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Increased transglutaminase activity was associated with IL-6 release in cultured human gingival fibroblasts exposed to dental cast alloys

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Summary. Molecular mechanisms underlying gingival and periodontal inflammation caused by dental alloys are still poorly understood. Recently, it has been demonstrated that tissue transglutaminase can be involved in inflammatory cell response. The aim of this study was to evaluate effects of exposure to orthodontic materials on transglutaminase in cultured human gingival fibroblasts. The incubation with Ni–Ti heat-activated (T3) or Ni–Ti super-elastic (T4), and with Ni–Cr–Co (T2) alloys produced respectively 2.5-fold and 8-fold increases in IL-6 release compared with control cultures. Transglutaminase activity was significantly increased in cells exposed to T3 and T4 alloys (about 170% of control; $p < 0.05$), where it was mainly localized close to inner part of cell membrane. The exposure to T3 and T4 specimens significantly up-regulated also tTG expression compared with control cultures. These data first show an association between IL-6 release and tissue transglutaminase increases, suggesting that TGase-mediated reactions may play a major role in periodontal inflammation.

Keywords: Inflammation – Tissue transglutaminase – Interleukins – Fibroblasts – Dental alloys

Introduction

Cast alloys used in dentistry are commonly source of metal ions released into the gingival and oral mucosa, which in turn often cause gingival and periodontal inflammation. In particular, nickel has been involved in pathological periodontal responses to nickel-containing crowns since nickel hypersensitivity is quite diffused in the general population. Different *in vitro* and *in vivo* models, such as cytotoxicity tests in cell cultures or mucosa contact test, have been exploited to assess the inflammatory potential of orthodontic materials. However, few information is still available on molecular mechanisms involved in inflammation triggered by dental cast alloys (Schmalz et al., 1998).

Cytokines, such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), have been demonstrated to play a central role in the inflammatory process observed in gingival and oral mucosa, often acting in a synergistic manner one with each other and with tumor necrosis factor-α (TNF-α), a powerful inducer of cytokine production (Feliciani et al., 1996). These inflammatory molecules are usually released during immune response by monocyte/macrophages and T lymphocytes, but endothelial cells and fibroblasts are also able to produce them. In particular, fibroblasts have recently been suggested to be important sentinel cells to the immune system, actively defining the structure of tissue microenvironments and regulating inflammatory response by the production of IL-1β and IL-6 (Dongari-Bagtzoglou et al., 1996; Ozen et al., 2005). The effects of cytokine release by fibroblasts involve induction of fibroblast proliferation, expression of adhesion molecules, bone resorption, and production of collagenase and prostaglandin E2 (Baker, 2000).

In recent years, it has been reported that tissue transglutaminase (tTG) induction may be part of biochemical mechanisms involved in the inflammatory response (Kuncio et al., 1998; Kim, 2005). tTG is an ubiquitous protein belonging to a family of Ca^{2+}-dependent enzymes, which catalyse the post-translational modification of proteins through the formation of N-ε(γ-glutamyl)-lysine isopeptide bonds between glutamine and lysine residues, or the incorporation of polyamines (Folk and Finlayson, 1977). Transamidating tTG activity is usually a marker of cell differentiation, but is also involved in cell

signalling and adhesion, playing an important role in the maturation and stabilization of extracellular matrix (ECM) (Fesus and Piacentini, 2002). Cross-linked products of several ECM proteins, such as fibronectin, collagen type I and osteonectin are easily detectable in dental pulpal and gingival fibroblasts (Martinez and Araujo, 2004). Further, it has recently been described in rat teeth the presence of high-molecular-weight form, deriving from tTG cross-linking activity, of the SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) proteins: dentin matrix protein 1 (DMP1), dentin phosphoprotein (DPP), and bone sialoprotein (BSP) (Kaartinen et al., 2005).

Indeed, the relationship between transglutaminase (TGase) activity and inflammatory process has not been investigated in periodontal tissue. In this study, we used as in vitro model of periodontal inflammation cultured human gingival fibroblasts exposed to titanium-containing orthodontic alloys, in order to first evaluate the involvement of TGase in cell response to inflammatory stimuli.

Materials and methods

Materials

The human gingival fibroblast cell line, HGF-1 (CRL 2014), was obtained from American Type Culture Collections (ATCC) (Rockville, Maryland, USA). Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS) and antibiotics were from Life Technologies (Milan, Italy). 3-[4,5-dimethyltiazol-2-il]2,5-diphenyl-tetrazolium bromide (MTT) and other chemicals of analytical grade were purchased from Sigma (Milan, Italy). [^{3}H]-putrescine dihydrochloride ([^{3}H]-PTC) (1 mCi/ml), horseradish peroxidase (HRP)-conjugated anti-mouse IgG, fluorescein-isothiocyanate (FITC)-conjugated streptavidin, and ECL kit for chemiluminescence were from Amersham Pharmacia Biotech (Milan, Italy). 5-(biotinamido)-pentylamine (BAPA) was from Pierce (Milan, Italy). Cyclin D1 mouse monoclonal antibody was from SantaCruz Biotech (DBA, Milan, Italy).

In this study, the biocompatibility of five commercial orthodontic samples of metallic and non-metallic composition was examined. The samples included archwires, brackets and molar bands. Four of the materials were metallic, made of nickel–titanium (Ni–Ti) or nickel–chromium–cobaltum (Ni–Cr–Co) cast alloys, while the non-metallic one was of ceramics. A list of the samples with details is given in Table 1.

Cell culture

Human gingival fibroblasts, HGF-1, were seeded on tissue culture plates at a density of 0.5×10^5 cells/cm^2, and grown in DMEM, supplemented with 12% (vol/vol) FBS, sodium pyruvate 1 mM, streptomycin (100 µg/ml) and penicillin (100 U/ml), at 37 °C in a 5% CO_2/95% air humidified atmosphere. Culture medium was renewed every 3 days, and fibroblasts were examined every 7 days up to 4 weeks. Contamination was excluded and viability assessed by visual control with light microscope.

In parallel with cell culturing, orthodontic samples were prepared for cytotoxicity tests. First, they were sterilized by autoclave. Thereafter, metal ions were extracted from the sample surface by incubating them for 14 days at 37 °C, in closed waterproof test tubes containing the same culture medium used for HGF-1 cells. The ratio between the sample weight and the volume of the extraction solution was 0.1 mg/ml because the surface area of the samples was not easily measurable.

When cell cultures reached a multi-layer conformation stage, the pure culture medium was replaced by the culture medium containing the release products of the five orthodontic materials (namely sample Ce, sample T1, sample T2, sample T3, and sample T4), and a 24–48 h incubation at 37 °C was carried out before performing cytotoxicity tests.

Cytotoxicity test

The biocompatibility of the five orthodontic samples after 24–48 h of incubation with the release medium from cast alloys was tested by MTT reduction assay (Wataha et al., 1991), evaluating the residual mitochondrial succinate dehydrogenase activity. Blue formazan crystals were dissolved with acidic isopropanol, and the rate of MTT reduction was determined by spectrophotometrical reading at 570 nm. Survival rates of the negative control tissues were set to represent 100% viability. Results were expressed as a percentage of the untreated control.

ELISA test

IL-6 release from treated and untreated cultures was measured using an ELISA test system according to Schmalz et al. (1998). Briefly, 100 µl aliquots were taken from exposed media and the amount of cytokine release was quantified against a standard curve of purified human IL-6 (0.01–1000 ng/ml). IL-6 secretion of the control cultures was set to 100%. Results of the tested materials were expressed as a percentage of the untreated cultures.

Transglutaminase activity

Cell ability to retain proliferating and differentiating features, after 48 h exposure to release products from orthodontic samples, was assessed by measuring TGase activity, a well known marker of differentiation. *In situ* enzyme activity was investigated according to Katoh et al. (1996) by adding 10 µM [^{3}H]-PTC to cell cultures one hour prior to termination of incubation with cast alloys. The radioactivity of [^{3}H]-PTC, incorporated

Table 1. List of materials tested

Sample name	Commercial name	Trademark	Composition	Type of material
Ce	Clarity™ Metal-Reinforced 3M-Unitek Ceramic Brackets	3M-Unitek	Ceramics	Brackets
T1	Nitinol	3M-Unitek	Ni–Ti	Archwires
T2	Micro Brackets	Advanced Orthodontics	Ni–Cr–Co	Brackets
T3	Nitinol (*h.a.*)*	3M-Unitek	Ni–Ti	Brackets, bands
T4	NeoSentalloy (*s.e.*)*	G.A.C. International	Ni–Ti	Archwires

[a] Dentaurum (Ispringen, Germany); 3M-Unitek (Saint-Paul, USA); G.A.C. (New York, USA). * *h.a.* heat-activated; *s.e.* super-elastic

by TGase into cell proteins, was measured using a liquid scintillation counter and expressed as relative percentage of control.

Confocal laser scanning microscopy analysis

Subcellular distribution of TGase activity was analysed by confocal laser scanning microscopy (CLSM) analysis, after addition of 2 mM BAPA to cells, cultured on slides, one hour prior to the end of incubation, according to Zhang et al. (1998). Then, cells were rinsed two times with phosphate buffered saline (PBS), fixed with sterile paraformaldehyde (4%), and incubated for one h with FITC-conjugated streptavidin (1:100).

TGase substrates labeled by BAPA, and marked by FITC-conjugated streptavidin, were examined, in control and treated cells, on a Leica DM IRB confocal laser scanning microscope (Laborlux K, Leica Microsystems GmbH Heidelberg, Mannheim, Germany) by green (FITC) fluorophore excitation at 488 nm, utilizing a He/Neon laser source. Image outputs electronically generated by the equal parameters setting, were measured and compared for intensity (treated vs control), after non-specific background subtraction.

Western blotting

After incubation, cells were rinsed two times with PBS, scraped and pelleted by centrifugation at $800 \times g$. Cell lysis was carried out by repeated cycles of *freezing-thawing*, followed by 2′ sonication, then, protein concentration in cell lysates was determined by Bradford method. Approximately 30 µg of proteins were loaded on a 10% polyacrylamide gel, and subjected to SDS-PAGE for 2 h; then, proteins were transferred to nitrocellulose membrane. tTG expression was analysed by Western blotting with specific mouse monoclonal antibody CUB 7402 (1:1000), followed by incubation with HRP-conjugated secondary antibody (1:2500). Bands were visualized by chemiluminescence using the kit ECL. Immunoblots were scanned and quantified by densitometric analysis with a AlphaImager 1200 System (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Analysis of variance was used to analyze differences among cultures exposed to different treatments. The Duncan multiple-range test was used to show differences between groups. Each material used was tested versus the untreated control. Data were expressed as means ± SEM.

Results

A not significant loss of cell viability was observed in fibroblast cultures exposed for 24–48 h to the medium containing the release products of ceramics (Ce) and Ni–Ti (T1, T3, T4) orthodontic alloys compared to control cultures, as monitored by MTT reduction assay (Fig. 1). The Ni–Cr–Co (T2) alloy was the most toxic material tested. Exposure to release products of this alloy caused a time-dependent decrease in cell viability of 25% compared to untreated cultures in repeated experiments (Fig. 1).

In parallel to cell survival assessment, changes in IL-6 secretion from fibroblasts cultured in presence or absence of cast alloys were evaluated by ELISA. Small amounts of IL-6 were produced by untreated fibroblasts after a 48 h period (Table 2). Likewise, exposure to specimens Ce and

Table 2. Release of IL-6 in the culture medium of fibroblasts exposed to orthodontic materials

Tested materials	IL-6 release (ng/ml)	
	24 h	48 h
Control	0.7 ± 0.068	1.260 ± 0.143
Ce (Ceramics)	0.732 ± 0.065	1.430 ± 0.170
T1 (Ni–Ti)	0.825 ± 0.093	1.570 ± 0.140
T2 (Ni–Cr–Co)	4.96 ± 0.257	10.08 ± 1.04**
T3 (Ni–Ti heat-activated)	1.12 ± 0.13	3.024 ± 0.32*
T4 (Ni–Ti super-elastic)	1.43 ± 0.095	3.15 ± 0.43*

Cells were cultured for 24–48 h in the presence of release products of tested specimens made of ceramic (*Ce*), Ni–Ti alloy (*T1*), Ni–Cr–Co alloy (*T2*), heat activated Ni–Ti (*T3*) and super-elastic Ni–Ti (*T4*). IL-6 levels were determined by ELISA and the results are expressed as alteration ratio in comparison with untreated control cultures. Data are given as mean values ± SEM from triplicates. $^{*}p < 0.05$; $^{**}p < 0.001$

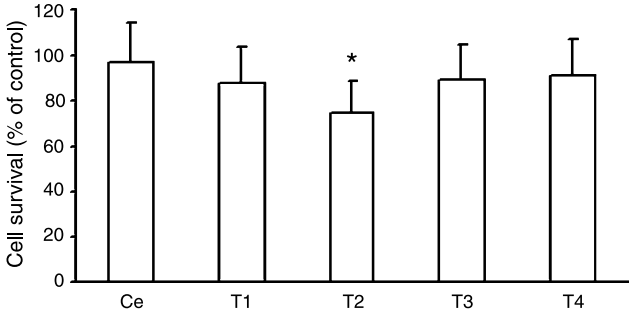

Fig. 1. Cell survival (MTT assay) of fibroblasts exposed to release products of cast alloys. Human gingival fibroblast (HGF-1) were exposed for 24 and 48 h to release medium of orthodontic materials, represented by specimens made of ceramic (Ce), Ni–Ti alloy (T1), Ni–Cr–Co alloy (T2), heat activated Ni–Ti (T3) and super-elastic Ni–Ti (T4). Values are means ± SEM from triplicates. Data are expressed as relative percentage of untreated control cultures. $^{*}p < 0.05$

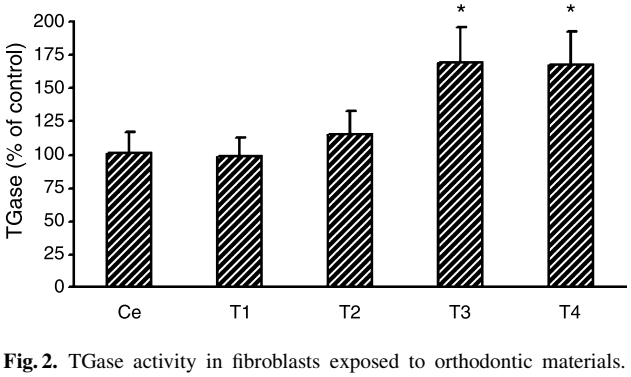

Fig. 2. TGase activity in fibroblasts exposed to orthodontic materials. Cells were cultured for 48 h in the presence of release products of tested specimens made of ceramic (Ce), Ni–Ti alloy (T1), Ni–Cr–Co alloy (T2), heat activated Ni–Ti (T3) and super-elastic Ni–Ti (T4). TGase activity was measured through the evaluation of radiolabeled putrescine incorporated into cell proteins. Values are means ± SEM from triplicates. Data are expressed as relative percentage of untreated control cultures. $^{*}p < 0.05$

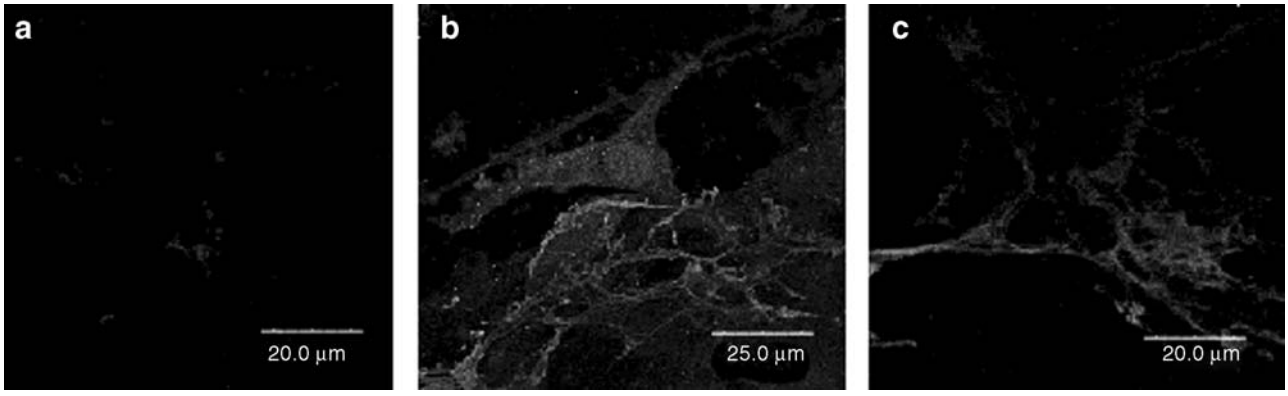

Fig. 3. Sub-cellular distribution of TGase activity in fibroblasts exposed to orthodontic materials in comparison with control cultures. After 48 h of incubation, BAPA was added for one h to cell cultures untreated (**a**) or exposed for 48 h to release products of T3 (**b**) and T4 (**c**) specimens. Then, fibroblasts were fixed and counter-stained with FITC-streptavidin. Analysis of TGase-mediated BAPA incorporation into cell proteins was carried out by confocal laser scanning microscopy, which showed a localization of TGase substrates close to cell membrane, with a more intense enzyme activity in fibroblasts exposed to cast alloys compared with control cells

T1 did not significantly change IL-6 levels (Table 2). In contrast, IL-6 secretion was 2.5-fold increased by 48 h exposure to release medium of T3 (h.a. Ni–Ti) and T4 (s.e. Ni–Ti) alloys, and 8-fold by exposure to T2 (Ni–Cr–Co) medium compared to control cultures (Table 2).

The measurement of transglutaminase activity through the evaluation of radiolabelled putrescine incorporated into cell proteins showed that after a 48 h exposure to orthodontic alloys enzyme activity was significantly increased (about 170% of control; $p < 0.05$) in T3- and T4-exposed fibroblasts, and at a lesser extent (115% of control) in T2-exposed cultures, while did not change in the presence of T1 and Ce release medium (Fig. 2). Confocal laser scanning microscopy analysis showed that increased enzyme activity in fibroblasts exposed to T3- and T4-release medium was localized in the cytosol (Fig. 3b, c), as showed by a more intense green fluorescence, due to BAPA incorporation into cell proteins, compared to control fibroblasts (Fig. 3a). Interestingly, the highest fluorescence was observed in the close proximity of cell membrane in treated cultures (Fig. 3b, c).

In order to determine whether tTG expression was affected by the exposure to release medium of cast alloys, changes in protein levels were evaluated by Western blot analysis. Figure 4 shows a strong tTG up-regulation in T3- and T4-exposed fibroblasts and, at a lesser extent, in T2-exposed cells compared to control cultures. Less significant were, in contrast, the differences in protein expression evidenced in cells exposed to Ce and T1 specimens.

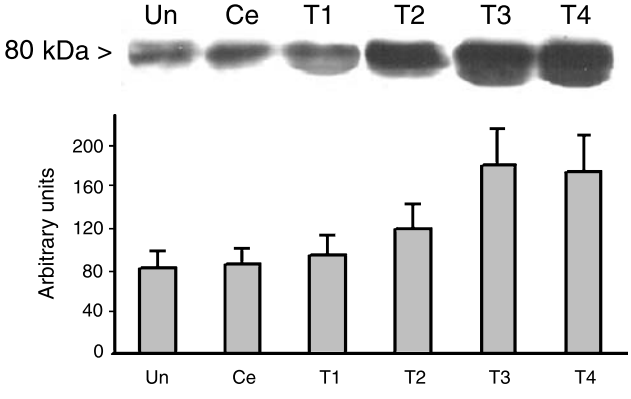

Fig. 4. Analysis of tTG expression in fibroblasts exposed to orthodontic materials. Cell cultures were exposed for 48 h to release medium of orthodontic materials, represented by specimens made of ceramic (*Ce*), Ni–Ti alloy (*T1*), Ni–Cr–Co alloy (*T2*), heat activated Ni–Ti (*T3*) and super-elastic Ni–Ti (*T4*). tTG expression was evaluated by Western blot analysis with specific monoclonal antibody. Data are from triplicate experiments

Discussion

Interleukins play a crucial role in gingival and periodontal inflammation. Biopsies of inflamed gingival tissues contain significantly higher IL-1 and IL-6 activities than those from healthy ones (Kamagata et al., 1989). IL-6 protein is mainly localized in fibroblasts, endothelial cells, and macrophages of inflamed gingival tissues, but not in healthy ones (Takahashi et al., 1994). IL-6 functions vary depending on cell type: it up-regulates the expression of adhesion molecules in endothelial cells, and modulates proliferation in a dose-dependent manner. Our results are in agreement with previous observations demonstrating that the exposure to cast alloys caused increases in interleukin release (Kamagata et al., 1989; Schmalz et al., 1998; Ozen et al., 2005). However, low concentrations have a stimulating effect, whereas high concentrations are inhibiting (Gonzáles et al., 2001). It is well known

that a consensus motif for IL-6 is present in the promoter sequence of tTG, and an inducing effect on tTG expression has been reported for IL-1β and IL-6, and TNF-α (Kuncio et al., 1998). Indeed, in our study we observed a good correlation between increased TGase activity and tTG expression and increased IL-6 release in fibroblast cultures exposed to release medium of T3 and T4 specimens (both alloys contain Ni–Ti modified by further treatments). Surprisingly, despite the highest IL-6 secretion measured in cell cultures exposed to T2 (Co–Cr–Ni) specimen, TGase activity was lower than in T3- and T4-exposed fibroblasts. This could be most likely explained by the higher cytotoxic effect of T2 compared with T3 and T4, and by a possible anti-proliferating effect of high levels of IL-6. In contrast, low levels of the cytokine usually stimulate cell proliferation in fibroblasts, characterized by cytoskeleton and ECM remodelling events. The involvement of tTG in these events is well characterized, and it is known that tTG externalized from cells becomes tightly bound to fibronectin and forms ternary complexes with collagens that function as a cementing substance in the ECM. Indeed, in this study the analysis of the distribution of TGase activity evidenced that BAPA-labelled proteins were mainly localized close to cell membrane in T3- and T4-exposed fibroblasts, suggesting that proliferation events were active.

Interleukins have been determined to enhance various immune response in vitro (Winkler, 2003; Takashiba et al., 2003). There is no direct evidence that an increased cytokine release after exposure to dental alloy specifically indicates inflammatory reaction in vivo. However, first we demonstrated that the secretion of IL-6 was associated with a continuous increasing pattern throughout 48 h of tTG expression. Considering the distribution of TGase-catalyzed reaction, it's possible to hypothesize that fibroblasts had successfully been stimulated to adhere to their substrates. Evidence for the association with interleukin release did not demonstrate that tTG expression might directly be caused by inflammatory response to orthodontic materials. However, one could speculate about some tTG-catalyzed reactions occurring in the in vivo response in fibroblasts exposed to cast alloys.

In summary, we have demonstrated for the first time that transglutaminase activity is significantly elevated in fibroblasts exposed to Ni–Ti alloys. These effects were associated with IL-6 release and were dependent on the presence of different elements in the cast alloy. Our results suggest that further studies are necessary to establish the role of transglutaminase reaction in the inflammatory response. However, the evaluation of protein substrates of tTG activity may be a powerful biochemical marker of post-translational induced changes associated with inflammatory response.

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