Original Article

CD40-CD40L Expression During Orthodontic Tooth Movement in Rats

Najat Alhashimi, BDS, MDS, Dr Odont[a]; Lars Frithiof, PhD[b]; Pongsri Brudvik, PhD[c]; Moiz Bakhiet, PhD[a]

Abstract: The aim of this study was to investigate the expression of the costimulatory molecules CD40 and CD40L in periodontal and bone cells in orthodontically treated and untreated teeth using immunohistochemistry. The upper first molars were moved mesially by a fixed appliance. In the experimental group, $CD40^+$ cells were detected at both the tension and the resorption sides in fibroblast-, macrophage-, and dendritic-like cells. The staining was more pronounced on the resorption side. The strongest expression was observed on day 3, decreased on day 7, and reached a low level on day 10 after application of orthodontic force. In contrast, in the treated animals CD40 ligand was expressed on day 3, the expression was enhanced on day 7, and was more pronounced on day 10. CD40L-expressing cells were found predominantly around hyalinized tissue in the resorption zone and the tension areas of the distal root. CD40L was expressed in the bone marrow cells in the pressure zone. In the tension side, some cells of the cellular cementum expressed CD40L. The expression of CD40 and CD40L was low in untreated teeth. These results suggest that CD40-CD40L interaction appears to be an active process during orthodontic tooth movement and that orthodontic force induces T-cell activation. Such activation may be involved in the induction of inflammatory mediators and subsequent bone remodeling. In addition, this may lead to the generation of anti-inflammatory mediators that support defense mechanisms against root resorption, which depend on the type of immune response that is induced regarding CD40-CD40L expression. (*Angle Orthod* 2004;74:100–105.)

INTRODUCTION

Periodontal and alveolar bone remodeling that take place during orthodontic tooth movement is a cell-mediated process. There will be a chain of vascular and cellular changes, as well as interaction between nervous, immune, and endocrine cells, that mediate the process. The transmission of mechanical stimuli into specific cellular activity is still not fully understood. The early phase of these responses is characterized by periodontal vasodilatation and migration of leukocytes out of periodontal ligament (PDL) capillaries.[1] The teeth move as a result of the biological response of the periodontal tissues to applied mechanical forces.

Thereafter, alterations in blood flow associated with pressure applied to the PDL, formation and release of biological messengers, and activation of cells occur.[2] Bone remodeling is controlled by a balance between formation and resorption processes that are regulated by a wide variety of systemic humoral factors such as parathyroid hormone (PTH[3]) and calcitonin, neurotransmitters such as substance P,[4] vasoactive intestinal peptide,[5] and calcitonin gene–related peptide[6] cytokines or monokines such as IL-1α,[1] IL-1β,[7] and chemokines.[8] Release of these messengers results in the activation of cells participating in resorption of the hyaline zone, alveolar bone, and root surface leading to remodeling of the compressed periodontium. Within the immune system, complex regulatory networks exist, which include both communication through soluble mediators like cytokines and direct cellular contacts through specific surface receptors. An efficient immune response is critically dependent on the interaction of such receptor-ligand pairs, for example, CD40-CD40L.

CD40 is a cell surface receptor, which belongs to the tumor necrosis receptor family (TNF-R). Initially, it was seen as a B-cell–specific receptor,[9–11] but it has now been found to be widely distributed and expressed on other cells, including monocytes, dendrite cells, and IL-6 or IL-8 secretion by ligation of endothelial cells, basophiles, epithe-

[a] Division of Infectious Diseases, Department of Medicine, Karolinska Institutet, Huddinge University Hospital, SE-141 86 Stockholm, Sweden.

[b] Department of Periodontology, Dental Faculty, Karolinska Institutet, Huddinge, Sweden.

[c] Department of Orthodontics and Facial Orthopedics, Dental Faculty, University of Bergen, Bergen, Norway.

Corresponding author: Najat Alhashimi, BDS, MDS, Dr Odont, Consultant Orthodontist, Department of Orthodontics, Hamad Medical Corporation, PO Box 3050, Doha, Qatar
(e-mail: najah-hash@qatar.net.qa).

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lial cells, and fibroblasts.[12–14] CD40 is a type I transmembrane protein with a molecular weight of 48 kDa, which is involved in multiple biological responses. It has been shown that human fibroblasts from a variety of tissues express CD40.[15] Cellular responses mediated by CD40 are triggered by its counterreceptor, the CD40L, which is defined as a type II membrane protein of 33 kDa, which is also a member of the TNF gene family.[9]

CD40-CD40L interactions are critical for the activation of $CD4^+$ T-cell–dependent effectors functions.[16] Moreover, humans with a defective CD40L develop hyper-IgM syndrome, which is a rare immunodeficiency disease characterized by low or absent IgG, IgA, and IgE to normal or elevated levels of IgM with increased susceptibility to opportunistic infections, mainly *Cryptococcus*, *Pneumocystis*, and histoplasma.[17] CD40L plays a central role in the perpetuation of rheumatoid synovitis because rheumatoid arthritis peripheral blood and synovial fluid T cells stimulated CD40L-dependent B-cell immunoglobulin production and dendritic cell IL-12 expression in the absence of prolonged in vitro T-cell activation.[18] The frequency of IL-6– and IL-8–secreting cells mirrors the frequency of cells expressing high levels of CD40 in cultures of gingival fibroblasts; this indicates that there is a direct functional relationship between CD40 expression molecule and gingival fibroblasts. In particular, $CD40^+$ fibroblast subsets upregulate secretion of these cytokines in vitro.[19] Signaling through CD40 regulates inflammatory cytokine secretion in many cell types. Because of this (the critical role of CD40-CD40L interaction in vivo and in vitro), little information is known about the role of this interaction during orthodontic tooth treatment. The objective of this investigation was to study the expression of CD40 and CD40 ligand during the process of orthodontic tooth movement.

MATERIALS AND METHODS

Animals

Fifteen Wistar male rats, weighing about 170–180 g and 40–45 days old, were used in this study as described.[20] The animals were fed a standard pellet diet with tap water ad libitum (801157 W Expanded Pellets, Stepfield Witham, Essex CM8 3AB, UK). The maxillary right first molar was moved mesially by a closed-coil spring (Elgiloy spring, F-0.008 × 0.032, Rocky Mountain Dental Products CO, USA) ligated to the mesial aspect of the first molar and through the eyelet on an incisor band. A strain of 400–500 mN was applied (Figure 1). There was no reactivation during the experimental period.

The animals were divided into three groups. Group-I (n = 4) appliances were left on for three days; group-II (n = 4) appliances were left on for seven days; group-III (n = 4) appliances were left on for 10 days. Group-IV (n = 3) served as a control. In the control group, the rats had no appliances. One rat was killed on day 3, one on day 7, and another on day 10. The weight was recorded on the day of the operation (day 0) and again before the animals were sacrificed. All operations were performed under general anesthesia (subcutaneous injection of Dormicum-Hypnorm solution, dosage 0.15–0.2 mL/100 g body weight).

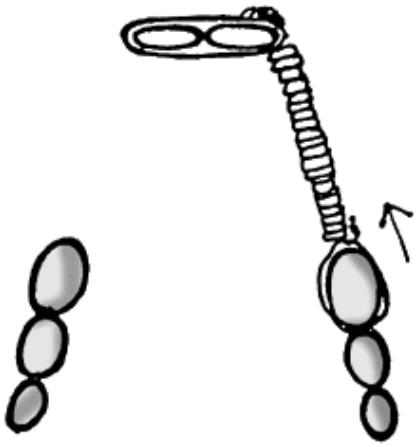

FIGURE 1. Rat appliance, a closed-coil spring ligated between an eyelet on the incisor band and the upper first molar. The arrow indicates direction of tooth movement.

On the day of the sacrifice, the rats were given an overdose of anesthesia (4% paraformaldehyde in 0.2% picric acid solution) and perfused through the left heart ventricle. After perfusion, the right and the left halves of the maxillae, including the first, second, and third molars, were dissected out and placed in a mixture of 4% paraformaldehyde and 0.2% picric acid for 24 hours at 4°C and rinsed in 0.1 M phosphate buffer pH 7.4. The maxillae were then decalcified in 10% ethylenediamine-tetraacetic acid (EDTA) for 2–4 weeks (the EDTA was changed every second day). The progress of demineralization was evaluated radiographically. The specimens were then rinsed in phosphate buffer with 30% sucrose overnight and placed in Tissue Tek (O.C.T compound, embedding medium, SAKURA, Zoeterwoude, The Netherlands), after which they were frozen in dry ice and kept at −70°C until use. The specimens were oriented in the embedding medium so that the mesiodistal sections parallel to the long axis of the teeth could be made. Serial freeze sections (5–6 μm thick) at −21°C were cut and collected on SuperFrust®/Plus slides (Menzel-Glaser, Braunschweig, Germany).

Immunohistochemistry

The staining technique was modified from the original protocol.[21] Frozen sections were rehydrated and nonspecific staining blocked by incubation with 1% H_2O_2 in 1× Earl's balanced salt solutions (BSS) (GIBCO) supplemented with 0.01% saponin (Riedel-de Haen, Seelze, Germany) for 30 minutes. To reduce risks for nonspecific antibody and hydrophobic interactions, the following precautions were taken: incubation with 5% normal horse serum for 30 minutes

TABLE 1. Mean ± SDs of CD40-Positive Fibroblast-, Macrophage-, and Dendritic-like Cells in the Periodontal Ligament of the First Maxillary Molars in the Control and Experimental Groups at 3, 7, and 10 Days

Experimental Period (days)		CD40-Positive Fibroblast-like Cells	Macrophage-like CD40-Positive Cells	CD40-Positive Dendritic-like Cells
Day 3	Experimental	170 ± 20	240 ± 30	115 ± 15
	Control	75 ± 10	100 ± 14	70 ± 10
Day 7	Experimental	165 ± 10	235 ± 12	113 ± 9
	Control	80 ± 10	110 ± 14	73 ± 10
Day 10	Experimental	150 ± 12	220 ± 13	105 ± 11
	Control	77 ± 10	105 ± 14	69 ± 10

at 37°C. CD40 and CD40L were observed using a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) directed to rat CD40 and CD40L. Optimal staining was obtained by incubating the sections with the antibody, at a concentration of 1 μg/mL in 2% normal horse serum–BSS-saponin at room temperature overnight. After several washes in BSS-saponin, biotinylated donkey anti-goat antibodies (Jackson Immunoresearch Labs, West Grove, PA) at a dilution of 1:600 in BSS-saponin were applied. The slides were then incubated with avidin-biotin-peroxidase complex (ABC, Vectastain Elite Kit, Vector—Burlingame, CA). Staining was performed using 3,3-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2. After DAB reaction and rinsing in water, some slides were counterstained with Harris hematoxylin, dehydrated, and mounted with Aquamount (Lerner Lab, New Haven, CT). As control, primary antibodies were omitted or irrelevant primary antibody was used. Labeled cells were counted by image analysis (Quantimet QW 550 Leica, Cambridge, UK).

RESULTS

CD40

In the present study, immunohistochemical analysis revealed the distribution of CD40 molecules in the periodontal tissues in the control and treated teeth. Anti-CD40 Ab specifically stained fibroblast-, macrophage-, and dendritic-like cells. Staining was evenly distributed throughout the periodontal tissues in the control group during the entire experimental period. In the experimental group, CD40-positive cells were increased at both the tension and resorption sides. The staining was more pronounced on the resorption side. Fibroblast-, macrophage- and dendritic-like cells were the immunolabeled cells. The strongest expression was observed on day 3 after application of orthodontic force. That was decreased on day 7 and showed slight decrease on day 10 (Table 1, Figure 2A,B).

CD40L

Interestingly, the cellular response of CD40 triggers its counterreceptor, the CD40 ligand, which was expressed on day 3 in the experimental group. The expression was enhanced on day 7 and did show further increase on day 10. CD40L-expressing cells were found in close proximity to CD40-positive cells in the resorption zone, tension areas of the distal root. CD40L expression was also seen in the resorption lacunae in the resorption side on day 7. In some sections, CD40L-expressing cells were seen even on day 3. CD40L was expressed in the bone marrow cells in the pressure zone. In the tension side, some cells of the cellular cementum expressed CD40L (Table 2, Figure 2C,D). In the control group, CD40L-immunolabeled cells were not detected.

DISCUSSION

The data of the present study showed that CD40 was constitutively expressed on fibroblast-, macrophage-, and dendritic-like cells in untreated periodontal tissues and hyperexpressed in treated periodontal tissues. Furthermore, CD40L was upregulated after the application of orthodontic force. Immunohistochemical methods are well accepted for studies of cell and tissue antigens. They are simple, provide quick results, and have the advantage of preserving tissue morphology. ABC was the method of choice because this method appears to be the most frequently used method because it is more sensitive than the direct method and other indirect methods.

Immunohistochemical markers were used to detect CD40-positive and CD40L-positive cells. CD40 is a transmembrane protein, expressed by cells of hematopoietic and nonhematopoitic origins, including human fibroblasts, which have the ability to control the maintenance and repair of the connective tissues.[22] PDL fibroblast attaches the tooth to the alveolus. The initial removal of the tissues is dominated by phagocytosis of cellular and connective tissue remnants and the precementum by macrophage- and fibroblast-like cells. Later removal of the connective tissue seemed to be dominated by fibroblast-like cells.[23] After examining inflamed gingival fibroblast cultures, the frequency of IL-6– and IL-8–secreting cells mirrored the frequency of cells expressing high levels of CD40. There was a direct relationship between CD40 expression and IL-6 or IL-8 secretion.[19]

Another study showed that the expression of CD40 was significantly higher in inflamed periodontal tissues than in uninflammed tissues and this was substantiated by CD40

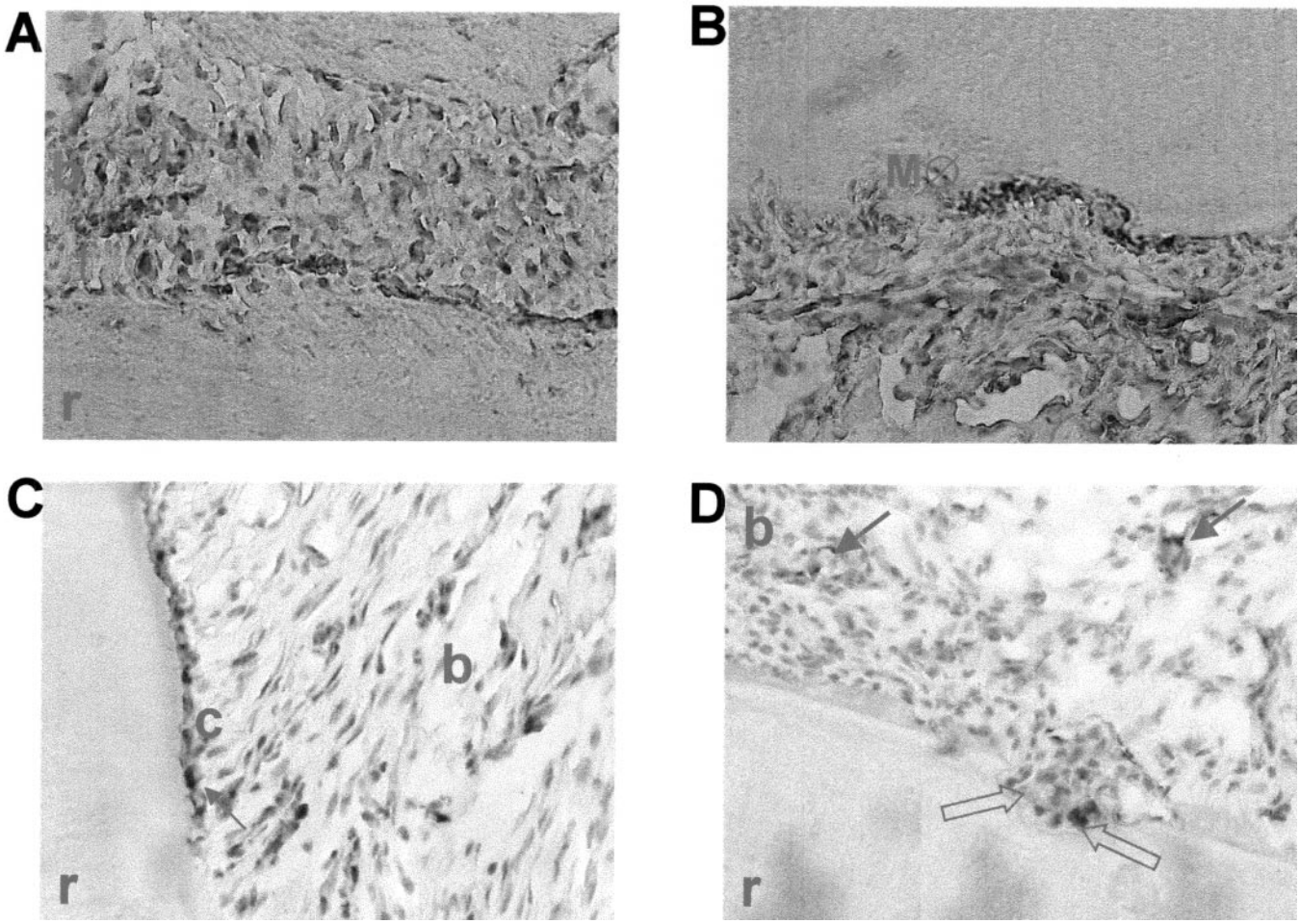

FIGURE 2. (A) CD40 expression of in the pressure zone on day 3. b indicates alveolar bone; r, root; f, fibroblast-like cell; d, dendritic-like cell. (B) CD40L expression in the pressure zone on day 3. Mϕ = macrophage-like cell. (C) CD40L-expressing cells in the tension zone on day 10. c indicates cellular cementum; r, root; b, bone. Solid arrows show positive cells of cellular cementum. (D) CD40L expression in the bone marrow cells in the pressure zone on day 3 (solid arrows). r indicates root; b, alveolar bone. Open arrows show positive cells in the resorption lacunae.

TABLE 2. Mean ± SDs of CD40L-Positive Cells in Resorption and Tension Zones in the Control and Experimental Groups at 3, 7, and 10 Days

Experimental Period (days)		CD40L-Positive Resorption Zone	CD40L-Positive Tension Zone
Day 3	Experimental	60 ± 5	50 ± 5
	Control	10 ± 5	5 ± 1
Day 7	Experimental	90 ± 10	60 ± 5
	Control	10 ± 5	5 ± 1
Day 10	Experimental	92 ± 10	61 ± 5
	Control	10 ± 1	5 ± 1

engagement stimulated by IL-6 production by gingival fibroblasts but not PDL fibroblasts.[15] We have shown CD40-positive cells with macrophage- and dendritic-like appearance. Dendritic cells are the most antigen-presenting cells for exogenous proteins, as well as presenting endogenous antigens to T cells.[24] Necrotic hyalinized tissue probably acts as potentially autoimmunogenic and therefore attracts dendritic-like cells. Cells with dendritic-like appearance and expressing Class II molecules were increased after orthodontic tooth movement.[25] Multinucleated cells and mononucleated macrophage–like cells were responsible for the removal of the necrotic hyalinized tissue and for the resorption of the surface parts of root cementum. Multinucleated clastlike cells were detected in the resorption lacunae on root and bone surfaces.[26]

The presence of CD40-positive cells in close proximity to the resorption lacunae and at some cells of the cellular cementum may indicate that the presence of CD40 molecules on these cells is an antigen, which can be triggered by CD40L expressed on infiltrating cells. The vascular blood flow and vascular proliferation have been shown to be increased on days 3 and 7 after experimental tooth movement.[27] The proliferation and perforation of blood vessels may lead to involvement of cells expressing CD40L. The CD40-CD40L signaling pathway could play a role in the defensive mechanism against root resorption. We pos-

tulate that secretion of inflammatory cytokines by periodontal cells during orthodontic tooth movement may be related to high expression of CD40 by these cells. Another possible mechanism for CD40-CD40L hyperexpression is that local cells as ligament fibroblasts may have been stimulated by the applied force to induce a costimulatory effect for the initiation of immune response and further inflammation.

Our results indicate that CD40 is an active signaling pathway in periodontal cells and that CD40-CD40L interaction can stimulate rat periodontal cell activation and synthesis of proinflammatory cytokines during orthodontic tooth movement. For example, IL-1β, IL-6, and TNF-α are proinflammatory cytokines that play a potential role in the process of bone remodeling, resorption, and new bone deposition.[28,29] Although these individual cytokines have multiple activities,[30] they are also concerned with bone remodeling, resorption, and new bone deposition.[31,32] IL-1β, IL-6, and TNF-α have been shown to be increased after orthodontic movement.[33,34] Thus, generation of inflammatory cytokines by orthodontic movement may directly induce production of proinflammatory cytokines and expression of costimulatory molecules. Both surface markers for costimulation and proinflammatory cytokines may enhance the induction of each other and further contribute to immune activation and the inflammatory processes that lead to bone resorption, deposition, or remodeling and may take part in the process of root resorption.

CONCLUSIONS

The data of the present work show that fibroblast-, macrophage- and dendritic-like cells expressed CD40 in normal rat PDL. This is the first report illustrating that CD40 expression in these types of cells was markedly expressed after application of orthodontic force. Moreover, CD40-CD40L interactions appear to play a role in the immune responses mediated by periodontal cells during orthodontic tooth movement. This might induce T-cell activation and hence generation of harmful inflammatory reactions with consequent bone resorption or a beneficial immune response with anti-inflammatory mediators that may play a defensive role against root resorption. To address this question, double staining for intracellular cytokine and the surface receptors CD40-CD40L are currently initiated at our laboratory to investigate the type of immune mediators that are generated by orthodontic force regarding CD40-CD40L expression.

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