2}) and high (over 15 blood vessels mm^{-2}). The degree of epithelial proliferation in cysts well was analyzed in respect of number of the epithelial layer cells and described as low, moderate and high. Cyst wall thickness was described as thick (over 1.5 mm) and thin (below 1.5 mm).

Immunohistochemical analysis
For immunohistochemistry, following monoclonal antibodies were used: anti-CD3, anti-CD20 and anti-CD68 (all from Sigma, St. Louis, MO, USA) by standard techniques, previously described on tissue section. Paraffin-embedded tissues were sectioned (3 μm) and collected in serial sections on glass slides coated with 2% 3-aminopropzltriethzlsilane (Sigma Chemicals). The sections were deparaffinized by immersion in xylene, and this was followed by immersion in alcohol and then incubation with 3% hydrogen peroxide diluted in Tris buffered saline (TBS) (pH 7.4) for 40 min. Next, the sections were immersed in citrate buffer (pH 6.0; Sigma, P4809) for 20 min at 95° for antigen retrieval. Soon afterwards, the sections were blocked by incubation with 3% normal goat serum diluted in distilled water, at room temperature, for 20 min. The slides were then incubated with primary antibodies in a humidified chamber.

After washing in TBS, the sections were treated with the labelled streptavidin-biotin kits (K0492; Dako, Glostrup, Denmark). The sections were then incubated in a 3.3% diaminobenzidine in a chromogen solution (Dako, K3468) for 2–5 min at room temperature. Finally, the sections were stained with Mayer's hematoxylin and were covered. Negative controls were obtained by omitting the primary antibodies.

Quantitative analysis
Microscopically, we determined the number of cells showing staining for CD3, CD20 and CD68 as a percentage of these cells of the total cell population. Proportions of $CD3^+$, $CD20^+$ and $CD68^+$ cells in inflammatory infiltrate were enumerated in 10 representative and consecutive microscopic high-power fields (×400). Cell subsets are expressed as mean percentage of positive cells per 100 cell of total cell population ± standard deviation (s.d.).

Statistical analysis
Statistical significance between observed parameters analyzed in the study was calculated by Mann–Whitney *U*-test and ANOVA using Excel and SPS software for windows 2000 on Compaq computer.

Results

Values of TNF-α in respect of cyst size

This investigation demonstrated the presence of TNF-α in all cysts (Figure 1). The mean value for TNF in 43 cysts was 99.3 ± 27.3 pg ml^{-1} of cystic fluid. We here compared TNF-α in respect of the cystic size and obtained different results. Higher values of TNF-α were associated with smaller radicular cyst (Figure 1). Influence of protein concentration in the cystic fluid analyzed at the level of cystic opalescence showed that cysts with higher protein concentration and higher opalescence have a higher concentration of TNF-α (Figure 2). We further investigated the role of cysts wall on TNF-α concentration in intracystic fluid. Radicular cysts with thick cystic wall (Figure 3) have significantly higher (Mann–Whitney *U*-test, $P < 0.05$) concentration of TNF-α (88 ± 12 pg ml^{-1}) in comparison with thin cystic wall (29.5 ± 9 pg ml^{-1}).

Values of TNF-α in respect of presence of degree of vascularization and epithelial proliferation

Further, we estimated the role of cyst wall on production of TNF-α in respect of presence of inflammatory cells, degree of epithelial proliferation and degree of vascularization, analyzed on histology H&E section. TNF-α significantly increased in cysts with higher degree of cell infiltration (Mann–Whiteney *U*-test, $P < 0.05$) in comparison to cysts with a smaller count of infiltrative cells (Figure 4). A similar trend was found in respect of vessel count analyzed in pericystic wall on tissues section (Figure 5) (Mann–Whitney *U*-test, $P < 0.05$). However, count of inflammatory cells and degree of vascularization rather than epithelial characteristics in these cysts (Figure 5a,b) showed a stronger association with increased TNF-α (ANOVA, $P < 0.05$).

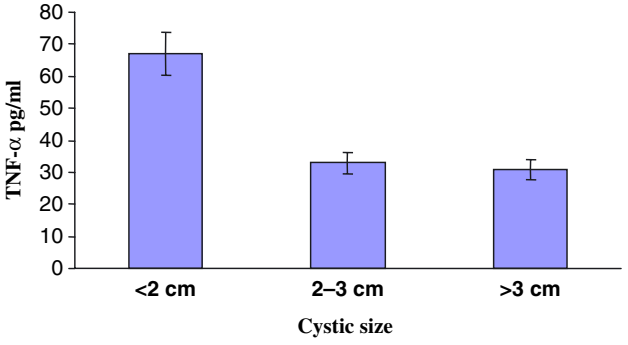

Figure 1 Tumor necrosis factor-alpha concentration depending on cystic volume

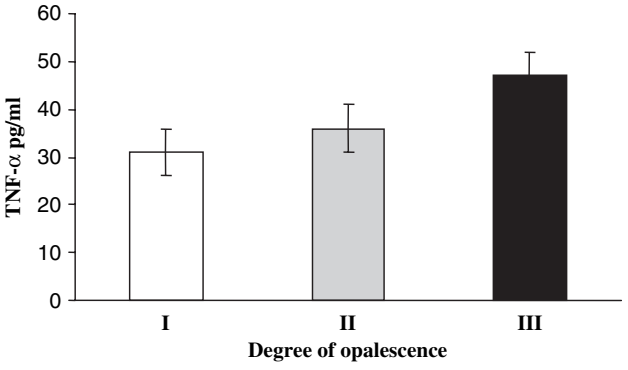

Figure 2 Tumor necrosis factor-alpha concentration significantly increased in terms of degree of fluid opalescence (Mann–Whitney *U*-test, $P > 0.05$)

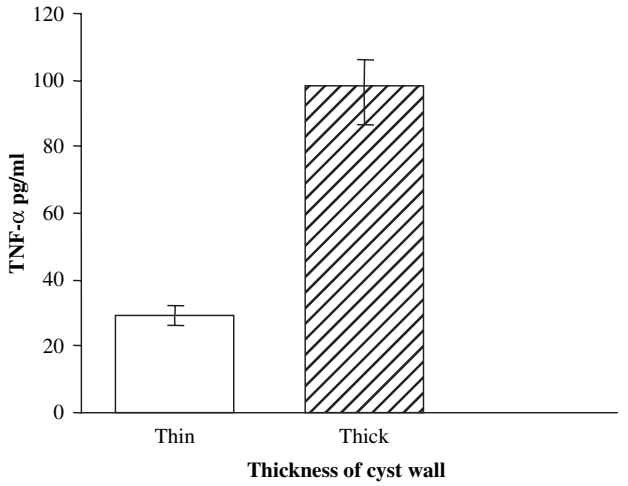

Figure 3 Tumor necrosis factor-alpha concentration was significantly higher in cysts with thick wall in comparison with thin wall (Mann–Whiteny *U*-test, $P < 0.05$)

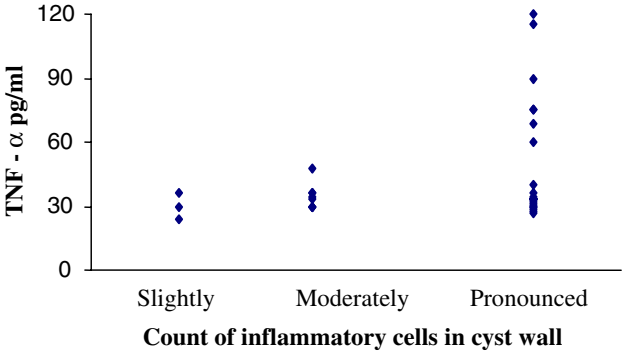

Figure 4 The individual values of tumor necrosis factor-alpha (TNF-α) concentration in cysts fluid in respect of count of inflammatory cells presenting in cyst wall. TNF-α significantly increased in cysts with a high number of inflammatory cells in comparison with low number (Mann–Whiteney *U*-test, $P < 0.05$)

Values of TNF-α in respect of presence of cell subtypes in cyst wall

Investigation of presence of several cell subtypes in cyst wall was also included in this research. The plasma cells were enumerated on the Giemsa section, while CD3, CD20 and CD68 cells by immunohistochemistry. Figure 6 shows representative sections for each subsets in thin (Figure 6a–c) and thick (Figure 6d–f) cyst wall. In addition, in Figure 7, we show comparative analysis of each cell subtype.

There exists a difference in the cell subset in inflammatory infiltrate presented in thin and thick cyst wall.

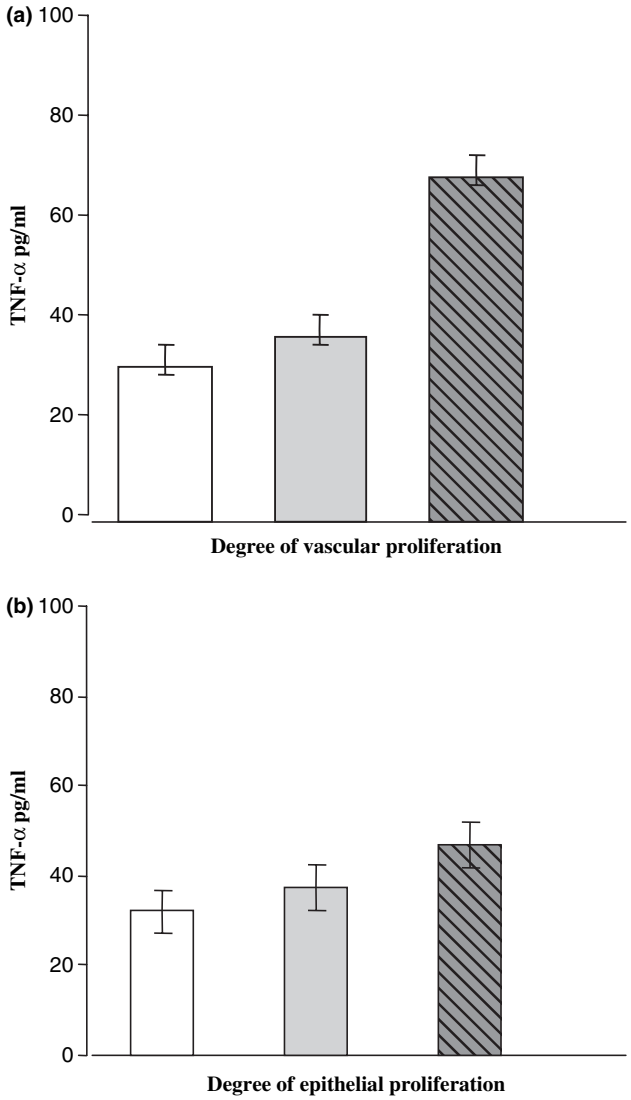

Figure 5 (**a**) Tumor necrosis factor-alpha (TNF-α) concentration in cystic fluid in respect of degree of vascularization. Open bar reflects low (below 10 blood vessels mm^{-2}), grey bar moderate (11–15 blood vessel mm^{-2}), and dark bar high vascularization (over 15 blood vessels mm^{-2}). Significant difference exists between low and high vascularization (Mann–Whitney *U*-test, $P < 0.05$). (**b**) Concentration of TNF-α in respect of degree of epithelial proliferation. Open bar reflects low, grey reflects moderate and shaded bar reflects high epithelial layers. There is no significant difference regarding these parameters (ANOVA, $P > 0.05$)

Thin cyst wall contains 73.5 ± 15.2% T cells ($CD3^+$), 17.8 ± 8.1% B cells ($CD20^+$), 10 ± 4% macrophages ($CD68^+$) and 4 ± 1% plasma cells. Contrary to this, thick cyst wall contains diverse ratios of cell subset, showing predominance of plasma cells 55 ± 16% and macrophages 56 ± 15%, but only 7 ± 4% T cells and 12.2 ± 6.1% B cells.

Discussion

The role of cytokines was extensively investigated during the last decade in inflammation and bone remodeling (Bertolini *et al*, 1986; Thomson *et al*, 1987; Kakaranatza-Angelopoulon and Nicolaton, 1990; Birkedal-Hansen, 1993; Meghji *et al*, 1996; Silva *et al*, 2004; Kaneyama *et al*, 2005; Bletsa *et al*, 2006). However, this investigation gives new results by showing different concentrations of TNF-α in cysts fluid of the radicular cyst depending on several clinical and histological parameters including also the count of inflammed cells and the degree of vascularization that have not been so much investigated simultaneously.

We here report that a higher concentration of TNF-α in respect of cysts size is more accumulated in small radicular cyst accompanied by a higher degree of fluid opalescence representing the total amount of different proteins, cell detritus and other cytokines released into cystic fluid, as we determined this parameter on semi-quantitative analysis. The larger cyst, usually with some orocystic communication has a lower amount of TNF-α probably by dilution into higher cyst volume or release from cystic fluid.

We here also analyzed the role of cystic epithelium on the amount of the released TNF-α into cystic fluid. Previous studies showed that polimorphonuclear leukocytes isolated and cultured from chronically inflammed tissues can produce inflammatory cytokines, such as TNF-α (Bando *et al*, 1993; Honma *et al*, 1998; Ohsima *et al*, 2000; Ninomiya *et al*, 2002; Colic *et al*, 2006). The histopathological examination in our study shows that radicular cysts contained a significant number of inflammed cells including neutrophiles, eosinophyls, plasma cells, lymphocytes and macrophage. Further, we report here that concentration of TNF-α is significantly increased in cysts in terms of cyst wall thickness, count of inflammatory cells, degree of vascularization per mm^2 and other epithelial characteristics. Histomorphometric analyses in this study showed that a majority, i.e. 35 (77%) from our 43 radicular cysts have wall thickness composed of diverse ratios of cell subtypes in comparison to thin wall. These cysts with thick wall have a higher concentration of TNF-α in comparison with those without thickness. This finding correlates also with increase in count of inflammatory cells in cyst wall, indicating that from all investigated radicular cysts, most (74.4%) have a pronounced account of infiltrated cells in cyst wall. Both these findings were closely associated with amount of TNF-α produced.

We further investigated the association of inflammed cell subtypes with TNF-α accumulated in cystic fluid. Activated macrophages and lymphocytes in comparison to fibroblasts also presenting in inflammatory cyst wall can be majority in cell population. We here obtained different ratios of cell subsets presented in inflammatory infiltrate in cyst with thin (Figure 6a–c) and thick cyst wall (Figure 6d–f). Cysts with thin wall contained mostly T cell subsets (Figure 6a), while cysts with thick wall showed predominantly plasma cells and macrophages (Figure 6d). In spite of great number of T cells (which is not activated) in thin cyst wall, concentration of TNF-α is low. On the basis of these results, we suppose that increased TNF-α in cyst with thick wall is a consequence of greater number of macrophages than in thin cyst wall. Therefore, the production of TNF-α

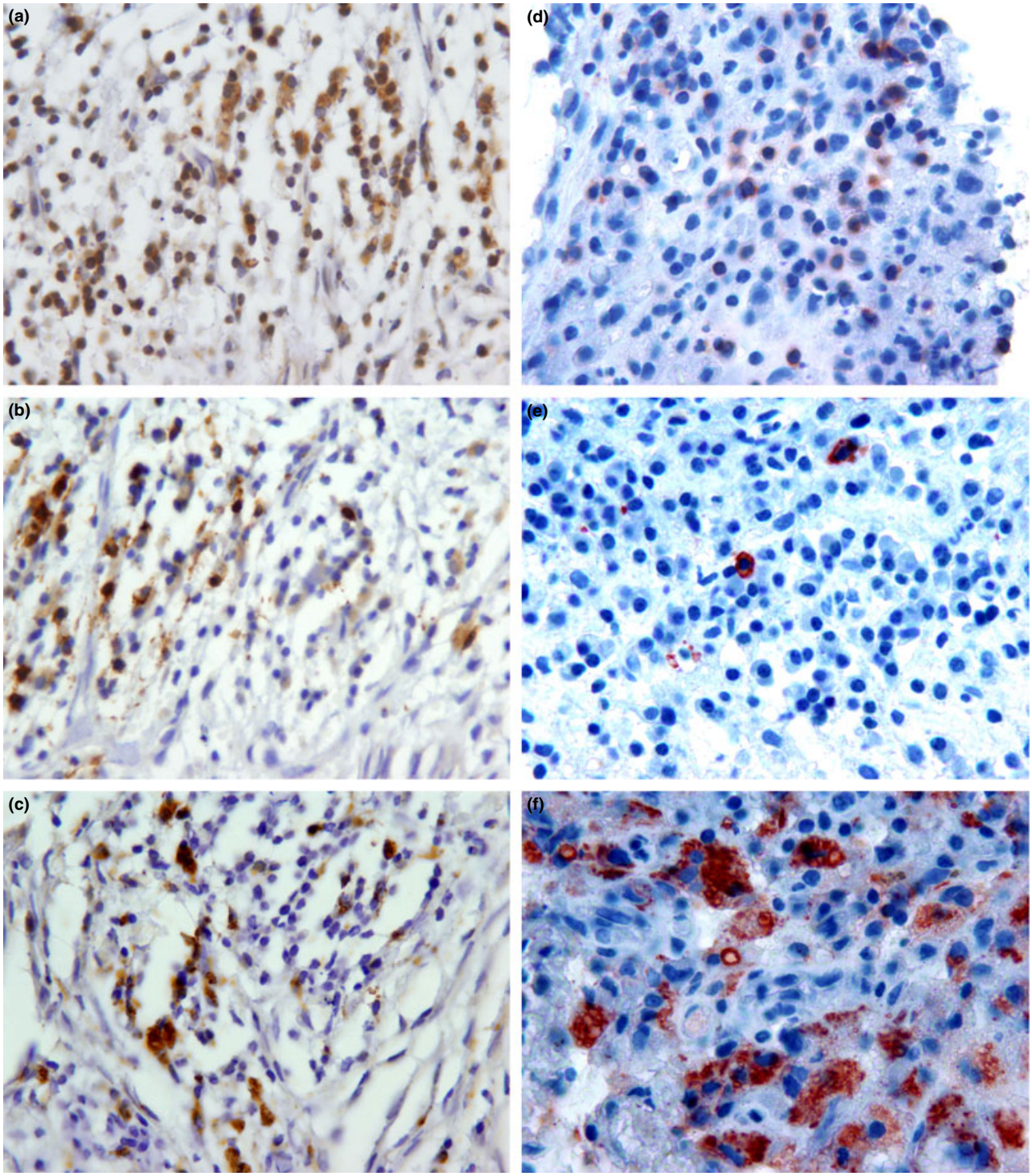

Figure 6 The representative photography for T cells ($CD3^+$), B-cells ($CD20^+$) and macrophages ($CD68^+$) presented in cysts with thin wall (**a**, **b**, **c**) and thick wall (**d**, **e**, **f**) respectively, analyzed by immunohistochemistry, (immunoperoxidase ×1000)

correlates with a great number of macrophages (CD68 cells) but not with number of T cells.

This is a consequence of the consideration that a major source for production of TNF-α was macrophages and that other cells subtypes including T cells, produced this cytokine, but mostly after stimulation. This finding is also in accordance with previously reported literature data, showing that cultured epithelial cells, after *in vitro* exposure to diverse stimuli, can produce TNF-α and other cytokines like GM-CSF and M-CSF, IL-1, IL-6 involved in inflammation, cell adhesion and cyst formation (Takeichi *et al*, 1996; Ninomiya *et al*, 2002; Danin *et al*, 2003).

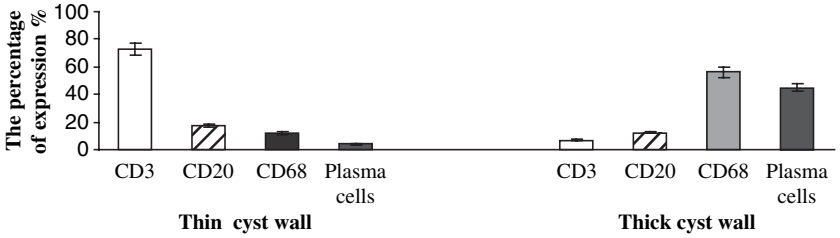

Figure 7 The mean values ± s.d. for several cell subtypes in inflammatory infiltrate of cysts with thin and thick wall. A significant difference was found (Mann–Whitney *U*-test, $P < 0.05$) between these cell subsets in respect of cyst wall thickness

Honma *et al* (1998) indicated that expression of mRNA analyzed by *in situ* hybridization for several pro-inflammatory cytokines exhibited variation in cell types, including keratocytes, macrophages and fibroblasts. We here clearly indicated the presence of correlation between the concentration of produced and accumulated TNF-α in cystic fluid and the amount of presenting macrophages in pericystic tissues in thick wall.

As angiogenesis is an essential part of a variety of pathological processes, including inflammation, controlled by numerous different bioactive molecules (Takeichi *et al*, 1996), we report for the first time correlation between production of TNF-α and degree of vascularization in pericystic tissues. We found a significant association between these parameters indicating that our cytokines among numerous different bioactive molecules have also an important role in angiogenesis and inflammation.

In conclusion, these results show increase in TNF-α concentration in radicular cysts based on several clinical parameters including cysts volume, the protein concentration in cystic fluid as well as on histological findings including cyst wall thickness, count and subtype of inflammatory cells and degree of vascularization analyzed in cyst wall.

We believe that determination of TNF simultaneously with other diagnostic procedures can be a useful parameter in clinical diagnosis of inflammatory cysts and recommend use of this parameter for routine analyses.

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