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ORIGINAL ARTICLE

Transglutaminase 2 up-regulation is associated with RANKL/OPG pathway in cultured HPDL cells and THP-1-differentiated macrophages

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Abstract Recent evidence emphasized that transglutaminase 2 (TG2), a protein cross-linking enzyme, may play a role in the early phase of inflammation. High levels of TG2 have been associated with the constitutive activation of nuclear factor-kappa B (NF-κB) that is considered the main regulator of inflammation. In this context, the receptor activator of NF-kappa B ligand (RANKL) and receptor activator of NF-κB have extensive functions in the regulation of cytokine secretion associated with different pathological conditions. The human periodontal ligament (HPDL) cells, which express and secrete osteoprotegerin (OPG) and RANKL, represent an useful "ex vivo" model for monitoring cell response in inflammatory microenvironments, such as periodontitis-dependent tissue response. Thus, we evaluated TG2 expression and alterations in RANKL/OPG ratio occurring in cultured HPDL cells. The HPDL cells were obtained from patients with chronic periodontitis (CP) and healthy subjects. We observed the up-regulation of some inflammatory markers, such as IL-6, TNF-α, and HMGB-1, and at the same time an increase in TG2 mRNA levels in HPDL cells from CP patients compared with healthy subjects. We found a positive correlation between RANKL/OPG ratio and TG2 mRNA levels in HPDL cells from CP patients. In the parallel experiments, we demonstrated that TG2 inhibition reduced RANKL expression in both HPDL cells from CP patients and monocytes differentiated to macrophages by tetradecanoyl phorbol acetate treatment. Given the RANKL key role in NF-κB pathway and the observed up-regulation of pro-inflammatory cytokines, our data suggest that TG2 may be involved in molecular mechanisms of inflammatory response occurring in periodontal disease.

Keywords RANKL/OPG · Chronic periodontitis · Transglutaminase · Monocytes/macrophages · Inflammation · Human periodontal fibroblasts

Abbreviations

ALP	Alkaline phosphatase
BAPA	5-(Biotinamido)pentylamine
CP	Chronic periodontitis
FBS	Fetal bovine serum
FMOD	Fibromodulin
HPDL	Human periodontal ligament
HMGB1	High mobility group box 1
IL-6	Interleukin-6
LUM	Lumican
NF-κB	Nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
OPG	Osteoprotegerin
PDL	Periodontal ligament

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TNF-α	Tumor necrosis factor-α
TG2	Tissue-type transglutaminase
TG	Transglutaminase

Introduction

The process of bone formation at any body site, including the alveolar bone of the periodontium, is orchestrated by osteoblasts under the systemic control of hormones and by cytokines or local factors residing in bone extracellular matrix (Dzink et al. 1985). More specifically, several proteins participate in cell attachment, spreading and signaling in osteoblasts, osteocytes, and osteoclasts, regulating mineralization of the collagen-rich extracellular matrix, thereby contributing to the overall strength of bone tissue (McKee and Sodek 2000). In this context, tissue-type transglutaminase (TG2) protein, a member of transglutaminase family of enzymes, has been implicated in hard tissue development, matrix maturation and mineralization (Lorand and Graham 2003). In particular, TG2 is a multifunctional calcium-dependent enzyme which catalyzes post-translational protein modifications leading to the formation of intra- or inter-molecular epsilon(gamma-glutamyl)lysine bonds (cross-links), or polyamine incorporation into proteins (Griffin et al. 2002).

Given the different functions of this protein, TG2 has been involved in various biochemical mechanisms associated with cell proliferation, cell differentiation, signal transduction, apoptosis, and wound healing (Telci and Griffin 2006; Ientile et al. 2007; Currò et al. 2014b). Furthermore, other results show that TG activity in osteoblast cultures is required for osteoblast differentiation (Al-Jallad et al. 2006).

In periodontal diseases, infiltrated leukocytes produce inflammatory mediators which affect the expression of both receptor activator of nuclear factor (NF)-kappa B ligand (RANKL) and osteoprotegerin (OPG) in osteoblasts and periodontal ligament (Nagasawa et al. 2007; Matarese et al. 2012, 2013). Changes in the relative RANKL/OPG expression ratio are considered indicative of cell or tissue capacity to regulate bone resorption in different pathological conditions, including periodontitis (Bostanci et al. 2007).

Periodontal ligament (PDL) tissue anchors the tooth root to jaw alveolar bone and plays an important role in tooth maintenance and mobility, cementum formation as well as in tissue defense against inflammation (Parlar et al. 2005). In addition, human PDL (HPDL) cells express and secrete OPG and RANKL, likely playing a role in alveolar bone metabolism (Crotti et al. 2003). Indeed, some results demonstrated that the expression of RANKL can be modified in HPDL cells in response to different substances, including H_2O_2, nicotine, and substance P (Lee et al. 2009). In particular, the alterations induced throughout the infection diseases, such as periodontitis, can result in PDL destruction and, subsequently, in tooth loss.

In this context, evidence showed that, similarly to various inflammatory diseases, RANKL plays a key role in inducing bone resorption by osteoclasts (Verdrengh et al. 2010). Noteworthy, the role of RANKL combined with OPG appears relevant in periodontal tissues for the regulation of bone remodeling during orthodontic tooth movement as well as root resorption. On the other hand, it is known that nuclear factor-kappa B (NF-κB) plays a central role during inflammation also by regulating RANKL expression in different cell types (Vardar-Sengul et al. 2009; Li et al. 2010).

Given these observations, the aim of our study was firstly to investigate whether TG2 can be involved in inflammatory response occurring in chronic periodontitis (CP). Thus, we used primarily cultured HPDL cells and human THP-1 cells, differentiated toward macrophages, to evaluate whether changes in TG2 expression and TG activity may be related with osteoclast specific RANKL/OPG pathway.

Materials and methods

Materials

Immortalized human gingival fibroblasts, HGF-1 (ATCC CRL-2014), were purchased from ATCC (American Type Culture Collections, Rockville, MD, USA). The human pre-monocytic cell line, THP-1, was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen-Braunschweig, Germany). RPMI-1640, DMEM, Hanks buffered salt solution, penicillin/streptomycin mixture, amphotericin B, L-glutamine, HEPES, sodium pyruvate, glucose, complete protease inhibitor cocktail, 12-O-tetradecanoylphorbol-13-acetate (TPA), dispase, developer, fixer, Kodak X-ray film, and other chemicals of analytical grade were from SIGMA Aldrich (Milan, Italy). Fetal bovine serum (FBS), M-SFM, as well as TRIzol for RNA extraction were from Invitrogen Life Technologies (Milan, Italy).

5-(Biotinamido)pentylamine (BAPA) was from Pierce Biotechnology Inc. (Rockford, IL, USA).

ECL Chemiluminescence detection kit was from GE Healthcare Life Sciences (Milan, Italy).

Oligonucleotide probe containing the NF-κB consensus sequence present in tTG promoter was synthesized by MWG Biotech (Monza, Italy). The Biotin 3′-End DNA Labeling kit, LightShift Chemiluminescent EMSA kit, Biodyne Nylon+ membranes, and Super-Signal West Pico Chemiluminescent Substrate System were from Pierce Biotechnology (Milano, Italy).

High-capacity cDNA archive kit, TaqMan Gene Expression Mastermix, SYBR green PCR Mastermix, TaqMan Gene Expression assays (Assays-on-Demand) for human β-actin (ID: Hs99999903_m1), TGM2 (ID: Hs00190278_m1), IL-6 (ID: Hs00985639_m1), TNF-α (ID: Hs00174128_m1), HMGB1 (ID: Hs01590761_g1), RANKL (ID: Hs00243519_m1), and OPG (ID: Hs00900358_m1) were from Applied Biosystems (Life Technologies, Milan, Italy).

1,3-Dimethyl-2[(oxopropyl)thio]imidazolium (R283) was a generous gift from Professor Martin Griffin of School of Life & Health Sciences at Aston University in Birmingham (UK).

Collection of periodontal ligament specimens and cell isolation

The biopsies of CP patients ($n = 14$) were obtained from the site with severe periodontal destruction and inflammation during extraction of teeth attributable to CP. The collection of PDL specimens was carried out during extraction for advanced caries and orthodontic indications for the healthy control group ($n = 14$). Six sites (mesio-buccal, buccal, disto-buccal, and disto-lingual, lingual and mesio-lingual) per tooth were used to derive periodontal disease status, according to the Centers for Disease Control and Prevention and the American Academy of Periodontology (CDC-AAP) definition for severe and moderate periodontal disease (Page and Eke 2007). All patients and healthy subjects gave written informed consent; the ethical approval was obtained for the experimental procedures applied in humans, in accordance with the provisions of the World Medical Association's Declaration of Helsinki of 1975, as revised in 2000.

The collection of PDL was carried out from the root surface of the tooth extracted and contained only periodontal ligament.

HPDL cells were then isolated as previously described (El-Awady et al. 2010). Briefly, teeth were rinsed in Hanks buffered salt solution containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 mg/ml amphotericin B, and after removing gingival tissue, were incubated in dispase (SIGMA Aldrich, Milan, Italy) for 1 h. Tissues obtained by scraping the middle third of the roots were placed in tissue culture dishes and allowed to dry briefly to enhance attachment. Then, dishes were filled with culture medium supplemented with 20 % fetal bovine serum (FBS), non-essential amino acids, penicillin–streptomycin, and 0.5 mg/ml amphotericin and incubated at 37 °C in 95 % air/5 % CO2. At confluence, the HPDL cells were used for subsequent analyses.

In parallel experiments, HPDL cells were incubated with the site-directed TG2 inhibitor R283 (250 μM) in fresh culture medium with 10 % heat-inactivated FBS for 24 h. Then, the cells were used for further analyses.

Immortalized human gingival fibroblasts, HGF-1, were cultured according to the supplier instructions.

Cell culture and treatment

THP-1 cells (DSMZ, Germany) were cultured as previously reported (Currò et al. 2014a). To allow monocyte to macrophage differentiation, THP-1 cells were seeded in 6-well culture plates at a density of 5×10^5 cell/ml in M-SFM with 200 nM TPA and incubated for 24 h at 37 °C in a 5 % CO_2/95 % air humidified atmosphere.

In a parallel experimental setting, TPA-differentiated THP-1 macrophages were washed twice with PBS and treated with the transglutaminase (TG) activity inhibitor R283 (250 μM) in fresh RPMI with 10 % heat-inactivated FBS for 24 h. Then, the cells were used for further analyses.

Quantitative real-time PCR

Total RNA was isolated from HPDL and HGF-1 cell cultures as well as TPA-differentiated THP-1 macrophages using TRIzol reagent. Two micrograms of total RNA were reverse transcribed with High-Capacity cDNA Archive kit according to the manufacturer's instructions. Then, TG2, interleukin-6 (IL-6), tumor necrosis factor (TNF-α), and High Mobility Group Box 1 (HMGB1) as well as RANKL and OPG mRNA levels were analyzed by Real-Time PCR using TaqMan gene expression assays. β-Actin was used as endogenous control.

Fibromodulin (FMOD), lumican (LUM), and alkaline phosphatase (ALP) mRNA levels were quantified by Real-time PCR using SYBR green-based gene expression analysis according to the manufacturer's instructions. The primer sequences used were the following: 5′-ccacttcacccactc-cactt-3′ (forward) and 5′-gtctcctcccacatcaggtc-3′ (reverse) for FMOD; 5′-tgatctgcagtggctcattc-3′ (forward) and 5′-aaaa-gagcccagctttgtga-3′ (reverse) for LUM; 5′-acaagcactcc-cacttcatctgga-3′ (forward) and 5′-tcacgttgttcctgttcagctcgt-3′ (reverse) for ALP; and 5′-ttgttacaggaagtcccttgcc-3′ (forward) and 5′-atgctatcacctcccctgtgtg-3′ (reverse) for β-actin, which was used as housekeeping gene (endogenous control). Quantitative PCR reactions were set up in duplicate in a 96-well plate and were carried out in 20 μl reactions containing 1x SYBR green PCR Mastermix, 0.1 μM specific primers, and 25 ng RNA converted into cDNA. Real-Time PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following profile: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). For Sybr green assays a standard dissociation stage was added to assess primer specificity.

Data were collected with SDS 2.3 software (Applied Biosystems, Foster City, CA) and analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method.

Western blotting

For Western blot analysis, HPDL cells were homogenized on ice in RIPA supplemented with complete protease inhibitor cocktail. Aliquots of 30 μg of total proteins were loaded on a 10 % denaturing SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Blotted membranes were incubated with mouse antibodies against TG2 (Neomarkers, Fremont, CA, USA) and β-actin (SIGMA Aldrich, Milan, Italy) (respectively, diluted 1:500 and 1:1000 in TBS-T), and rabbit antibodies against RANKL and OPG (Millipore, Milan, Italy) (diluted 1:1000 in TBS-T) at 4 °C overnight. After washing, blotted membranes were incubated for 2 h with HRP-conjugated anti-mouse (1:2000 and 1:5000 in TBS-T) or anti-rabbit (1:3000 in TBS-T) secondary antibodies (SIGMA Aldrich, Milan, Italy). Immunoblots were developed with ECL Plus chemiluminescent detection system kit using Kodak film. The bands were scanned and quantified by densitometric analysis with ImageJ 1.47 (http://imagej.nih.gov/ij/), after normalization against β-actin.

Transglutaminase activity

To measure in situ TG enzyme activity in HPDL cell cultures and THP-1 macrophages, the incorporation of 5-(biotinamido)pentylamine (BAPA) into proteins was quantified using a colorimetric assay as previously described (Currò et al. 2014a). In brief, BAPA (1 mM) was added to the culture medium 1 h prior to the end of incubation period. Then, cells were homogenized and ten micrograms of protein, diluted with coating buffer, were loaded into each well of a 96-well microtiter plate and incubated overnight at 4 °C. After blocking non-specific binding sites, 100 μl of HRP-conjugated streptavidin (1:1000) in 1 % BSA and 0.01 % Tween 20 in borate saline buffer were added to each well and incubated at room temperature for 1 h. After washing, 200 μl of substrate solution (0.4 mg of o-phenylenediamine dihydrochloride/ml of 0.05 M sodium citrate phosphate buffer, pH 5.0) were added to each well. The reactions were stopped by 3 N HCl, and the presence of biotinylated proteins was quantified by measuring the absorbance at 492 nm on a microplate spectrophotometer (Tecan). All measurements were done in triplicate and repeated at least three times.

Nuclear extracts and gel mobility shift assay

The presence of NF-κB DNA binding activity in cell nuclear extracts was evaluated by EMSA as described by Caccamo et al. (2005). Nuclear proteins (2 μg) were incubated with the double-stranded biotin-labeled NF-κB DNA probe (5′-GTT TTG GAA AAA GTG CCG GGG AAG CCC CGT GGG CCT CTG T-3′ and 5′-ACA GAG GCC CAC GGG GCT TCC CCG GCA CTT TTT CCA AAA C-3′), and then the protein/DNA complexes were separated on a non-denaturing 6 % polyacrylamide gel. For the competitive experiments, nuclear extracts were simultaneously incubated with the biotin-labeled probe and a 100-fold molar excess of unlabeled oligonucleotide (cold probe). After transferring onto a nylon membrane, detection was performed using streptavidin-HRP and a chemiluminescent substrate. The membrane was developed by exposure to film and the bands were scanned.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using Student's *t* test for comparisons between two groups, or one-way ANOVA followed by Bonferroni post hoc test, with *p* values less than 0.05 considered significant. Correlation analysis was used to describe the relationship between TG2 with RANKL/OPG ratio mRNA levels.

Results

In this work, we used HPDL cell cultures from chronic periodontitis patients and healthy subjects as fibroblast model, given that PDL fibroblasts are prominent cell type in periodontium, with central roles in periodontal tissue homeostasis and remodeling (El-Awady et al. 2010). According to previous observations (Lallier et al. 2005), first we compared the expression of FMOD (specific gene transcript for HPDL fibroblasts) to those of LUM and ALP in HPDL cell preparation and HGF-1 cell line in order to ascertain the prevalence of HPDL fibroblasts in the cell preparation (Fig. 1). Under our conditions, FMOD mRNA transcript was expressed at 40-fold higher levels than HGF-1, while LUM and ALP transcript levels were about three-fold higher in comparison with those in gingival fibroblasts. These results confirm that obtained HPDL cells were fibroblast-enriched cultures.

HPDL cells from patients with periodontitis and healthy subjects were examined for the levels of inflammatory markers, such as IL-6, TNF-α, and HMGB-1. Based on the results of quantitative Real-Time PCR, the most relevant increase was observed in the expression of both IL-6 and TNF-α, which were increased fifteen and five-fold, respectively, in periodontitis compared with controls, whereas HMGB-1 mRNA transcript levels were three-fold higher than control levels (Fig. 2a). Moreover, significant increases

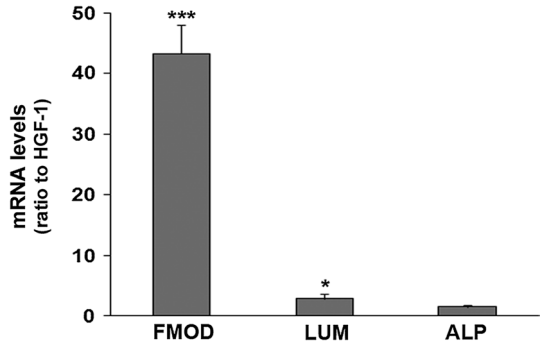

Fig. 1 Analysis of expression levels of FMOD, LUM, and ALP in HPDL cells. Results obtained by Real-time PCR are expressed as ratio to HGF-1 cell line. *Error bars* represent standard deviation. $*p < 0.05$ and $***p < 0.001$ significant differences in comparison with HGF-1

were observed in both TG2 mRNA levels (seven-fold higher in comparison with healthy subjects) and protein content in HPDL cells from periodontitis patients (Fig. 2a, b). These results suggest that in this pathological condition, TG2 levels may be modulated in response to inflammatory stimulus. In addition, we also showed that under our experimental conditions, TG enzyme activity increased by 36 % in HPDL cells from periodontitis patients compared with controls (Fig. 2c).

In HPDL cells from CP patients, we also observed a marked increase in RANKL mRNA transcript levels, which were 2.5-fold higher compared with healthy subjects. The addition of TG activity inhibitor R283 to HPDL cells from CP patients significantly reduced RANKL expression levels. On the other hand, no significant changes were observed for OPG gene expression (Fig. 3a). These findings

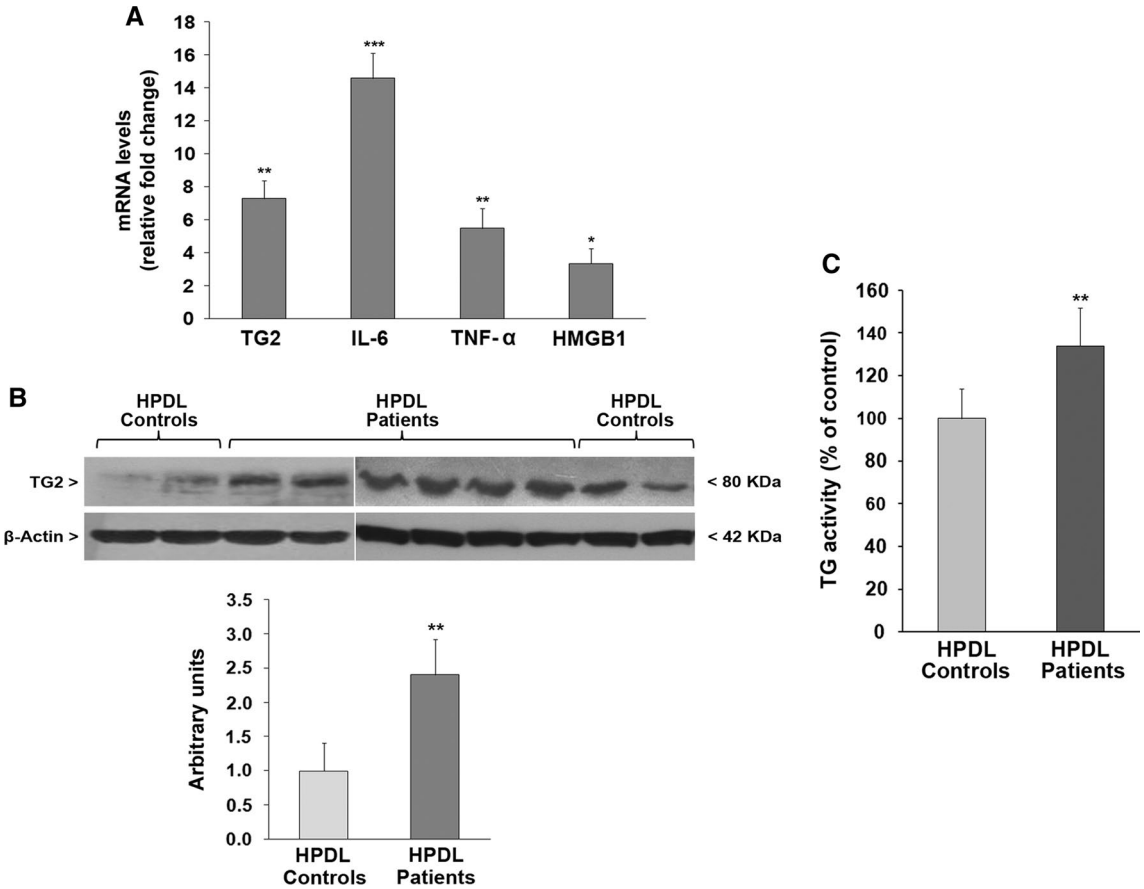

Fig. 2 Analysis of expression levels of inflammatory markers and TG2, and measurement of TG activity in HPDL cells from healthy ($n = 14$) and periodontitis subjects ($n = 14$). **a** Results obtained by Real-time PCR are expressed as relative fold change compared with control subjects. *Error bars* represent standard deviation. **b** Western blot analysis of TG2 protein amounts. This picture is representative of PDL cells obtained from different patients and controls. A representative densitometric analysis of all samples is also reported (*bottom*). **c** TG activity was determined by measuring the incorporation of 5-(biotinamido)pentylamine (BAPA) into proteins as described in "Materials and methods". The results are expressed as mean ± S.D. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significant differences in comparison with controls

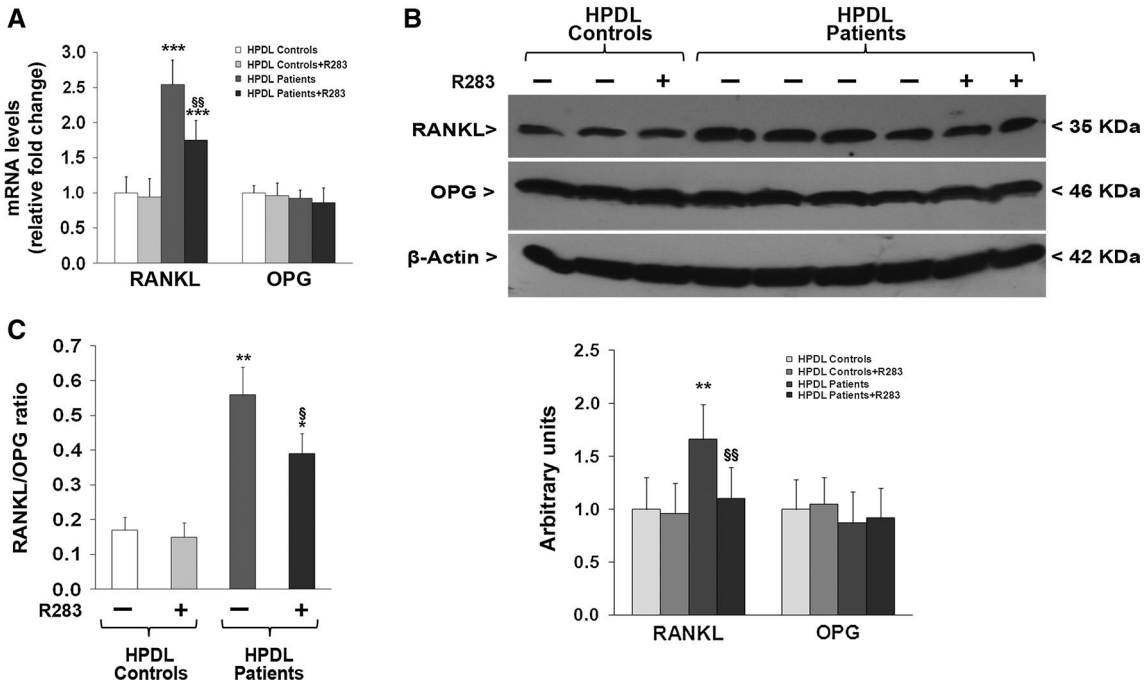

Fig. 3 RANKL and OPG mRNA and protein levels in HPDL cells from periodontitis patients ($n = 14$) in comparison with control subjects ($n = 14$), incubated in presence or absence of R283. **a** Data obtained by Real-time PCR analysis are expressed as relative fold change compared to control subjects. *Error bars* represent standard deviation. **b** Western blot analysis of RANKL and OPG protein amounts. The picture is representative of HPDL cells obtained from different patients and controls. A representative densitometric analysis of all samples is also reported (*bottom*). The results are expressed as mean ± SD. **c** RANKL/OPG ratio in HPDL cells from patients and controls, incubated in presence or absence of R283. The data are presented as the mean ± SD for the ratios of RANKL/β-actin to OPG/β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ significant differences in comparison to HPDL controls, $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ significant differences in comparison to HPDL patients

were confirmed by evaluating protein expression by Western blot analysis (Fig. 3b). In HPDL cells from patients, RANKL protein significantly increased in comparison with healthy subjects, whereas in all samples OPG was expressed in a constitutive manner. Since the RANKL/OPG balance may be a useful biomarker of bone remodeling, RANKL/OPG ratio was calculated from mRNA transcript levels assessed in all patients by quantitative Real-Time PCR taking into account that semi-quantitative analysis of proteins by Western blotting. The mean ratio RANKL/OPG in HPDL cells from CP patients was three-fold higher than that in healthy subjects; notably, in HPDL cells from CP patients incubated with the TG activity inhibitor R283, the ratio RANKL/OPG was significantly reduced (Fig. 3c).

In order to emphasize the involvement of TG2 expression in the inflammatory response induced by periodontal disease, we evaluated a possible association of RANKL/OPG ratio with TG2-mRNA transcripts. A non-parametric analysis of correlation between RANKL/OPG and TG2 mRNA levels showed that TG2 and RANKL/OPG in HPDL cells from periodontal disease patients were significantly and positively correlated ($r = 0.711$, $p = 0.004$) (Fig. 4).

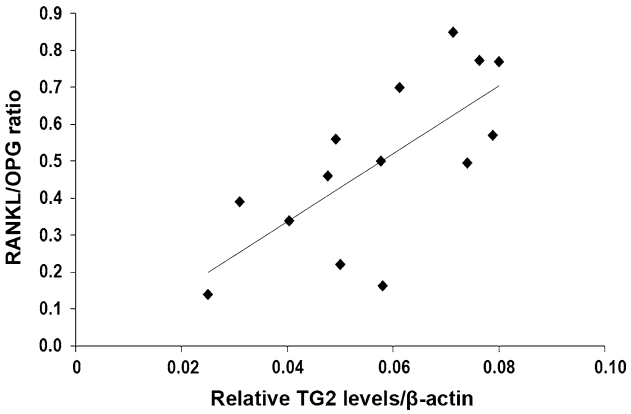

Fig. 4 Relationship between the RANKL/OPG ratio and TG2 mRNA levels in HPDL cells from periodontitis patients. TG2 mRNA transcript amount showed a positive correlation with the RANKL/OPG ratio ($r = 0.711$; $p = 0.004$)

Given that leukocytes, including monocyte/macrophages, play an important role in the RANKL/OPG pathway, in a subset of experiments using human THP-1 cells differentiated toward macrophages by TPA treatment,

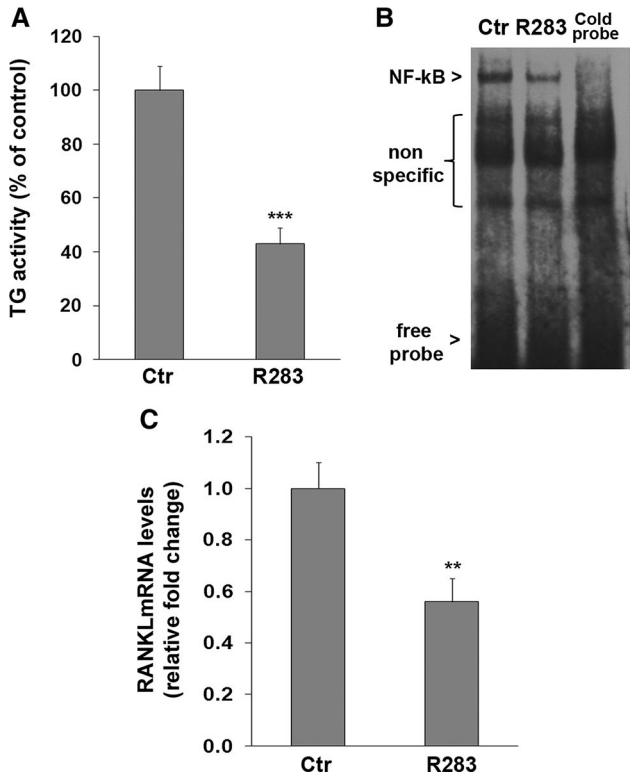

Fig. 5 Effect of TG2 inhibition on NF-κB activation and RANKL mRNA levels in human THP-1 cells differentiated to macrophages. After differentiation, THP-1 cells were incubated for 24 h in presence or absence of 250 μM R283, a specific inhibitor of intracellular TG2 activity. **a** TG activity was determined by measuring the incorporation of BAPA into proteins. **b** DNA binding activity of NF-κB in nuclear extract was assessed by EMSA analysis. **c** RANKL mRNA levels were evaluated by quantitative real-time PCR, after normalization against β-actin as endogenous control. $^{**}p < 0.01$ and $^{***}p < 0.001$ significant differences in comparison to controls

we also investigated the relationship between TG2 and RANKL/OPG. We evaluated RANKL expression after addition of R283, a specific inhibitor of intracellular TG activity. Under these conditions, THP-1 macrophages treated with R283 showed a significant reduction of TG activity (Fig. 5a), which was concomitant with a significant reduction in NF-κB activation (Fig. 5b), and a significant decrease in RANKL mRNA transcript level (Fig. 5c).

Discussion

In this study, we demonstrated the concomitant increase of TG2 mRNA transcript and cytokine levels in HPDL cells cultures obtained from periodontitis patients. The HPDL cells are responsive to biochemical and molecular stimuli that regulate their phenotype according to functional needs of tissue, so that tissue width is constantly maintained at steady dimensions (El-Awady et al. 2010).

Some results demonstrated the presence of IL-6 in human gingival tissues and cells involving this lymphokine as a participant in the molecular events associated with inflammatory periodontal diseases (Belibasakis et al. 2005). Moreover, in periodontitis lesions, macrophages and T lymphocytes produce inflammatory mediators, including IL-1, IL-6, TNF-α, and prostaglandin E2, which can induce bone resorption indirectly by stimulating osteoblasts to produce RANKL (Taubman and Kawai 2001).

Accumulating evidence showed that TG2 expression may play a relevant role in inflammatory states. In particular, several results emphasized the involvement of TG2 in the initial phase of wound healing and inflammation (Mehta et al. 2010). Indeed, cytokines and growth factors secreted during early phases of cell injury regulate TG2 expression (Mehta et al. 2010). In this context, we here show evidence for TG2 up-regulation concomitant with the increase in cytokine mRNA transcript levels. These results agree with our previous results demonstrating that exposure to orthodontic materials promoted both IL-6 release and TG2 increases in human gingival fibroblasts (Matarese et al. 2006).

In normal and transformed cells, TG2 is able to promote the cell–ECM interaction, and fibroblasts possessing high TG2 levels show increased cell attachment and spreading, whereas TG2-deficient fibroblasts exhibit decreased adhesion and spreading (Gross et al. 2003).

As the periodontal ligament plays important roles in alveolar bone formation and resorption in orthodontic or periodontal treatment, it is an important issue whether periodontal ligament fibroblasts, as well as osteoblasts, can support osteoclastogenesis. PDL fibroblasts produce OPG, preventing the pre-osteoclast differentiation and functions, and OPG inactivation may play a key role in osteoclast formation by PDL fibroblasts. Moreover, the expression and secretion of OPG and RANKL by HPDL cells suggest that they play a role in alveolar bone metabolism (Parlar et al. 2005). Clear evidence also suggests that OPG-RANKL system is involved in the pathogenesis of bone and mineral metabolism diseases as well as in periodontitis (Belibasakis and Bostanci 2012). Our data demonstrated the RANKL/OPG balance is disturbed in periodontal disease in favor of RANKL.

The activation of NF-κB pathway plays a pivotal role in the modulation of cell response (Oeckinghaus and Ghosh 2009). Nevertheless, underlying mechanism modulating NF-κB activation should be further clarified. Previous results demonstrated the occurrence of a TG2/NF-κB interplay (Ientile et al. 2007; Kuncio et al. 1998). On the other hand, TG2 is able to promote the activation of NF-κB by cross-linking of I-kBα (Kim 2006). In human THP-1 cells differentiated to macrophages, the reduction of TG enzyme activity was concomitant with the loss in NF-κB activation

and also caused a reduction in RANKL expression. These results confirm the occurrence of an TG2/NF-κB interplay and suggest that this latter plays a role in activated cells involved in the inflammatory response by increasing RANKL expression in PDL from periodontitis patients.

Our results agree with the observations of Liu et al. (2003) showing that RANKL mRNA levels assessed by semi-quantitative RT-PCR were highest in advanced periodontitis compared with the moderate periodontitis or healthy subjects. It has been suggested that increased RANKL/OPG ratio occurring in periodontitis may be useful marker of the molecular mechanisms of bone resorption. Furthermore, relevant studies have investigated the effect of periodontal treatment on the RANKL-OPG system. Indeed, increased availability of OPG reduced RANKL/OPG ratio and represents an important therapeutic strategy for the prevention of progressive alveolar bone loss (Jin et al. 2007).

In particular, we show clear evidence for a positive correlation between TG2 and RANKL/OPG mRNA ratio, suggesting that increase in TG2 expression may be considered an early event in tissue changes induced by periodontal disease. Moreover, results from experimental inhibition of TG activity demonstrate the involvement of TG enzyme activity in RANKL expression increase, likely mediated by NF-κB activation.

In conclusion, our results demonstrate that increased TG2 expression in HPDL cells from periodontitis patients could be associated with high levels of pro-inflammatory markers promoting the interaction between molecular mechanisms involved in bone remodeling and resorption.

Conflict of interest This work has been performed with Departmental funding only. The authors declare that they have no conflict of interest to declare.

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