British Journal of Pharmacology (2007) 150, 286–297
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Effects of etanercept, a tumour necrosis factor-α antagonist, in an experimental model of periodontitis in rats

R Di Paola[1,2,6], E Mazzon[1,2,6], C Muià[1], C Crisafulli[1], D Terrana[1], S Greco[1], D Britti[3], D Santori[4], G Oteri[5], G Cordasco[5] and S Cuzzocrea[1,2]

[1]*Department of Clinical and Experimental Medicine and Pharmacology, Torre Biologica, Policlinico Universitario, Messina, Italy;* [2]*IRCCS Centro Neurolesi 'Bonino-Pulejo', Messina, Italy;* [3]*Università della Magna Grœcia di Catanzaro (UMG), Catanzaro, Italy;* [4]*Dipartimento di Scienze Cliniche Veterinarie, Università degli Studi di Teramo, viale Crispi, Teramo, Italy and* [5]*Istituto Policattedra di Odontostomatologia Universita degli Studi di Messina, Messina, Italia*

Background and purpose: Etanercept is a tumour necrosis factor antagonist with anti-inflammatory effects. The aim of our study was to evaluate, for the first time, the therapeutic efficacy of *in vivo* inhibition of TNF-α in an experimental model of periodontitis.
Experimental approach: Periodontitis was induced in adult male Sprague-Dawley rats by placing a nylon thread ligature around the lower 1st molars. Etanercept was administered at a dose of 5 mg kg^{-1}, s.c., after placement of the ligature.
Key results: Periodontitis in rats resulted in an inflammatory process characterized by oedema, neutrophil infiltration and cytokine production that was followed by the recruitment of other inflammatory cells, production of a range of inflammatory mediators, tissue damage, apoptosis and disease. Treatment of the rats with etanercept (5 mg kg^{-1}, s.c., after placement of the ligature) significantly reduced the degree of (1) periodontitis inflammation and tissue injury (histological score), (2) infiltration of neutrophils (MPO evaluation), (3) iNOS (the expression of nitrotyrosine and cytokines (eg TNF-α)) and (4) apoptosis (Bax and Bcl-2 expression).
Conclusions and Implications: Taken together, our results clearly demonstrate that treatment with etanercept reduces the development of inflammation and tissue injury, events associated with periodontitis.
British Journal of Pharmacology (2007) **150**, 286–297. doi:10.1038/sj.bjp.0706979; published online 2 January 2007

Keywords: TNF-α antagonist; periodontitis; neutrophils infiltration; cytokine expression; apoptosis

Abbreviations: IL-1, interleukin-1; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NO, nitric oxide; PBS, phosphate-buffered saline; PMN, polymorphonuclear; PMSF, phenylmethylsulphonyl fluoride; |ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; TBS, tris-buffered saline; TdT, terminal deoxynucleotidyltransferase; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labelling

Introduction

Periodontitis is one of the most common infectious diseases in the world (Slavkin, 1999). The disease is characterized by destruction of the tooth supporting tissues, ultimately leading to tooth loss and reduced mastication. The disease is caused by accumulation of bacteria on the tooth surface in the periodontal pockets. These bacteria form masses of sticky film called plaque, which is able to adhere to the surface of teeth and gums. Microbial plaque is recognized as the primary aetiological agent for periodontal disease initiation and progression (Haffajee and Socransky, 1994).

Plaque microorganisms may damage cellular and structural components of the periodontium by releasing their proteolytic and noxious waste products. Microorganisms produce a large variety of soluble enzymes to digest extracellular host proteins and other molecules; these enzymes include proteases capable of digesting collagen, elastin, fibronectin and various other components of the intercellular matrix of epithelial and connective tissues. Protective aspects of the host response include recruitment of neutrophils, production of protective antibodies and

Correspondence: Professor S Cuzzocrea, Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Torre Biologica, Policlinico Universitario Via C. Valeria, Gazzi, Messina 98100 Italy.
E-mail: salvator@unime.it
[6]These authors contributed equally to this work.
Received 13 October 2006; accepted 20 October 2006; published online 2 January 2007

possibly the release of anti-inflammatory cytokines including transforming growth factor-α, interleukin-4 (IL-4), interleukin-10 (IL-10) and IL- 12 (Page, 1998).

Constituents of the biofilm also stimulate host cells to produce pro-inflammatory cytokines including IL-1β and tumour necrosis factor-α (TNF-α). TNF-α mediates a number of biological processes that can induce connective tissue and alveolar bone destruction (Gemmell *et al*., 1997). These include stimulation of bone resorption, inhibition of bone formation, inhibition of proteoglycan synthesis and induction of collagen- and cartilage-degrading metalloproteinases and prostaglandin E_2, as well as further production of TNF and other pro-inflammatory cytokines such as IL-1 (Dayer *et al*., 1985; Bertolini *et al*., 1986; Saklatvala 1986; Shinmei *et al*., 1989). These cytokines are present in diseased periodontal tissues and gingival crevicular fluid (Stashenko *et al*., 1991).

The catabolic activities of these cytokines are controlled by endogenous inhibitors that include IL-1 and TNF-receptor antagonists. When administered for therapeutic purposes, these antagonists can reduce inflammation (Rosenbaum and Boney, 1991; Windsor *et al*., 1993; Mullarkey *et al*., 1994; Russel *et al*., 1995). The use of cytokine receptor antagonists to inhibit periodontal disease progression has been investigated in a ligature-induced periodontitis non-human primate model (Assuma *et al*., 1998; Delima *et al*., 2001). It was demonstrated that IL-1/TNF blockers partially inhibited disease progression (Delima *et al*., 2001).

Etanercept is a dimeric, soluble form of the 75-kDa TNF receptor. The anti-inflammatory effects of etanercept are owing to its ability to bind to TNF, preventing it from interacting with cell-surface receptors and rendering it biologically inactive. Etanercept can also modulate biological responses that are induced or regulated by TNF, including both expression of adhesion molecules responsible for leukocyte migration and serum levels of cytokines and matrix metalloproteinase-3.

This article investigated the role of TNF in the pathogenesis of periodontitis and evaluates the therapeutic effects of etanercept.

In particular, we have determined the following end points of the inflammatory response: (1) histological damage, (2) bone loss (radiography), (3) neutrophil infiltration, (4) cytokine expression (TNF-α), (5) nitrotyrosine, inducible nitric oxide synthase (iNOS) expression and (6) apoptosis (Bax and Bcl-2 expression).

Methods

Surgical procedure

Male Sprague–Dawley rats (280–400 g) were lightly anaesthetized with surgical doses of sodium pentobarbitone (35 mg kg^{-1}). Sterile, 2-0 black braided silk thread was placed around the cervix of the lower left first molar and knotted medially as described previously (Di Paola *et al*., 2004). After the rats had recovered from the anaesthetic they were allowed to eat commercial laboratory food and drink tap water *ad libitum*. Animal care and the study protocol were approved by the Institutional Animal Care and User Committee of the University of Messina.

Measurement of arterial blood pressure indirectly in conscious rat

Mean arterial blood pressure in conscious rats was measured by a blood pressure recorder (UGO BASILE, Biological Research Apparatus, Comerio, Italy), After a week, rats were treated as described below and blood pressure was measured 30 min before and after subcutaneously (s.c.) injection. To measure arterial blood pressure, rats were housed for 30 min in a warmed room (28–30°C) and then a tail cuff, consistently about 2 cm from the base of the tail, was applied and arterial blood pressure measured. Heart rate was detected by a pulse rate counter placed after the tail cuff.

Experimental groups

Rats were randomly allocated into the following groups: *Ligature + vehicle group:* rats were subjected to ligature-induced periodontitis and animals received vehicle s.c. *Ligature + etanercept group:* rats were subjected to ligature-induced periodontitis and animals received *etanercept* (5 mg kg^{-1} s.c. after ligature). At 8 days after the ligature induction of periodontitis, the rats ($n = 10$ from each group for each parameter) were killed in order to evaluate the various parameters described below. The doses of etanercept used here, 5 mg kg^{-1}, were based on those used in a previous *in vivo* study (Genovese *et al*., 2006).

Measurement of vascular permeability by Evans blue extravasations

Vascular permeability was determined as described previously (Gyorfi *et al*., 1994). Briefly, animals received Evans blue (2.5% dissolved in physiological saline, at a dose of 50 mg kg^{-1}) via the femoral venous catheter. Extravasated Evans blue in the excised gingivomucosal tissue samples was extracted with 1 ml formamide for 48 h at room temperature for spectrophotometric determination at 620 nm and expressed as μg g^{-1} gingivomucosal tissue (Gyorfi *et al*., 1994).

Measurement of alveolar bone loss

In the same set of experiments, the distance from the cementoenamel junction of first lower molars to the alveolar crest was measured by using a modification of the method used by Crawford *et al*. (1978). Recordings were made along the median axis of the lingual surface of the mesial and mediolingual roots of the lower first left and right molars as described previously (Di Paola *et al*., 2004). These measurements were performed by an independent investigator who was unaware of the treatment regimes. The alveolar bone loss induced by the ligature was expressed as a difference between the left and the right side.

Histological examination

For histopathological examination, biopsies of gingival and mucosa tissue from the buccal and lingual aspect of the teeth were taken 8 days after the ligature induction of periodontitis. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin and sectioned. The sections, orientated longitudinally from the

teeth crowns, were stained with trichrome stain. The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces from gingival and mucosa tissues were assessed quantitatively by counting the number of polymorphonuclear (PMN) cells in 20 high-power fields.

Radiography

Mandibles were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and legated mandibles was performed by X-ray machine (Philips X12 Germany) with a 40 kW exposure for 0.01 s. A radiographic examination of the mandibles 8 days after ligature placement revealed bone matrix resorption in the lower first left after ligation as described previously (Di Paola *et al*., 2004).

Malondialdehyde measurement

Malondialdehyde (MDA) levels were determined as an indicator of lipid peroxidation as described previously (Ohkawa *et al*., 1979). Gingivomucosal tissue, collected at the specified time, was homogenized in 1.15% ($w\,v^{-1}$) KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% ($w\,v^{-1}$) sodium dodecyl sulphate (SDS), 1.5 ml of 20% ($v\,v^{-1}$) acetic acid (pH 3.5), 1.5 ml of 0.8% ($w\,v^{-1}$) thiobarbituric acid and

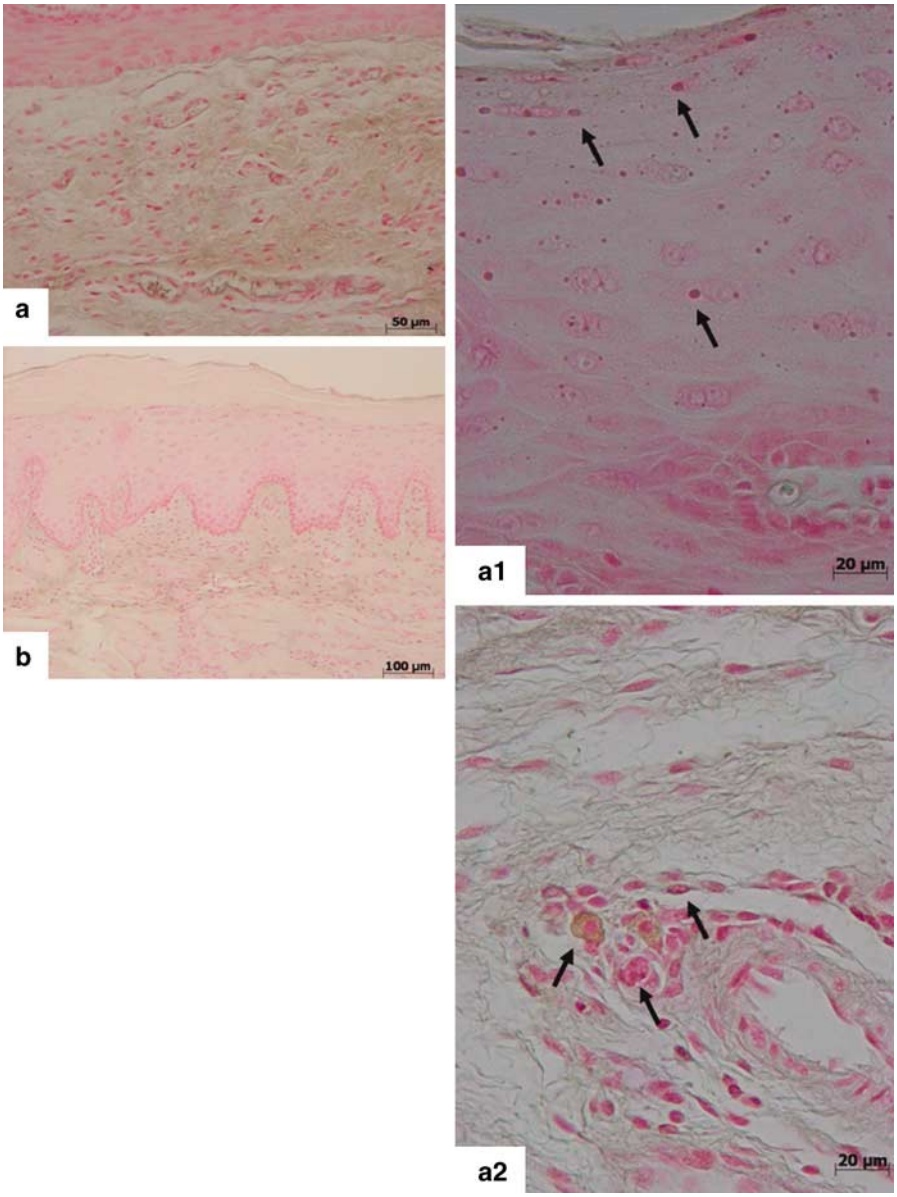

Figure 1 Immunohistochemical staining for TNF-α. Positive staining for TNF-α (**a**) was observed in gingivomucosal tissue after ligature mainly localized in the epidermis and in inflammatory cells in derma (see arrows particles in **a1** and **a2**). In gingivomucosal tissue of etanercept (5 mg kg^{-1} i.p after ligature) – treated rats no positive staining was observed for TNF-α (**b**). The figure is representative of at least three experiments performed on different experimental days.

700 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm.

Myeloperoxidase activity

Myeloperoxidase activity, an indicator of PMN leukocyte accumulation, was determined as described previously (Mullane *et al*., 1985). Gingivomucosal tissue, collected at the specified time, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20 000 *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol min^{-1} of peroxide at 37°C and was expressed in μg^{-1} of wet tissue.

Immunohistochemical localization of TNF-α, iNOS, nitrotyrosine

After the removal of paraffin, endogenous peroxidase was quenched with 0.3% (v v^{-1}) H_2O_2 in 60% (v v^{-1}) methanol for 30 min. The sections were then incubated overnight with primary anti-TNF-α (1:500 dilution) antibody, anti-nitrotyrosine antibody (1:1000 dilution), primary anti-iNOS (1:500 dilution) with control solutions including buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). The counterstain was developed with DAB (brown colour) and nuclear fast red (red background). A positive staining (brown colour) was found in the sections, indicating that the immunoreactions were positive. No positive staining (pink colour) was observed in the sections indicating that the immunoreactions were negative. Immunocytochemistry photographs ($n = 5$ photos from each samples collected from all rats in each experimental group) were assessed by densitometric analysis by using Optilab Graftek software on a Macintosh personal computer.

Total protein extraction and Western blot analysis for iNOS, TNF-α, Bax and Bcl-2

Tissue samples from ligature-operated rats were homogenized with a Ultra-turrax T8 homogenizer in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid pH = 7.9, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-tetraacetic acid, 1 mM dithiothreitol, 0.5 mM phenylmethyl-sulphonyl fluoride, 1.5 μg ml^{-1} trypsin inhibitor, 3 μg ml^{-1} pepstatin, 2 μg ml^{-1} leupeptin, 40 μM benzidamin, 1% NP-40, 20% glycerol. The homogenates were centrifuged at 13 000 r.p.m., for 15 min and at 4°C, the supernatant was collected to evaluate contents of Bax and Bcl-2. Protein concentration was determined with the Bio-Rad (Milan, Italy) protein assay kit. Proteins were mixed with gel loading buffer (50 mM Tris, 10% (w v^{-1}), SDS, 10% (w v^{-1}) glycerol, 10% (v v^{-1}) 2-mercaptoethanol, 2 mg ml^{-1} bromophenol), boiled for 3 min and centrifuged at 10 000 r.p.m. for few

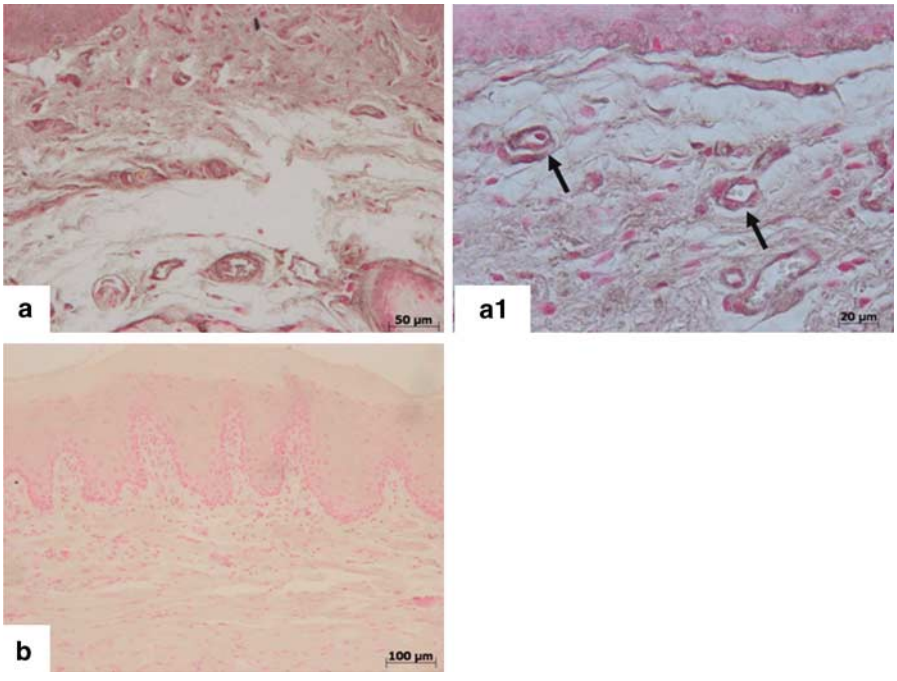

Figure 2 Immunohistochemical staining for iNOS. Positive staining for iNOS (**a**) was observed in gingivomucosal tissue after ligature mainly localized in the inflammatory cells in derma and around the vessels (see arrows particles in **a1** and **a2**). In gingivomucosal tissue of etanercept (5 mg kg^{-1} i.p after ligature)-treated rats no positive staining was observed for iNOS (**b**). The figure is representative of at least three experiments performed on different experimental days.

seconds. Protein concentration was determined and equivalent amounts (75 μg) of each sample were subjected to electrophoreses in a 12% (w v^{-1}) discontinuous polyacrylamide minigel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 10% non-fat dry milk in Tris-buffered saline (TBS) for 1 h and then, incubated with primary antibodies against iNOS, TNF-α, Bax and Bcl-2 (1:1000) overnight at 4°C. The membranes were washed three times for 10 min in TBS with 0.1% Tween20 and incubated with AffiniPure Goat Anti-Rabbit IgG coupled to peroxidase (1:2000). The immune complexes were visualized using the SuperSignal West Pico chemiluminescence Substrate (Pierce, Milan, Italy.).

Terminal deoxynucleotidyltransferase-mediated UTP end labelling assay

Terminal deoxynucleotidyltransferase-mediated UTP end labelling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (Apotag, HRP kit DBA, Milan, Italy). Briefly, sections were incubated with 15 μg ml^{-1} proteinase K for 15 min at room temperature and then washed with phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

Materials

The primary antibodies directed at Bax and Bcl-2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The secondary antibody was obtained from Jackson Immuno Research, Laboratories, Inc. (Jackson, Bar Harbor, Maine, USA). Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

Data analysis

All values in the figures and text are expressed as mean ± s.e.m. of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemistry colouration) performed on different experimental days on the tissue sections collected from all the animals in each group. The results were analysed by one-way analysis of variance followed by a Bonferroni *post hoc* test for multiple comparisons. A *P*-value <0.05 were considered significant. Individual group means were compared with Student's unpaired *t*-test. A *P*-value <0.05 was considered significant.

Results

Effects of etanercept on TNF-α and iNOS expression in periodontitis

Sections of gingivomucosal tissues from the contralateral side did not reveal any immunoreactivity for TNF-α and

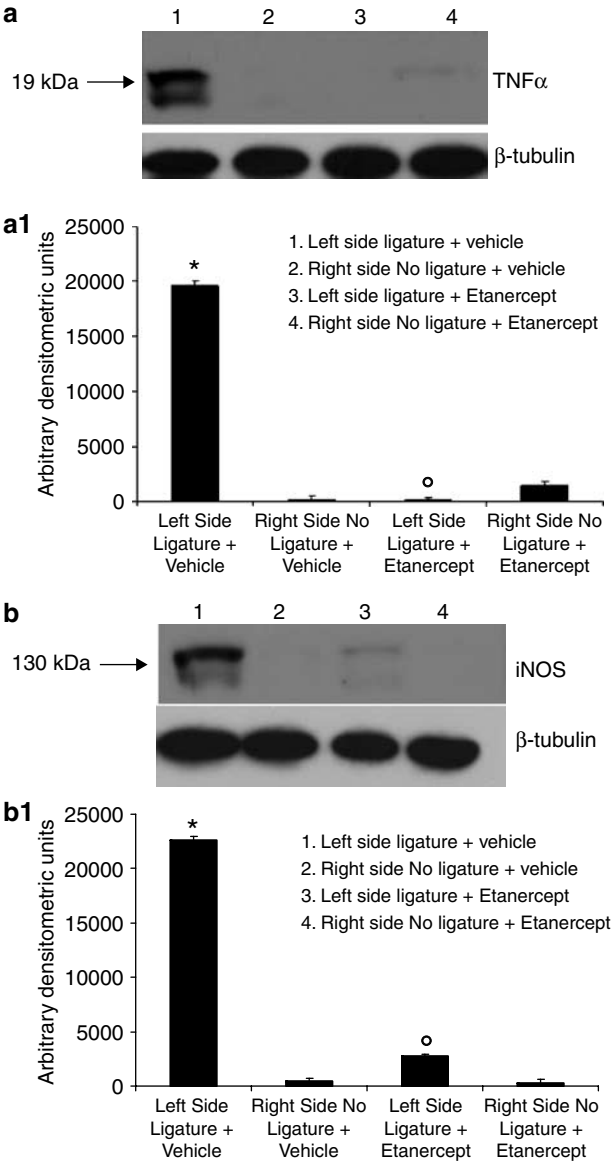

Figure 3 Representative Western blot of TNF-α and iNOS. A representative blot of TNF-α (**a**) and iNOS (**b**) expression in gingivomucosal tissue collected 8 days after injury. A significant increase in TNF-α (**a**) and iNOS (**b**) expression, assayed by Western blot analysis, was detected in the tissue from ligature-treated rats. The treatment with etanercept 5 mg kg^{-1} significantly reduced TNF-α (**a**) and iNOS (**b**) expression in the gingivomucosal tissues. A representative blot of lysates (**a** and **b**) obtained from five animals per group is shown and densitometry analysis of all animals is presented. The results in (**a1**) and (**b1**) are expressed as mean ± s.e.m. from $n=5/6$ tissues for each group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

iNOS within the normal architecture (data not shown). At 8 days following ligation, positive staining for TNF-α (Figure 1a see densitometry analysis in Figure 4a) was found in the gingivomucosal tissues from ligature-operated rats mainly localized in the epidermis and in inflammatory cells in derma (see arrows particles in Figure 1a1 and a2. Similarly, at 8 days following ligation, positive staining for iNOS (Figure 2a see densitometry analysis in Figure 5a) was found in the gingivomucosal tissues from ligature-operated rats mainly localized in the inflammatory cells in derma and around the vessels (see arrows particles in Figure 2a1 and a2). Etanercept (5 mg kg^{-1}) abolished the staining for TNF-α and iNOS (Figures 1b and 2b respectively, see densitometry analysis in Figure 5a). A significant increase in iNOS and TNF-α expression 8 days following ligation, as assayed by Western blot analysis, was also detected in gingivomucosal tissues obtained from ligature-operated rats (Figure 3a and b see densitometry analysis in Figure 3a1 and b1. Etanercept (5 mg kg^{-1}) treatment significantly attenuated the iNOS and TNF-α expression (Figure 3a and b see densitometry analysis in Figure 3a1 and b1).

Effects of etanercept on nitrotyrosine formation and lipid peroxidation in periodontitis

Immunohistochemical analysis of gingivomucosal tissues from the contralateral side did not reveal any immunoreactivity for nitrotyrosine (data not shown). In contrast, 8 days following ligation, positive staining for nitrotyrosine was found in the gingivomucosal tissues from ligature-operated rats (Figure 4a see densitometry analysis in Figure 5a) mainly localized in the inflammatory cells in derma and around the vessels (see arrows particles in Figure 4a1 and a2). In contrast, no positive staining for

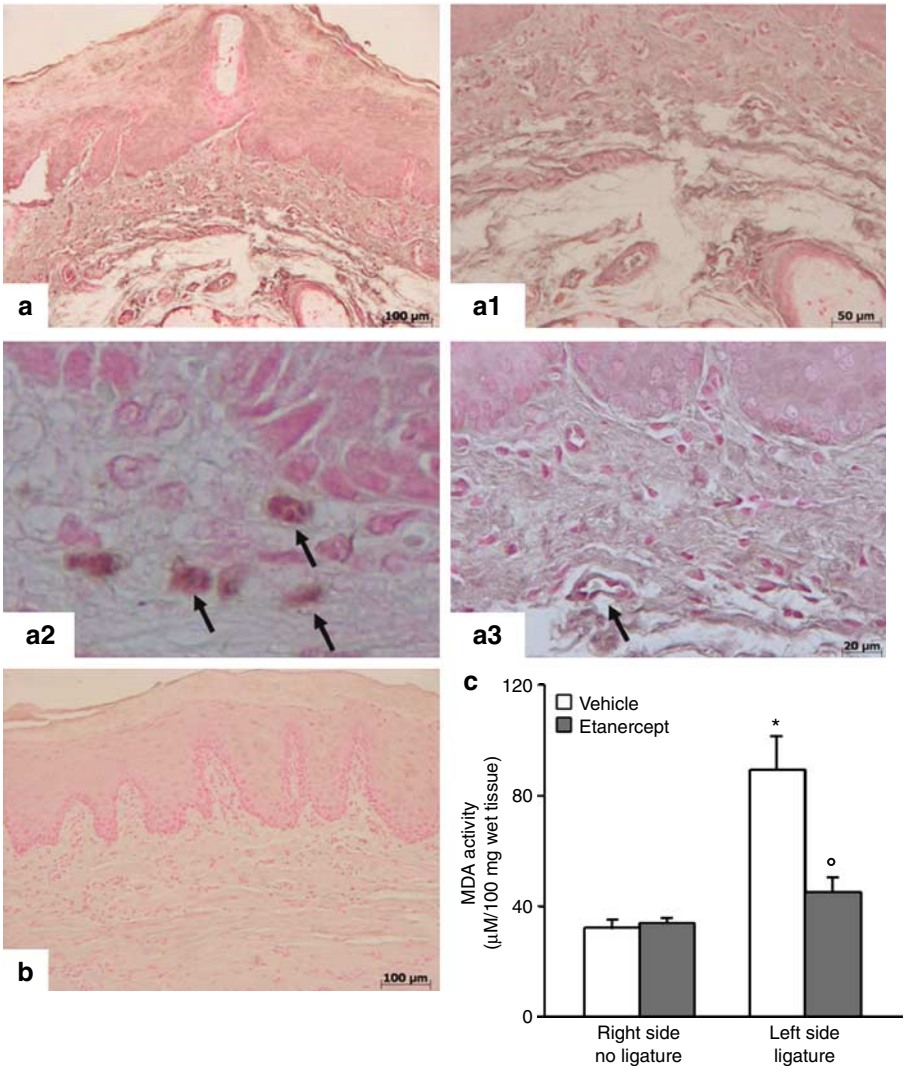

Figure 4 Immunohistochemical staining for nitrotyrosine. Positive staining for nitrotyrosine (**a**) was observed in gingivomucosal tissue after ligature mainly localized in the inflammatory cells in derma and around the vessels (see arrows particles **a1** and **a2**). In gingivomucosal tissue of etanercept (5 mg kg^{-1} i.p after ligature)-treated rats no positive staining was observed for nitrotyrosine (**b**). MDA levels, an index of lipid peroxidation, were significantly increased in gingivomucosal tissue collected 8 days after injury (b). Etanercept (5 mg kg^{-1} i.p.) significantly reduced the increase of MDA tissues levels (**c**). The figure is representative of at least three experiments performed on different experimental days. Data are expressed as mean ±s.e.mean from $n=10$ rats for each group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

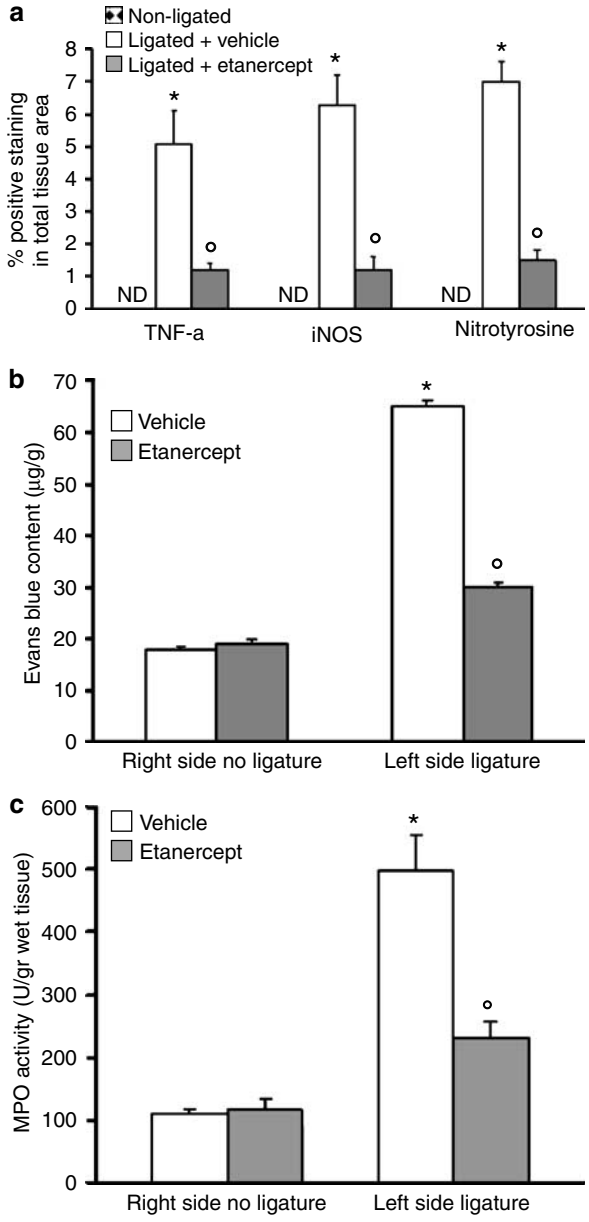

Figure 5 Densitometry analysis of immunocytochemistry photographs (**a**) $n=5$ photos from each sample collected from all rats in each experimental group) for TNF-α, iNOS and nitrotyrosine from gingivomucosal tissue. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Evans blue content (**b**) and myeloperoxidase activity (**c**) in gingivomucosal tissue were significantly increased by ligature compared to the contralateral side. Etanercept (5 mg kg^{-1} i.p after ligature) significantly reduced Evans blue content and myeloperoxidase activity levels. Densitometry data are expressed as % of total tissue area. Data are means ± s.e.m. from $n=10$ rats for each group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

nitrotyrosine was found in the gingivomucosal tissues from ligature-operated rats, which had been treated with etanercept (5 mg kg^{-1}) (Figure 4b see densitometry analysis in Figure 5a). In addition, at 8 days following ligation, MDA levels were also measured in the gingivomucosal tissues as an indicator of lipid peroxidation. As shown in Figure 4c, MDA levels were significantly increased in the gingivomucosal tissues from ligature-operated rats. Lipid peroxidation was significantly attenuated by the intraperitoneal injection of etanercept (Figure 4c).

Effects of etanercept on plasma extravasation and neutrophils infiltration in periodontitis

Before the measurement of Evans blue extravasation, mean arterial pressure of vehicle-treated and etanercept-treated animals was recorded. Etanercept treatment did not affect mean arterial blood pressure (vehicle-treated: 124 ± 3 mm Hg; $n=10$ and etanercept treated: 122 ± 4 mm Hg; $n=10$). Ligation significantly increased Evans blue extravasation in gingivomucosal tissue compared to the contralateral side (Figure 5b). Etanercept treatment prevented this increase in Evans blue extravasation, but did not change the Evans blue content of the contralateral side (Figure 5b). Myeloperoxidase activity was significantly elevated ($P<0.001$) at 8 days after the ligature (Figure 5c) and etanercept treatment significantly reduced these levels (Figure 5c). No significant changes of myeloperoxidase activity were observed in the gingivomucosal tissues from the contralateral side (Figure 5c).

Effect of etanercept on tissue damage and bone destruction

When compared to gingivomucosal tissue sections taken from the contralateral side (Figure 6a), histological examination of gingivomucosal tissue sections of ligature-operated rats showed oedema, tissue injury (Figure 6b) as well as infiltration of the tissue with inflammatory cells (Figure 6b see arrows particles in Figure 6b1). Etanercept treatment reduced the degree of injury or the gingivomucosal tissues (Figure 6c). Quantification of infiltrating PMN cells into gingivomucosal tissue showed that there were only a minimal number of PMN cells in tissue from the contralateral side (Figure 6d). However, a large number of infiltrating PMN cells was observed in the gingivomucosal tissue of ligated rats (Figure 6d). Etanercept administration significantly reduced the numbers of PMN cells infiltrating into gingivomucosal tissue (Figure 6d). A radiographic examination of the mandibles, 8 days after ligature placement, revealed bone matrix resorption in the lower left first molar region after ligation (Figure 7a). There was no evidence of changes in pathology in the right first molar (data not shown). Etanercept markedly reduced the degree of bone resorption in the lower left first molar region after ligation (Figure 7b). In addition, a significant alveolar bone loss, between the lower first left and the right first molars induced by the left side ligature, was observed in vehicle-treated rats (Figure 7c). Etanercept treatment resulted in a significant inhibition of alveolar bone loss after ligation (Figure 7c).

Western blot analysis and immunohistochemistry for Bax and Bcl-2

The appearance of Bax and Bcl-2 in homogenates of gingivomucosal tissues was investigated by Western blot analysis after ligature. A basal level of Bax was detectable

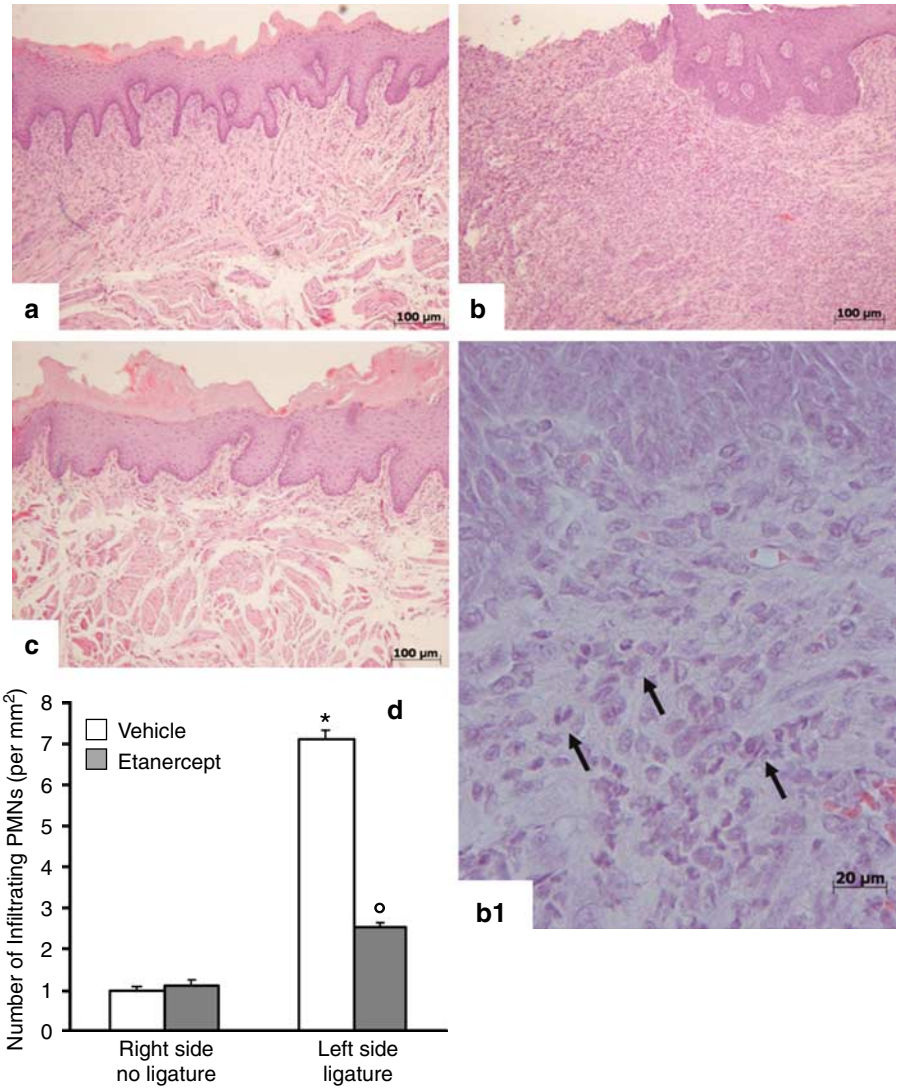

Figure 6 When compared to gingivomucosal tissue sections taken from the contralateral side (**a**), oedema, tissue injury (**b**) as well as infiltration of the tissue with inflammatory cells (see arrows particles **b1**) were observed in gingivomucosal section from ligature-treated rats. Significantly less oedema and inflammatory cell infiltration was observed in gingivomucosal sections from ligature-treated rats that had been administered etanercept (5 mg kg^{-1} i.p after ligature) (**c**). The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in gingivomucosal tissue was assessed quantitatively by counting the number of PMN cell in 20 high-power fields (**d**). The results presented are representative of at least three experiments performed on different experimental days. The tissue sections, orientated longitudinally from the teeth crown, were stained with trichrome stain. Data represent the mean ± s.e.m. for 20 counts obtained from the gingivomucosal tissue of each treatment group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

in the homogenized gingivomucosal tissues from sham-operated animals (Figure 8). Bax levels were substantially increased in the gingivomucosal tissues of saline-treated rats (Figure 8). In contrast, etanercept treatment (5 mg kg^{-1} s.c. after ligature) prevented the periodontitis-mediated Bax expression (Figure 8). A low basal level of Bcl-2 expression was detected in gingivomucosal homogenates from tissue of sham-operated rats (Figure 9). The expression of Bcl-2 was significantly diminished in whole extracts obtained from gingivomucosal tissues of vehicle-treated rats after ligature (Figure 9). Treatment of rats with etanercept (5 mg kg^{-1} s.c. after ligature) significantly reduced the ligature-induced inhibition of Bcl-2 expression (Figure 9).

Effect of etanercept on apoptosis in gingivomucosal tissue after ligation-induced periodontitis

To investigate whether periodontitis is associated with apoptotic cell death we measured TUNEL-like staining in gingivomucosal tissues. Eight days following ligation, a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments was found in the gingivomucosal tissues from ligature-operated rats (Figure 10b, see particles in b1 and b2). In contrast, no apoptotic cells or fragments were observed in the gingivomucosal tissues from ligature-operated rat treated with etanercept 5 mg kg^{-1} (Figure 10c). Similarly, no apoptotic cells were observed in gingivomucosal tissue sections taken from the contralateral side (Figure 10a).

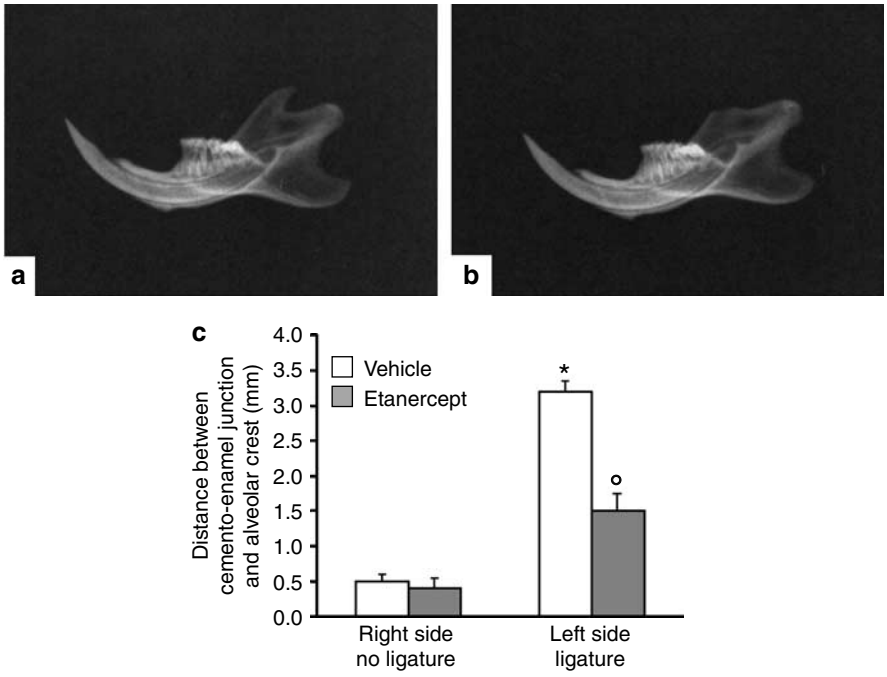

Figure 7 The alveolar bone from ligated (8 days) rats demonstrated bone resorption (**a**). Etanercept treatment suppressed these changes in alveolar pathology in the rat alveolar bone (**b**). A significant increase in the distance between cemento-enamel injunction and alveolar crest at mediolingual root of the first molar was observed in ligature-treated rats. Etanercept treatment significantly reduced the increase in the distance between cemento-enamel injunction and alveolar crest. (**c**) The radiograph is representative of at least three experiments performed on different experimental days. Data represent the mean from 20 counts obtained from the gingivomucosal tissue of each treatment group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

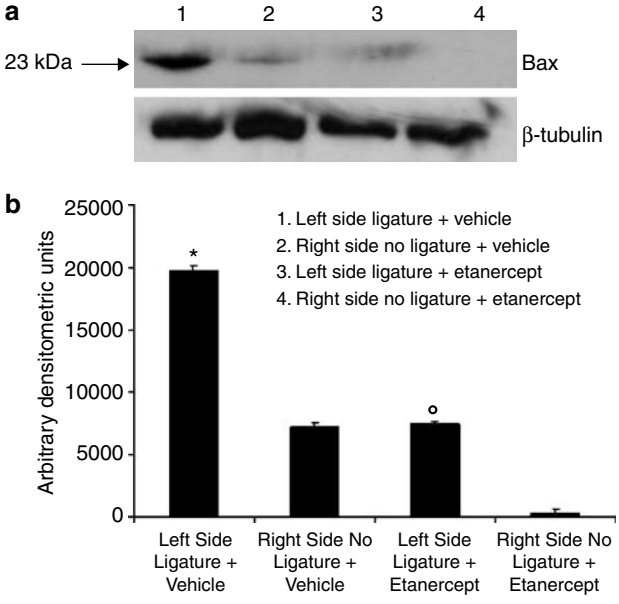

Figure 8 Representative Western blot of Bax levels. Western blot analysis was performed on gingivomucosal tissue collected 8 days after injury. Basal level of Bax was present in the tissue from-sham-operated rats. Bax band is more evident in the tissue from ligature-treated rats. The Bax band disappeared in the tissue from ligated rats that received etanercept. The immunoblot in (**a**) represents one tissue of the 5–6 analyzed. The results in (**b**) are expressed as mean ± s.e.m. from 5–6 tissues. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

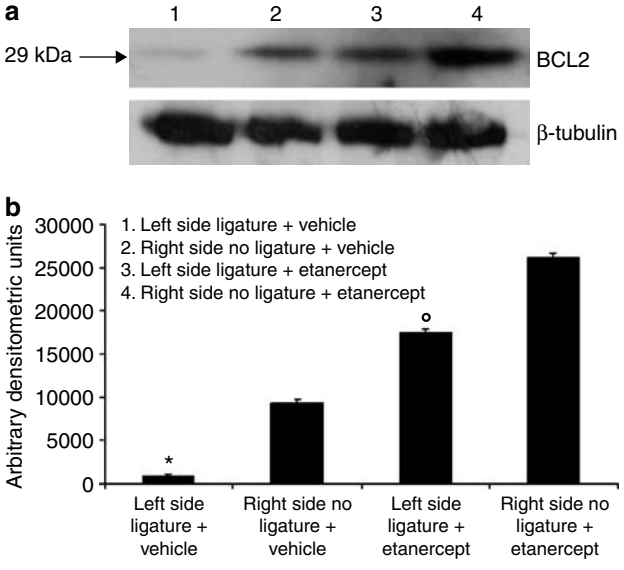

Figure 9 Representative Western blot of Bcl-2 levels. Western blot analysis was performed in gingivomucosal tissue collected 8 days after injury. A basal level of Bcl-2 was present in the tissue from sham-operated rats. The Bcl-2 band disappeared in the tissue from rats subjected to ligation. The Bcl-2 band is more evident in the tissue from ligated rats that received etanercept. The immunoblot in (**a**) is representative of one tissue out of 5–6 analyzed. The results in (**b**) are expressed as mean ±s.e.m. from 5–6 tissues. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

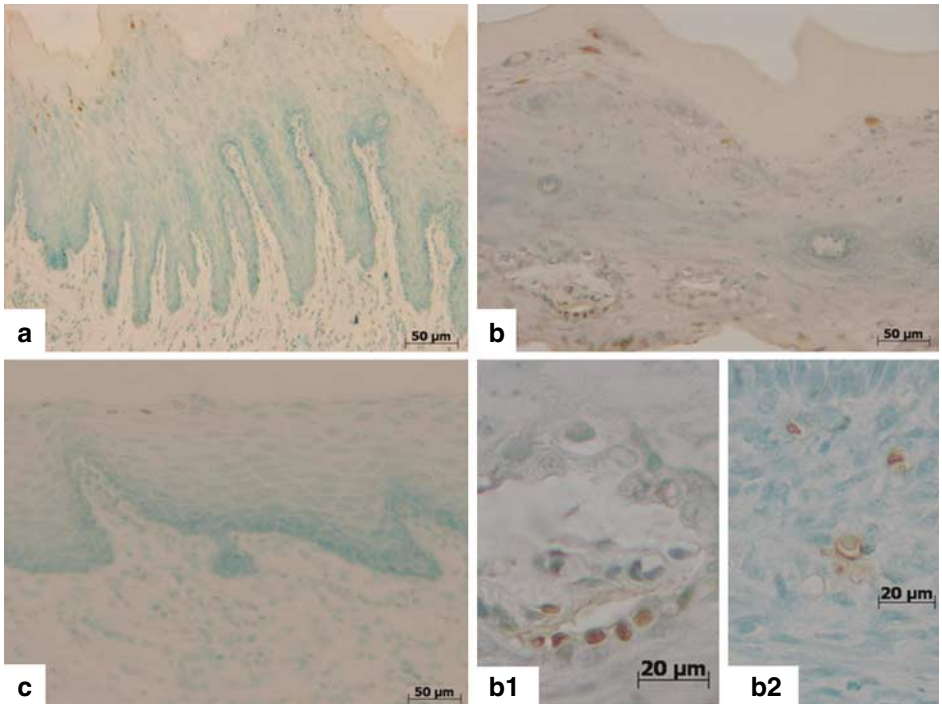

Figure 10 Effect of etanercept on apoptosis in gingivomucosal tissue after ligation-induced periodontitis. This was evaluated by TUNEL-like staining. At 8 days following ligation, a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments was found in the gingivomucosal tissues from ligatured rats. (**b**, see particles in **b1** and **b2**). In contrast, no apoptotic cells or fragments were observed in the gingivomucosal tissues from ligatured rats treated with etanercept 5 mg kg^{-1} (**c**). Similarly, no apoptotic cells were observed in gingivomucosal tissue sections taken from the contralateral side (**a**). The figure is representative of at least three experiments performed on different experimental days.

Effect of periodontitis on body weight

The rate and the absolute gain in body weight were significantly different between the sham-operated rats and ligature-operated rats that developed periodontitis (Figure 11). Beginning 24 h after ligation, the ligature-operated rat gained significantly less weight than the sham-operated rats, and this trend continued through to day 7 (Figure 11). After administration of etanercept, ligature-operated rats exhibited a significant increase in weight ($P<0.0001$) when compared with the respective control group (Figure 11).

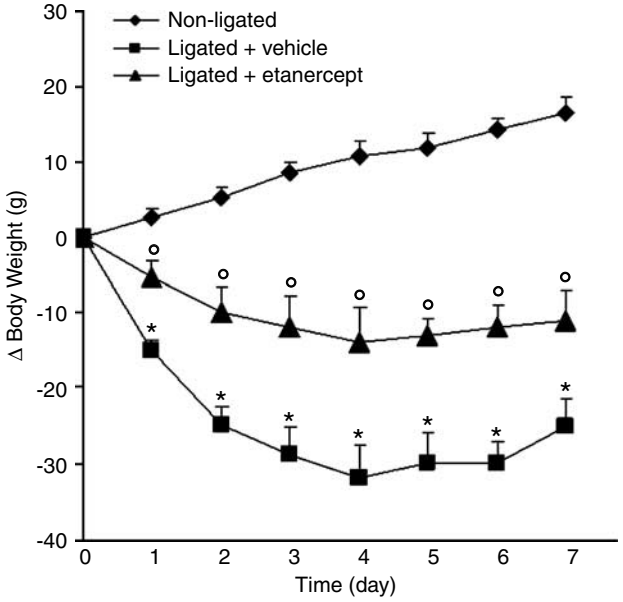

Figure 11 Effect of etanercept on body weight gain. The rate and the absolute gain in body weight were significantly different between the sham-operated rats and ligature-operated rats that developed periodontitis. Beginning 24 h after the ligature, the ligature-operated rats gained significantly less weight than the sham-operated rats, and this trend continued through to day 7. After administration of etanercept, ligature-operated rats exhibited a significant weight gain when compared with the respective control group. Data are expressed as mean ± s.e.m. from $n=10$ rats for each group. *$P<0.01$ vs non-ligated. °$P<0.01$ vs ligated.

Discussion

In this study, we demonstrate that a TNF antagonist etanercept, which inhibits TNF activity by competitively binding to it and preventing interactions with its cell surface receptors, exerts beneficial effects in a rat model of periodontitis. The main findings are that treatment with etanercept attenuates: (i) TNF-α activity, (ii) the infiltration of neutrophils, (iii) cell apoptosis, (iv) the iNOS, (v) nitrotyrosine formation and (vi) the injury of gingivomucosal tissues in rats subjected to ligature-induced periodontitis. All of these findings support the view that TNF-α has a detrimental role in the development of injury associated with periodontitis in rats.

Our results demonstrated that etanercept exerted a significant inhibitory effect on plasma extravasation during

periodontitis. Our study also confirmed earlier findings, that one of the characteristic signs of inflammation, Evans blue extravasation, was higher on the ligated side on the eighth day, than on the opposite side (Gyorfi *et al*., 1994).

The role of pro-inflammatory cytokines in periodontal tissue destruction is well documented in animal models (Assuma *et al*., 1998). In particular there is good evidence that TNF-α helps to propagate the prolongation of periodontitis (Ikezawa *et al*., 2005).

In the present study, we have clearly demonstrated, by immunohistochemistry, a significant increase of positive staining for TNF-α in periodontitis. In contrast, no significant expression of TNF-α was observed in tissue sections obtained from etanercept-treated rats. There is a large amount of evidence that TNF-α, also plays an important role in the induction of iNOS which is known to be essential for the development of periodontitis (Di Paola *et al*., 2004). In this study, we observed that etanercept treatment reduced the expression of iNOS in ligated rats. Hence, we propose that the attenuation of the induction of iNOS expression, observed in ligated rats treated with etanercept, is secondary to a reduced formation of endogenous TNF-α. This observation is in agreement with those from other studies where it has been clearly demonstrated that iNOS expression is reduced by TNF-α-antagonists (Genovese *et al*., 2006).

Initial host responses to bacterial infections in periodontitis include activation and recruitment of neutrophils and macrophages. These cells subsequently release mediators including reactive oxygen species (ROS), which are antagonistic to plaque biofilms, but which in excess may initiate inflammation. (Wahl *et al*., 1994). For example, nitric oxide (NO) is a free radical involved in host defence that can be toxic when present at high levels and it has been implicated in a variety of inflammatory conditions (Nathan, 1992; Boughton-Smith *et al*., 1993; Wahl *et al*., 1994; Miller *et al*., 1995; Brahn *et al*., 1998). Several studies have implicated the role of ROS and reactive nitrogen species in tissue destruction associated with inflammatory periodontal diseases (Waddington *et al*., 2000).

It has been demonstrated, in fact, that several chemical reactions, involving nitrite, peroxynitrite, hypochlorous acid and peroxidases can induce tyrosine nitration and may contribute to tissue damage including gingivomucosal tissues (Lohinai *et al*., 1998). In addition to NO, peroxynitrite is also generated in ligature-induced periodontitis (Lohinai *et al*., 1998). To confirm the pathological contributions of peroxynitrite to tissue damage after ligature, we evaluated nitrotyrosine formation, an index of 'increased nitrosative stress' in the injured tissue. We observed that the immunostaining for nitrotyrosine is reduced in rats treated with etanercept.

Apoptosis, or programmed cell death, is a form of physiological cell death. It is increased or decreased in the presence of infection, inflammation or tissue remodelling. Previous studies have suggested that apoptosis is involved in the pathogenesis of inflammatory periodontal disease (Bascones *et al*., 2004). It has also been demonstrated that the higher frequency of Bcl-2 expression results in progressive periodontal destruction (Bulut *et al*., 2006). A novel signal-transduction pathway, which is involved in mediating apoptotic effects of TNF-α, has been identified in several cell types (Chae *et al*., 2000). Activation of this pathway appears to occur early in the response to TNF-α, within seconds or minutes, and is closely coupled to the receptor complex, as this event can be reconstituted in a cell-free system. As apoptosis is an exceedingly complex process involving a large variety of signalling molecules, we have focused our attention on a few selective major players. From the results, we identified proapoptotic transcriptional changes, including upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2, using a Western blot assay. This is the first study to show that treatment with etanercept in periodontitis inhibits TNF-α and prevents the loss of the antiapoptotic pathway and, also, reduces activation of the proapoptotic pathway by an, as yet, unidentified mechanism.

In conclusion, this study provides the first evidence that etanercept causes a substantial reduction of ligature-induced periodontitis in the rat. Finally, our findings suggest that interventions, which may reduce the generation or the effects of TNF-α, may be useful in conditions associated with local or systemic inflammation.

Acknowledgements

This study was supported by a Grant from PRA 2003 from University of Messina. We thank G Pergolizzi and CL Spada for their excellent technical assistance during this study, Mrs C Cutrona for secretarial assistance and Miss V Malvagni for editorial assistance with the manuscript.

Conflict of interest

The authors state no conflict of interest.

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