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Transforming growth factor beta-1 expression in macrophages of human chronic periapical diseases

Z.-Z. Liang[1]*, J. Li[2]* and S.-G. Huang[3]

[1]Department of Dentistry, Faculty of Dentistry, Jinan University, Guangzhou, China
[2]Department of Dentistry, Faculty of Dentistry, Jinan University, Guangzhou, China
[3]Department of Dentistry, Faculty of Dentistry, Jinan University, Guangzhou, China

*These authors contributed equally to this study.
Corresponding author: S.-G. Huang
E-mail: huangsg_06@163.com

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ABSTRACT. The objective of this study was to observe the distribution of macrophages (MPs) expressing transforming growth factor beta-1 (TGF-β1) in tissue samples from patients with different human chronic periapical diseases. In this study, samples were collected from 75 volunteers, who were divided into three groups according to classified standards, namely, healthy control (N = 25), periapical granuloma (N = 25), and periapical cyst (N = 25). The samples were fixed in 10% buffered formalin for more than 48 h, dehydrated, embedded, and stained with hematoxylin and eosin for histopathology. Double immunofluorescence was conducted to analyze the expression of TGF-β-CD14 double-positive MPs in periapical tissues. The number of double-positive cells (cells/mm^2) were significantly higher in the chronic periapical disease

tissues ($P < 0.01$) compared to that in the control tissue; in addition, the density of TGF-β1-CD14 double positive cells was significantly higher in the periapical cyst group than in the periapical granuloma group ($P < 0.01$). The number of TGF-β1 expressing macrophages varied with human chronic periapical diseases. The TGF-β1-CD14 double-positive cells might play an important role in the pathology of human chronic periapical diseases.

Key words: Chronic periapical disease; Transforming growth factor beta-1; Expression; Macrophages; Inflammation; immune regulation

INTRODUCTION

The TGF-β superfamily consists of more than 40 members, which are similar in structure and function. TGF-β is secreted as a homodimer, and its ligands include TGF-β, activin, and bone morphogenetic protein (BMP), which are widely expressed growth factors playing important roles in maintenance of matrix homeostasis and regulation of inflammation (van Dijk et al., 2012). In addition, the members of the TGF-β super family are involved in proliferation, differentiation, reconstruction of the extracellular matrix, embryonic development, apoptosis, and so on (Yue and Mulder, 2001; Rahimi and Leof, 2007; Wrighton et al., 2009; Matsuura et al., 2010).

TGF-β1-3 is a mammalian TGF-β1 subtype. TGF-β1 is the founding molecule of this subclass, with a molecular weight of 25kDa, and is produced by diverse cells in the body, such as active macrophages (MPs) and eosinophils (Yue and Mulder, 2001). TGF-β1 is a cell activity factor with multiple functions; it also acts as a growth factor, which promotes tissue repair and is widely applied for tissue engineering.

Studies on TGF-β1 have mainly focused on its roles in distraction osteogenesis, salivary gland neoplasms, orthodontic teeth movement, and periodontal tissue repair in stomatology (Kuru et al., 2004; Delatte et al., 2005; Knabe et al., 2005). However, reports on the effect of TGF-β1 in periapical inflammation are scarce (Khalil et al., 1989). Fujii et al. (2010) suggested that TGF-β1 is involved in the incidence, development, and tissue repair in rat experimental periodontal disease; however, the expression and the possible mechanism via which TGF-β1 effects MPs in human periodontal disease is unknown. In this study, double immunofluorescence (DIF) was used to determine the levels of TGF-β1 in MPs of human periodontal disease, and its role in the pathology of the disease and tissue repair was also investigated.

MATERIAL AND METHODS

Subjects

Sample selection

The seventy-five people who visited the Department of Stomatology of Zhongshan City People's Hospital in the postgraduate culture base of the Jinan University from March 2015 to August 2015 included 39 males (aged 38 ± 18.32 years old) and 36 females (aged 31

± 14.12 years old), who did not differ statistically in age and gender. Volunteers who were pregnant, took pills, had systemic diseases, and were prescribed antibiotics in the last three months were excluded from the study. An informed written consent was obtained from all the individuals. The Human Subjects Research Committee (HSRC) approved the study protocol and the informed consent forms.

Groups and sample collection

Based on the results of clinical X-ray inspection, operative investigations, and histopathological examination, the subjects were divided into three groups of 25 cases each, in which the healthy control subjects were age and gender matched with the patients with periapical granulomas or radicular cysts; the two patient groups were also age and gender matched. The cases in the healthy control group were characterized by periodontal ligament with healthy periapex, and premolars, the fully developed roots of which were removed by orthodontic surgery. The inclusion criteria of the healthy control group were the presence of vital teeth, absence of transmission shadows with low-density in the apical area in X-ray exposure, smooth and continuous periodontal membrane and unbroken periodontal ligament of the apical area after tooth extraction, and the absence of capillary proliferation or inflammatory cell infiltration in pathological observation. The characteristics of the periapical granuloma group were the presence of periapical lesions in the periodontal ligaments, which had to be treated exclusively by periapical curettage and not by any non-invasive method. The inclusion criteria for this group were the presence of non-vital teeth, round or oval transmission shadow with low-density in the apical area (diameter $\leq$ 1 cm) in X-ray exposure, the presence of soft tissue lesion in the apical area during the operation, and the presence of lesion tissues with granulation tissue mass accompanied by capillary proliferation and inflammatory cell infiltration in pathological observation. The radicular cyst group was characterized by the presence of periapical lesions in the periodontal ligaments, which had to be treated exclusively by periapical curettage and not by any non-invasive method. The inclusion criteria were presence of non-vital teeth, round or oval transmission shadow with low-density in the apical area, whose edges showed ray resistance (diameter $\leq$ 1 cm) in X-ray exposure, and the presence of soft tissue lesion in the apical area, which contained liquid or semi-solid cystic tissues in the operation, and the presence of lesion tissues with collagen fibrils, which were cysts or tissues covered fully or partially with non-keratinized stratified squamous epithelium (Teixeira-Salum et al., 2010).

Tissue collection, treatment, and observation

The periodontal tissues were fixed in 10% buffered formalin for more than 48 h, washed thrice in PBS (phosphate buffer saline; Mingwang Biological Company, Guangzhou, China), dehydrated in ethanol gradient, embedded in paraffin, and processed into 5 μm continuous slices using the Leica ultra-thin semiautomatic microtome. The slices were stained with hematoxylin and eosin (HE) for microscopic analysis (Costa et al., 2009). DIF was performed to analyze the expression of TGF-β1-CD14 double-positive cells (MPs) in periapical tissues.

Double immunofluorescence imaging of TGF-β1-CD14

Rabbit-anti-TGF-β1 antibody (Santa Cruz Biotechnology, USA), mouse-anti-CD14 antibody (Santa Cruz Biotechnology, USA), anti-rabbit-alexa fluor®555-conjugated secondary antibody (Cell Signaling technology, USA), anti-mouse-alexa fluor®488 conjugated-secondary antibody (Cell Signaling Technology, USA), and diamidino-phenylindole (DAPI) (MP Biomedicals, USA) were purchased. The tissue slices were repaired with saline sodium citrate buffer, pH 6.0, as described previously (Gammon et al., 1982), blocked with secondary anti-serum at 37°C for 1 h, then incubated with the first antibody at 4°C (1:200 dilution), stained with the secondary antibody (1:500 dilution) in dark at 25°C for 1.5 h, followed by staining with DAPI for 15 min, and immediately observed under the fluorescence microscope (Leica DM750, Beijing, China). The TGF-β1 and CD14 signals were visualized using red (the wavelength is 555nm) and green(the wavelength is 488nm) lights, respectively, to detect cell membrane-bound or cytoplasmic localization; the signal appeared orange when the red and green signals were merged in the same field; the nucleus was stained with DAPI, which appeared blue in the microscopy images. The slices were blindly observed under the fluorescence microscope by two pathologists according to the counting method reported by Batista et al. (2005). Ten typical continuous fields were selected (0.0725 mm^2/field), where DAPI-positive cells and CD14-TGF-β1 double-positive cells were counted, and the double-positive cell density (cells/mm^2) was obtained after averaging the data from all the images.

Statistical analysis

Data was analyzed using the SPSS 13.0 software (Chicago, America), and is represented as mean ± standard error of mean (SEM). One-way ANOVA was used to compare the CD14-TGF-β1 double-positive average density in each group, and the Levene's method was used for homogeneity test of variance. When variance was homogeneous, the Bonferroni method was used; otherwise, the Tamhane's T2 method was used. The two-tail $\alpha = 0.05$ was used as the analysis standard, and $P < 0.05$ was considered as statistically significant.

RESULTS

Histological observation

The periodontal membrane of the apical area was intact, rich with fibrous tissues, and lacked any obvious capillary proliferation or inflammatory cell infiltration in the cases of the healthy control group (Figure 1A and B).

The periodontal membrane of the apical area was destroyed, with abundant capillary proliferation and inflammatory cell infiltration in the periapical granuloma group. Additionally, the epithelial cord was observed in parts of the apical area (Figure 1C and D).

The periodontal membrane of the apical area was destroyed and showed angiotelectasis in the radicular cysts group. The cyst tissues were covered with stratified squamous epithelium and had abundant inflammatory cell infiltration under the epithelium. The fibrous tissues contained mainly collagen fibrils and few inflammatory cells. In addition, hemosiderin and cholesterol crystals were also found in parts of the cyst wall (Figure 1E and F).

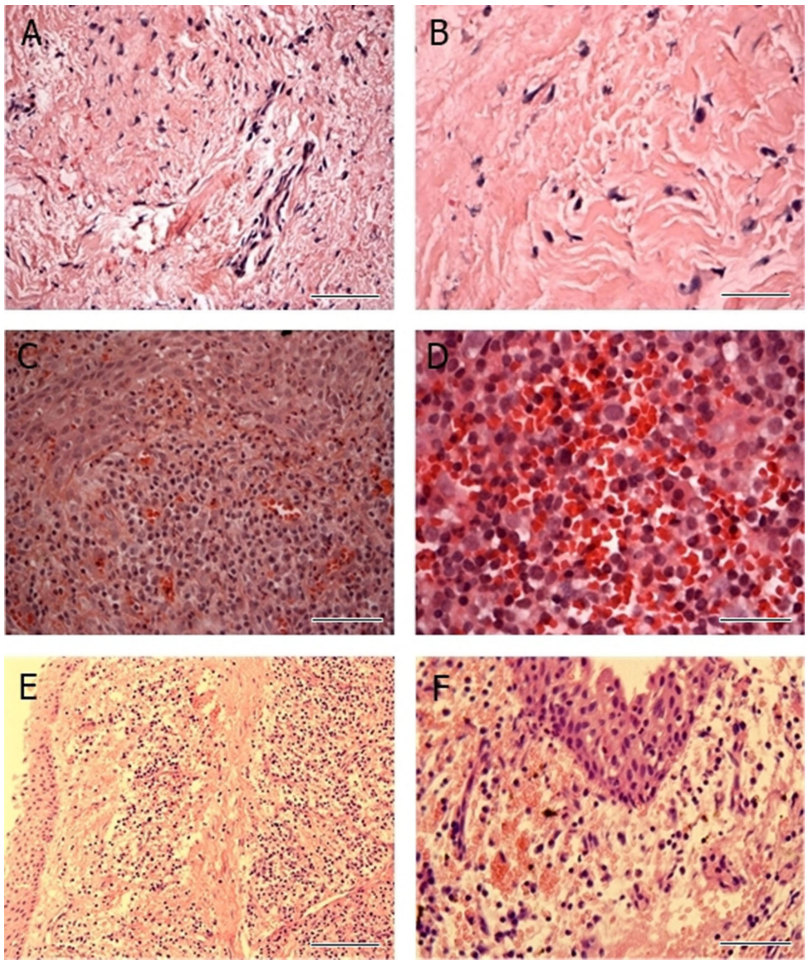

Figure 1. Hematoxylin-Eosin staining. **A. B.** Healthy control; **C. D.** periapical granulomas; **E. F.** radicular cysts. (A, C, and E scale bars =100 μm, original magnification, 200X; B, D, F scale bars = 50 μm, original magnification, 400X).

Microscopic observation of CD14-TGF-β1 double-positive cells

The results of DIF for each group are shown in Figures 2, 3, and 4. In the healthy control group, few scattered CD14-TGF-β1 double-positive cells were observed (Figure 2). The number of cells increased significantly in the periapical granuloma group (Figure 3). Infiltration of CD14-TGF-β1 double-positive cells was observed in the radicular cysts, with few mature erythrocytes and epithelial structures (Figure 4). The negative control group did not express CD14 or TGF-β. Figure 5 shows a comparative analysis of the number of CD14-TGF-β1 double-positive cells in all the groups, which revealed that the number of CD14-TGF-β1 double-positive cells increased significantly in the two chronic periapical disease tissues than in the healthy control group ($P = 0.000$, $P < 0.01$). The density of the CD14-TGF-β1 double-positive cells was dramatically higher in the radicular cyst group than in the periapical granuloma group ($P = 0.000$, $P < 0.01$).

The method of specific staining is simple, specific and sensitive.

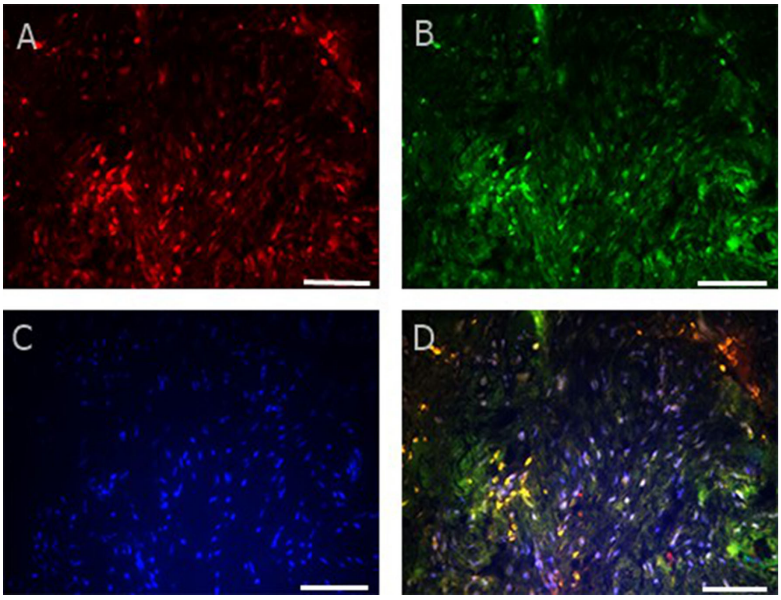

Figure 2. Double immunofluorescence staining for TGF-β1 and CD14 in tissues from the healthy control group. **A.** TGF-β1^{+}; **B.** CD14^{+}; **C.** DAPI^{+}; **D.** Merged. (staining scale bars = 50 μm, original magnification, 400X).

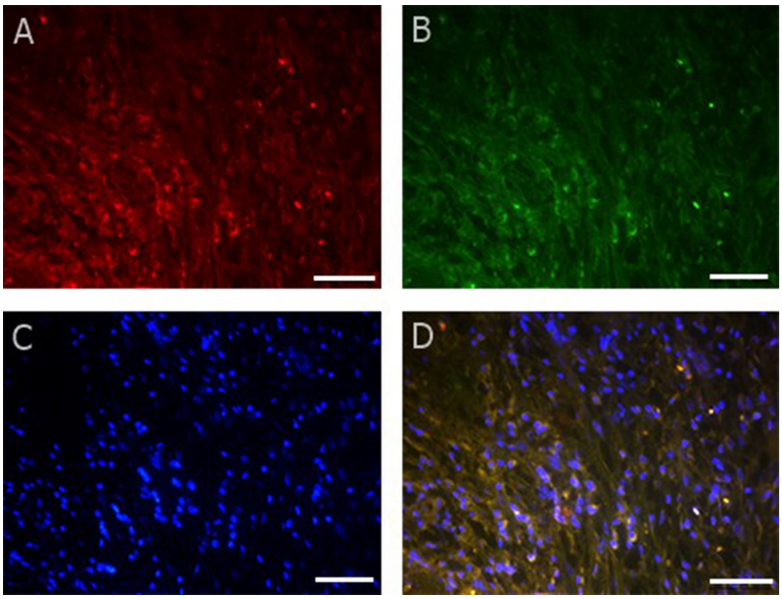

Figure 3. Double immunofluorescence staining for TGF-β1 and CD14 in tissues from periapical granulomas. **A.** TGF-β1^{+}; **B.** CD14^{+}; **C.** DAPI^{+}; **D.** Merged. (staining scale bars = 50 μm, original magnification, 400X).

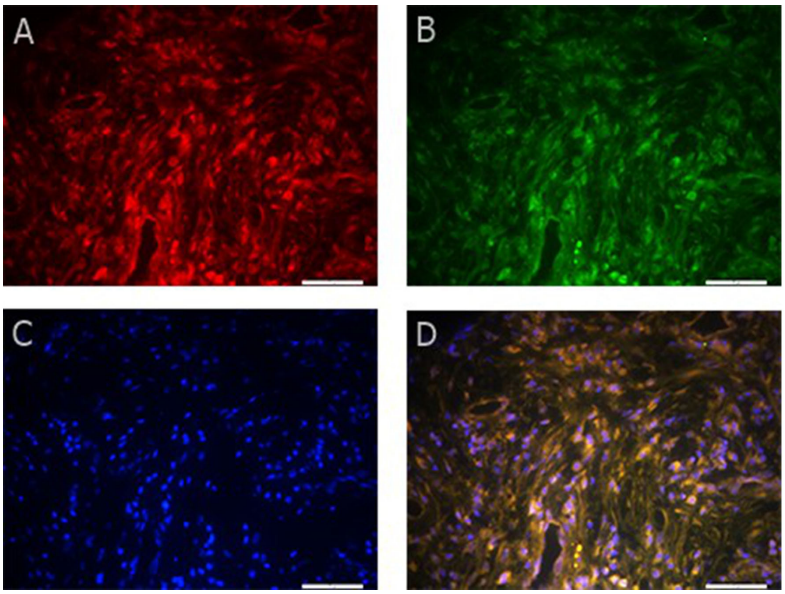

Figure 4. Double immunofluorescence staining for TGF-β1 and CD14 in tissues from radicular cysts. **A.** TGF-β1^{+}; **B.** CD14^{+}; **C.** DAPI^{+}; **D.** Merged. (scale bars = 50 μm, original magnification, 400X).

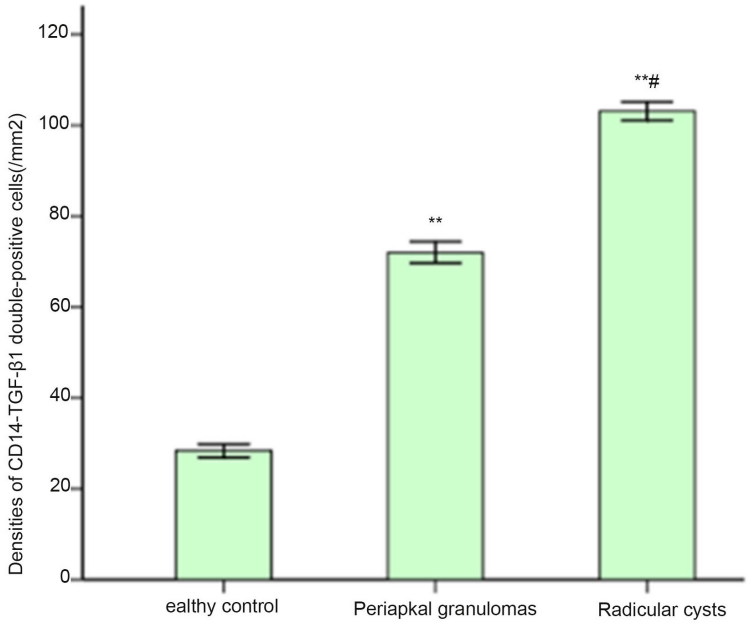

Figure 5. Analyses of the densities of CD14-TGF-β1 double-positive cells in periapical tissues of each group. Data are represented as mean ± SEM. Significant differences between groups were analyzed by Tamhane's T2 test. **P < 0.01 compared to healthy control group; #P < 0.05 compared to periapical granulomas.

DISCUSSION

As a cell activity regulatory factor with multiple functions, TGF-β1 participates in several important pathophysiological processes, and is currently studied extensively. TGF-β1 plays a bidirectional role in immune regulation, and has complex relationships with diseases. The dual role of TGF-β in modulating macrophage function is rapidly gaining recognition. In addition to its role as a 'macrophage-deactivating' agent, TGF-β functions as a monocyte activator, inducing cytokine production and mediating host defense (Ashcroft, 1999). It also exerts inhibitory and stimulatory effects on bone marrow cells (Jacobsen et al., 1993) TGF-β1 has high chemotactic activities on osteoblasts and can stimulate the proliferation and differentiation of mesenchymal stem cells to promote the synthesis of osteoblasts, chondroblasts, and extracellular matrix (Kingsley, 1994; Fox and Lovibond, 2005; Hu et al., 2011). TGF-β1 appears to play an important role in inducing fibroblastic differentiation of periodontal ligament (PDL) stem/progenitor cells and in maintaining the PDL apparatus under physiological conditions (Fujii et al., 2010). It also regulates the expression of collagen type I and promotes tissue healing (Okubo et al., 2003; Fujita et al., 2004; Ono et al., 2007). In addition, the gradual increase in connective tissue growth factor (CTGF) and TGF-β1 levels in hepatic tissue is associated with liver fibrosis. Early expression of CTGF and TGF-β1 in biliary epithelial cells may be involved in the pathogenesis of congenital biliary atresia (Li et al., 2016).

Chronic periapical disease is a chronic inflammation of periapical tissue, which is caused by long-term residence of bacteria in root canals. However, periapical lesions are not directly caused by bacteria, but are induced by various cells and cytokines. MPs are the main immune cells present in periapical lesions, which enter the periapical tissue at the earliest stage, suggesting that it is the first line of local defense for eliminating destructive stimuli and preventing infectious substance from invading periapical tissue (Metzger, 2000). MPs have high plasticity, local tissue function specificity, and are capable of abnormal differentiation induced by inflammatory factors. Therefore, the changes associated with different stages of the disease, tissue internal environment, and the cytokine milieu can induce various MPs (M1 and M2) to mediate pro- or anti-inflammatory reactions (Gordon and Taylor, 2005). In chronic inflammation, the changes in MP phenotype act as critical effectors (Koh and DiPietro, 2011). One study demonstrated that infiltration of the M1 type MPs removed necrotic tissue in the inflammatory lesion infected by bacteria, followed by tissue healing via the anti-inflammatory action of the M2 MPs (Rigamonti et al., 2014), which secrete TGF-β, vascular endothelial growth factor, and epidermal growth factor to promote wound healing and tissue fibrosis (Gordon and Martinez 2010; Wynn et al., 2011). The mechanism underlying the change in MP phenotype is unclear at present (Koh and DiPietro, 2011) and requires further investigation.

In this study, DIF was used to observe the distribution of MPs in different human chronic periapical disease tissues, and to analyze the effect of TGF-β1 on MPs. The results showed that the number of MPs was more in the radicular cyst group compared to that in the healthy control group. The density of TGF-β1-CD14 double-positive cells (cells/mm^2) was significantly higher in human periapical disease tissues ($P < 0.01$), which indicated that the number of TGF-β1 expressing macrophages varied with the different types of human periapical disease tissues. TGF-β1-CD14 double-positive cells might participate in the pathogenesis of chronic periapical disease, especially in fiber formation and tissue repair of radicular cyst. Furthermore, the TGF-β1-CD14 double-positive cells might participate in the immune regulation of periapical lesions, and doubly regulate the occurrence and development of this

disease. We speculate that TGF-β1and the M2 type MPs might play a key role in the tissue repair process of chronic periapical disease; however, further investigation is required to substantiate this hypothesis. The results of this study showed that there may be theoretical significance and clinical guidance value for understanding the mechanism of TGF-β1 in the incidence of periapical disease and its tissue repair process. The TGF-β1-CD14 double-positive cells might play an important role in the pathology of human chronic periapical disease, especially in the process of fibrous tissue formation and tissue repair of periapical cysts.

However, certain limitations exist in this study. First, our findings regarding the correlation between the number of CD14-TGF-β1 double-positive macrophages and the extent or type of periapical disease does not allow us to ascertain whether the increased number of macrophages is a causative, direct or indirect effect of inflammation, because binding of TGF-β1 to the cell surface receptors might yield a false-positive result or cause overestimation of the number of CD14 and TGF-β double-positive macrophages. Secondly, we cannot determine whether the presence of macrophages or TGF-β1 levels exacerbates or mitigates the extent of inflammation based on our results. Therefore, we cannot demonstrate a direct role for macrophages or TGF-β1 in the pathogenesis or healing of the disease. Additionally, we hypothesized a mechanism for macrophage-mediated repair of periodontal lesions based on the results of immunofluorescence assay, which may not be sufficient.

CONCLUSIONS

We used DIF to analyze the TGF-β1levels of MPs in different types of human chronic periapical disease tissues. First, the number of TGF-β1 expressing macrophages varied with the type of human chronic periapical disease. Second, the TGF-β1-CD14 double-positive cells might play an important role in the pathology of human chronic periapical, especially in the process of fibrous tissue formation and repair of periapical cysts. Finally, the specific role of the M2 type MPs in the repair process of periapical tissues is still unknown, and a larger sample size is required to address this question.

Conflicts of interest

The authors declare no conflict of interests regarding the publication of this paper.

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