DEL-1 restraints osteoclastogenesis and inhibits inflammatory bone loss in nonhuman primates

Jieun Shin,1* Tomoki Maekawa,1* Toshiharu Abe,1 Evlambia Hajishengallis,2 Kavita Hosur,1 Kalyani Pyaram,17 Ioannis Mitroulis,3 Triantafyllos Chavakis,36 George Hajishengallis1,3§

DEL-1 (developmental endothelial locus–1) is an endothelial cell–secreted protein that regulates LFA-1 (lymphocyte function–associated antigen–1) integrin–dependent leukocyte recruitment and inflammation in various tissues. We identified a novel regulatory mechanism of DEL-1 in osteoclast biology. Specifically, we showed that DEL-1 is expressed by human and mouse osteoclasts and regulates their differentiation and resorptive function. Mechanistically, DEL-1 inhibited the expression of NFATc1, a master regulator of osteoclastogenesis, in a Mac-1 integrin–dependent manner. In vivo mechanistic analysis has dissociated the anti-inflammatory from the anti–bone-resorptive action of DEL-1 and identified structural components thereof mediating these distinct functions. Locally administered human DEL-1 blocked inflammatory periodontal bone loss in nonhuman primates—a relevant model of human periodontitis. The ability of DEL-1 to regulate both upstream (inflammatory cell recruitment) and downstream (osteoclastogenesis) events that lead to inflammatory bone loss paves the way to a new class of endogenous therapeutics for treating periodontitis and perhaps other inflammatory disorders.

INTRODUCTION

Periodontitis is a chronic inflammatory disease that causes destruction of the bone and soft tissues that support the teeth (collectively known as the periodontium) and is associated with increased risk for certain systemic disorders, such as atherosclerosis and rheumatoid arthritis (1). Although the disease is initiated by local dysbiotic microbial communities, it is the host inflammatory response to this microbial challenge that ultimately causes tissue damage, including pathologic activation of osteoclasts to resorb bone (2). There is currently an unmet need for efficacious and safe therapeutics for chronic inflammatory diseases including periodontitis, which is often unresponsive to conventional mechanical treatment combined with antimicrobial therapy. The high prevalence of periodontitis, which affects >47% of U.S. adults with 8.5% experiencing severe forms of the disease (3), together with its economic burden (4), underscores the importance of implementing innovative treatment interventions. In this regard, we have investigated the therapeutic potential of developmental endothelial locus–1 (DEL-1), a potent anti-inflammatory protein (5–7) that importantly also exerted anti-osteoclastogenic effects, as shown in this study.

Although originally described for its role in the vascular system (8), DEL-1 has recently emerged as a homeostatic factor controlling unwanted inflammatory responses (5–7, 9–11). This endothelial cell–secreted 52-kD protein comprises three epidermal growth factor (EGF)–like repeats (E1–E3) at the N terminus followed by two discoidin I–like domains (C1–C2) at the C terminus (fig. S1A), also known as EGF-like repeats and discoidin I–like domain 3 (EDIL3) (8). The second EGF repeat of DEL-1 contains an RGD motif that mediates binding to \( \alpha_v \) integrins (8, 9), whereas its discoidin I–like domains interact with glycosaminoglycans and phosphatidylserine (12, 13). These interactions are functionally important. For instance, DEL-1 contributes to endothelial cell clearance of platelet microparticles by capturing them via phosphatidylserine and anchoring them to \( \alpha_v \beta_3 \) integrin receptors on endothelial cells for uptake (9). Moreover, DEL-1 promotes the efferocytosis of apoptotic cells by acting as a molecular bridge between the \( \alpha_v \beta_3 \) integrin on phagocytes and phosphatidylserine on apoptotic cells (13).

Recently, DEL-1 was shown to bind RGD-independent integrins. Specifically, we have shown that DEL-1 interacts antagonistically with leukocyte \( \beta_3 \) integrins, thereby suppressing inflammatory cell adhesion to intercellular adhesion molecule–1 (ICAM-1) on the endothelium and blocking inflammation in mucosal tissues or the central nervous system (5–7, 14). Accordingly, in animal models of periodontitis or experimental autoimmune encephalitis, DEL-1–deficient mice display uncontrolled lymphocyte function–associated antigen–1 (LFA-1; \( \alpha_v \beta_3 \)) integrin–dependent neutrophil recruitment and inflammatory tissue damage, which is prevented by administration of soluble recombinant DEL-1 (expressed as an Fc fusion protein; DEL-1–Fc) (5, 7). This anti-inflammatory role of DEL-1 is consistent with the identification of the DEL-1–encoding gene EDIL3 as a human disease–susceptibility gene in multiple sclerosis (MS) (15), Alzheimer’s disease (16), and ankylosing spondylitis (17).

Several inflammatory cell types contribute to the destruction of the periodontium, among which neutrophils play a key role through the release of inflammatory mediators and tissue-degrading enzymes (18). Consistent with this, unrestrained neutrophil recruitment to the periodontium of DEL-1–deficient mice leads to spontaneous periodontal inflammation and bone loss (5). Although DEL-1 and earlier established antagonists of LFA-1, such as anti–LFA-1 monoclonal antibody (mAb), exhibited comparable capacities to inhibit neutrophil transmigration, DEL-1 was significantly more effective in inhibiting periodontal bone loss in mice (5). A plausible interpretation of these findings is that DEL-1 might have LFA-1–independent mechanisms to inhibit inflammation-induced bone loss. Here, we have explored this.
possibility and identified DEL-1 as a new homeostatic regulator of osteoclasts—a major cellular player in the pathogenesis of bone loss disorders such as periodontitis—by using both mouse and human osteoclastogenesis systems. Moreover, DEL-1 blocked inflammatory bone loss in a nonhuman primate (NHP) model of periodontitis. These findings pave the way to a new class of endogenous anti-inflammatory and homeostatic therapeutics for the treatment of human periodontitis and other inflammatory bone loss disorders.

RESULTS

DEL-1 expression and regulatory function in osteoclasts

DEL-1 is expressed by endothelial cells in the gingival connective tissue of mice (5). Here, we focused on the expression of DEL-1 in the underlying alveolar bone in mice and detected DEL-1 in areas of osteoclastic activity. DEL-1 colocalized with cathepsin K, an osteoclast-secreted protease involved in bone resorption, and tartrate-resistant acid phosphatase (TRAP)—positive multinucleated cells (Fig. 1A). This finding suggested that osteoclasts might express DEL-1. To test this possibility, we generated receptor activator of nuclear factor κB (NF-κB) ligand (RANKL)–differentiated osteoclasts from bone marrow–derived osteoclast precursor cells (OCPs). In addition to established activation and functional markers [such as NFATc1, the heterodimeric αvβ3 integrin (CD51/CD61), and cathepsin K], RANKL-differentiated osteoclasts expressed Del-1 mRNA at ≈100-fold higher levels than OCPs (Fig. 1B). The generated osteoclasts also expressed DEL-1 protein (Fig. 1, C and D), DEL-1 mRNA and protein expression was shown also for osteoclasts generated from human CD14+ monocytes (Fig. 1E).

Given that DEL-1 is a homeostatic anti-inflammatory factor (5, 7), we hypothesized that DEL-1 could be a novel regulator of osteoclastogenesis. We silenced DEL-1 expression in the RANKL-stimulated murine RAW264.7 macrophages, which are used to model osteoclast differentiation and function. Maximal knockdown of Del-1 mRNA expression by small interfering RNA (siRNA) was seen after 24 hours, although diminished Del-1 mRNA and protein expression persisted for at least 96 hours (fig. S2). Remarkably, Del-1 siRNA–treated macrophages underwent more efficient osteoclastogenesis (higher numbers of TRAP-positive cells (Fig. 2A), consistent with higher mRNA expression of osteoclast markers Nfatc1, αvβ3 integrin, cathepsin K, and Trap (Fig. 2B).

We also noted enhanced resorption pit formation for Del-1 siRNA–treated cells (Fig. 2C). Furthermore, the addition of DEL-1–Fc (DEL-1 expressed as an Fc fusion protein) to murine osteoculture cultures in calcium phosphate [Ca3(PO4)2]–coated plates inhibited resorption pit formation (Fig. 2D); however, this inhibitory action may not necessarily reflect direct DEL-1–Fc inhibition of resorptive activity because the effect could be attributed to decreased osteoclastogenesis. Therefore, in a modified experiment, the differentiation of osteoclasts was first performed in plastic plates in the absence of DEL-1–Fc, and then the mature osteoclasts were transferred to Ca3(PO4)2–coated plates, where they were incubated for 12 hours together with DEL-1–Fc or Fc control. Treatment of already differentiated osteoclasts with DEL-1–Fc, but not Fc control, significantly inhibited resorptive pit formation (Fig. 2D). Therefore, DEL-1 inhibits not only RANKL-induced differentiation toward osteoclasts but also the resorptive function of mature osteoclasts.

To further explore elevated expression of NFATc1 upon knockdown of Del-1, we examined RANKL-stimulated RAW264.7 macrophages containing a luciferase reporter with an NFAT response element. Consistent with the earlier results, these cells displayed increased

---

![Fig. 1. DEL-1 is expressed by human and mouse osteoclasts.](http://stm.sciencemag.org)
luciferase activity upon treatment with Del-1 siRNA as compared with control siRNA or no treatment (Fig. 2E). Because ligation of the Mac-1 integrin (CD11b/CD18) inhibits the induction of NFATc1 expression (19) and DEL-1 binds MAC-1 as recently shown by our group (14), we hypothesized that this may represent a mechanism whereby DEL-1 inhibits NFATc1. Indeed, the ability of DEL-1–Fc to inhibit NFATc1 expression was significantly inhibited upon siRNA-mediated knockdown of Cd11b or Cd18, but not upon knockdown of Cd11a or when the cells were treated with control siRNA (Fig. 2F).

The expression of these β2 integrin subunits was inhibited by 75 to 80% by their corresponding specific siRNA treatments (fig. S3). The inhibition of Nfatc1 expression was associated with CD11b-dependent up-regulation of B cell lymphoma 6 (Bcl6) by DEL-1–Fc (Fig. 2G), consistent with the role of BCL6 as a transcriptional repressor of the NFATc1 gene (19). DEL-1 could also up-regulate BCL6 in human osteoclast precursors comparably to the Mac-1 ligand fibrinogen (Fig. S4).

Moreover, we examined the differentiation and function of primary mouse osteoclasts in the presence of increasing concentrations of DEL-1–Fc. DEL-1–Fc, but not Fc protein control, dose-dependently inhibited the expression of osteoclast differentiation and functional markers NFATc1, αβ3 integrin, cathepsin K, and TRAP (Fig. 3A), osteoclastogenesis (Fig. 3B), and resorption pit formation on Ca3(PO4)2-coated wells (Fig. 3C). In line with these results, OCPs from the bone marrow of Del-1 knockout mice underwent more efficient osteoclastogenesis than did OCPs from wild-type bone marrow (Fig. S5). In RANKL-treated human CD14+ monocytes, DEL-1–Fc dose-dependently inhibited osteoclastogenesis and resorption activity (Fig. 3, D to F).

**Fig. 2.** DEL-1 regulates mouse osteoclast differentiation and function. (A to C) Mouse RAW264.7 macrophages were stimulated with RANKL and treated with control or Del-1–specific siRNA. (A) Enumeration of TRAP-positive MNCs after 3 days. (B) After 3 days, the cells were assayed for the indicated mRNA. Data were normalized to Gapdh mRNA and presented relative to those of undifferentiated RAW264.7 cells (−RANKL), with the average set as 1. (C) Resorptive pit formation (dark spots in representative images) after culture under osteoclastogenic conditions on Ca3(PO4)2-coated wells. Data are relative to the control siRNA–treated group, set as 1. (D) RAW264.7 cells were cultured under osteoclastogenic conditions on Ca3(PO4)2-coated wells in the presence of DEL-1–Fc or Fc control (2 μg/ml). (Right) RAW264.7 cells were differentiated to osteoclasts in plastic plates in the absence of DEL-1–Fc, and the mature osteoclasts were transferred to Ca3(PO4)2-coated plates, where they were incubated for 12 hours together with DEL-1–Fc or Fc control (2 μg/ml). In both experiments, the total resorption area in each culture was measured and expressed relative to the control siRNA–treated group, set as 1. (E) Effect of Del-1 siRNA on NFAT response (luciferase activity) of RANKL-stimulated RAW264.7 cells. (F and G) Effect of DEL-1–Fc (2 μg/ml) on Nfatc1 (F) and Bcl6 (G) mRNA expression in RANKL-induced RAW264.7 osteoclasts that were treated with the indicated siRNA. Bcl6 and Nfatc1 expression were determined at 24 and 12 hours, respectively. Data were normalized to Gapdh mRNA and are relative to undifferentiated controls. All data are means ± SD (A, n = 5; B and E, n = 6; C, n = 5 to 8; D, n = 3; F and G, n = 5). P values were determined by one-way analysis of variance (ANOVA) or unpaired t test (for D). NS, not significant.
Fig. 3. DEL-1 dose-dependently inhibits mouse and human osteoclastogenesis and resorption pit formation. (A to F) RANKL-induced osteoclastogenesis and resorption pit formation were determined in mouse bone marrow–derived OCPs (A to C) and human CD14+ monocytes (D to F) in the presence of increasing concentrations of DEL-1–Fc or Fc control (2 μg/ml). (A and D) Osteoclasts were assayed for mRNA expression of the indicated molecules by RT-PCR. Results were normalized to those of GAPDH mRNA and are presented relative to those of undifferentiated (−RANKL) OCPs, set as 1. (B and E) Cells were stained for TRAP to detect osteoclasts, and TRAP-positive MNCs were counted. Representative images are shown. Scale bars, 200 μm. (C and F) OCPs on Ca3(PO4)2-coated wells were cultured under osteoclastogenic conditions, and resorptive areas (dark spots) were visualized by light microscopy. The total resorbed area in each culture was measured and expressed relative to the Fc control group, set as 1 (left). All data are means ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared to Fc control (one-way ANOVA).
Therefore, DEL-1 has similar regulatory roles in both mouse and human osteoclasts.

**DEL-1 domains and motifs involved in osteoclast formation and function**

Osteoclasts express high levels of $\alpha_{v}\beta_{3}$, which up-regulates their differentiation and resorptive function, whereas $\alpha_{v}\beta_{3}$-blocking antibodies and RGD peptide mimics inhibit osteoclast formation and bone resorption (20). The E2 repeat of DEL-1 contains an RGD motif (fig. S1A) that is critical for binding to the $\alpha_{v}\beta_{3}$ integrin (8). To determine the role of this RGD motif in osteoclastogenesis, we constructed a point mutant in which we substituted Glu [E] for Asp [D] in the RGD motif of DEL-1. This mutant, designated DEL-1[RGE]–Fc, was significantly less potent than the wild-type molecule in terms of inhibiting mouse and human osteoclastogenesis and resorption pit formation (fig. S6). Although these data indicate that the RGD motif is required for the full inhibitory activity of DEL-1 on resorption pit formation, it was not clear whether this merely reflected anti-osteoclastogenic activity or additionally involved direct anti-resorptive effects. The latter was confirmed by showing that DEL-1[RGE]–Fc was significantly less effective than the wild-type molecule in inhibiting resorption pit formation by mature mouse osteoclasts (that were previously differentiated on plastic plates in the presence of RANKL, without DEL-1–Fc or DEL-1[RGE]–Fc) (fig. S7). Nevertheless, DEL-1[RGE]–Fc retained significant anti-osteoclastogenic and anti-resorptive activity compared with the Fc control (figs. S6 and S7).

Thus, besides the RGD, additional components of DEL-1 contribute to its ability to regulate osteoclasts. Accordingly, a deletion mutant lacking the discoidin I–like domains (hence containing only the E1–E3 repeats and designated DEL-1[E1–3]–Fc) lost the ability to regulate osteoclastogenesis and resorption pit formation in the mouse assay system (fig. S6A). However, DEL-1[E1–3]–Fc retained significant inhibitory activity in the human assay system; it was only after the additional removal of the E3 repeat that the activity of the resulting molecule, DEL-1[E1–2]–Fc, was severely diminished (fig. S6B). The fact that the DEL-1[E1–2]–Fc retained only $\approx$15% of the anti-osteoclastogenic activity of the intact/wild-type molecule indicates that the E3 repeat and the discoidin-like domains C1 and C2 contain much of the regulatory activity of DEL-1 in human osteoclastogenesis. In sum, although the RGD motif in the E2 repeat contributes to the osteoclast regulatory activity of DEL-1, additional downstream domains are critical for full activity (C1 and C2 for mouse osteoclasts; E3, C1, and C2 for human osteoclasts).

**DEL-1 components involved in protection against bone loss in mice**

To determine whether the features of DEL-1 involved in osteoclastogenesis can regulate bone loss in vivo, we tested the various DEL-1 constructs for blocking ligature-induced periodontal bone loss in mice. As shown previously (5), wild-type DEL-1–Fc significantly inhibited bone loss compared with Fc control (Fig. 4A). Whereas DEL-1[RGE]–Fc retained almost full inhibitory activity, DEL-1[E1–3]–Fc and DEL-1[E1–2]–Fc completely lost the ability to inhibit bone loss when tested at equivalent molar quantities (12.3 pmol) as 1 µg of intact/wild-type molecule (Fig. 4A). DEL-1[E1–3]–Fc and DEL-1[E1–2]–Fc were tested also at higher doses (2.0 and 2.5 µg, respectively) so that they contained the same amount of DEL-1 protein (0.63 µg) as 1 µg of intact DEL-1–Fc molecule. At these higher doses,
DEL-1[E1–3]–Fc, but not DEL-1[E1–2]–Fc, displayed significant capacity to inhibit bone loss, although it was still less potent than the intact molecule (Fig. 4A). Therefore, consistent with in vitro data, the discoidin I-like domains are required for full capacity of DEL-1 to inhibit bone loss in vivo. The differential capacity of the various constructs to inhibit bone loss was associated with analogous differences in their anti-osteoclastogenic potential in the periodontal tissue; for instance, the numbers of TRAP-positive osteoclasts were lowest in DEL-1–Fc–treated mice and highest in DEL-1[E1–2]–Fc–treated mice (Fig. 4B), which exhibited maximal and no protection from bone loss, respectively (Fig. 4A).

Because DEL-1[E1–3]–Fc can inhibit bone loss in vivo without regulating murine osteoclastogenesis, it is possible that E3 supports (or is required for) anti-inflammatory activity that is not directly related to regulation of osteoclastogenesis. On the basis of the hypothesis that the E3 repeat is associated with an anti-inflammatory activity of DEL-1, and the fact that neutrophil recruitment is a major anti-inflammatory activity of DEL-1 (5, 6), we tested DEL-1[E1–3]–Fc and DEL-1[E1–2]–Fc in transmigration assays. DEL-1[E1–3]–Fc, but not DEL-1[E1–2]–Fc, inhibited neutrophil transmigration (Fig. 4C), implicating the E3 repeat in the regulation of neutrophil recruitment. This finding may account for the ability of DEL-1 [E1–3]–Fc to attenuate bone loss in vivo, a property lacking from DEL-1[E1–2]–Fc. DEL-1[RGE]–Fc displayed inhibitory capacity comparable to wild-type DEL-1 and LFA-1 inhibitors anti-LFA-1 mAb and LFA878 (Fig. 4C), consistent with the dependence of neutrophil transmigration on LFA-1 (an RGD-nonbinding integrin) and also with the strong capacity of DEL-1[RGE]–Fc to inhibit bone loss in vivo.

**Differential capacities of DEL-1 and LFA-1 antagonist to inhibit bone loss**

We have previously shown in a 5-day ligature-induced periodontitis study that most bone loss (~70%) occurs after day 3 (21). Here, neutrophil infiltration peaked at 24 hours and declined thereafter (Fig. 5A). DEL-1–Fc could significantly inhibit ligature-induced bone loss even when its administration started at day 3 (Fig. 5B) —2 days after the peak of neutrophil recruitment. In contrast, LFA878, a potent small-molecule LFA-1 antagonist, significantly inhibited bone loss but only when administered before neutrophil recruitment (from day −1) (Fig. 5B). Whereas both DEL-1–Fc and LFA878 inhibited the expression of inflammatory markers TNF (tumor necrosis factor), IL-1β (interleukin-1β), IL-6, IL-17, and RANKL when administered before neutrophil recruitment, they had no significant effect in this regard when administered late (from day 3) (Fig. 5C). These data suggest that DEL-1 can inhibit bone loss via mechanisms beyond LFA-1–dependent neutrophil recruitment and associated inflammation, consistent with its anti-osteoclastogenic and anti-resorptive properties that depend on interactions with other integrins, such as Mac-1 and αvβ3.

**Inhibition of periodontitis in NHPs by locally administered DEL-1–Fc**

The ability of DEL-1 to regulate both upstream (inflammatory cell recruitment) and downstream (osteoclastogenesis) events in periodontal disease pathogenesis suggests that this homeostatic molecule could find application for the treatment of human periodontitis. As proof of concept, we tested whether human DEL-1–Fc could inhibit inflammatory bone loss in a highly relevant preclinical model of periodontitis. Specifically, we induced ligature-induced periodontitis in the posterior maxillary teeth of cynomolgus monkeys and treated them using a split-mouth experimental design: one side of the maxilla...
(upper jaw) was locally injected in the gingiva with DEL-1–Fc and the other side with Fc control. Thus, each animal served as its own control. Treatments started 3 days after placing the ligatures and continued twice weekly throughout the 6-week-long study.

Treatments with DEL-1–Fc caused a reduction in clinical indices of periodontal inflammation and tissue destruction over the course of 6 weeks (Fig. 6A) and significantly inhibited bone loss compared with Fc control (Fig. 6B). Specifically, whereas all experimental and control sites had similar bone heights [distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC)] at baseline (Fig. 6B, left), the sites treated with DEL-1–Fc exhibited lower bone heights than their corresponding contralateral control sites at the end of the 6-week experimental period (Fig. 6B, middle and right). The inhibition of bone loss by DEL-1–Fc correlated with decreased osteoclast numbers in bone biopsy samples from DEL-1–Fc–treated sites (Fig. 6C).

Multicytokine analysis of collected gingival crevicular fluid (GCF) samples revealed that DEL-1–Fc treatment resulted in significantly lower levels of proinflammatory cytokines, including TNF, IL-1β, IL-17, and RANKL (Fig. 7A). In contrast, osteoprotegerin (OPG), a natural inhibitor of RANKL, was maintained at higher levels in DEL-1–Fc–treated sites than in control sites during the course of the study (Fig. 7A). These findings further support the RANKL/OPG ratio in the GCF as a potential indicator of periodontitis (22).

DEL-1–Fc–mediated inhibition of IL-17, a major bone-resorptive cytokine, and the differential effects of DEL-1–Fc on RANKL and OPG production were confirmed by immunohistochemistry of periodontal biopsy specimens (Fig. 7B). DEL-1–Fc also reduced neutrophil infiltration (elastase staining) in the gingival tissue compared to Fc control treatment (Fig. 7B). RANKL was detected in regions positive for cathepsin K and TRAP, linking it to osteoclastic activity (Fig. 7C). In summary, DEL-1–Fc treatment also decreased bone loss and inflammation in monkeys, correlating with lower levels of proinflammatory cytokines in the GCF and decreased numbers of osteoclasts in bone specimens.

**DISCUSSION**

The modular structure of DEL-1 endows it with functional versatility and homeostatic properties (5–7, 9, 12, 13). DEL-1 appears to prevent inflammatory bone loss by regulating both upstream events related to inflammatory cell recruitment and downstream processes at the level of osteoclastogenesis (fig. S1B). This two-pronged mechanism of action is potentially therapeutically important because local administration of human DEL-1 blocked inflammation and bone loss in an NHP model of periodontitis. These findings could translate to human periodontitis because the immune system and periodontal anatomy of the cynomolgus monkey is very similar to that of humans, and ligature-induced periodontitis in NHPs shares key clinical, microbiological, and immunohistological features with human disease (23).
NFATc1 expression in the periodontal tissue is elevated in periodontitis and is strongly correlated with RANKL expression and with clinical parameters, such as clinical attachment loss (CAL) (24). RANKL-induced expression of NFATc1 is critical for the differentiation and function of osteoclasts (25). However, through its interaction with the Mac-1 integrin (CD11b/CD18) on osteoclasts, DEL-1 upregulated the transcriptional repressor BCL6 and inhibited NFATc1 expression. Acting upstream of osteoclastogenesis, DEL-1 also inhibits periodontal tissue expression of IL-17 (5), which is known to induce RANKL in stromal/osteoblastic cells and thereby stimulate osteoclast differentiation (26). Therefore, our work collectively indicates that DEL-1 can regulate osteoclastogenesis by suppressing both IL-17 production and IL-17–dependent osteoclast activation through induction of RANKL.

DEL-1 inhibited neutrophil transmigration comparably with the LFA-1 antagonist LFA878. However, DEL-1 was more potent than LFA878 in inhibiting bone loss, especially when they were administered late, after the peak of neutrophil infiltration, at which time LFA878 was ineffective. This finding indicates that DEL-1 can inhibit bone loss through mechanism(s) that is independent of the LFA-1 integrin, in line with our demonstration here that DEL-1 uses Mac-1 (CD11b/CD18) but not LFA-1 (CD11a/CD18) to inhibit NFATc1 expression. When administered late, DEL-1–Fc could inhibit bone loss without significantly affecting inflammation, suggesting direct DEL-1 regulation of osteoclasts in vivo.

DEL-1–deficient mice are only mildly osteopenic (5), suggesting that the effects of the deficiency are context-dependent: In a mucosal tissue, such as the periodontium, that is constantly exposed to bacterial challenge, inflammation can lead to enhanced RANKL expression and osteoclastogenesis, which can be exacerbated in the absence of regulatory mechanisms, for example, lack of DEL-1. In contrast, the long bones and the vertebrae are not normally exposed to bacteria and, therefore, they can be protected from excessive osteoclastogenesis even if a specific regulatory mechanism is defective. Thus, although our findings are applicable to periodontal bone loss induced by inflammation associated with mucosal dysbiosis (2), we did not investigate distinct forms of bone loss in the present article, such as osteoporosis. A thorough analysis of osteoporosis and its different types (for instance, osteoporosis due to sex hormone deficiency or steroid-induced osteoporosis) merits detailed investigation in a future study. This could in-
volve further histomorphometric analysis of bones under steady-state conditions or upon sex hormone deficiency, for example, resulting from orchidectomy or ovariectomy; the latter model might be of particular interest because inflammation has been suggested to contribute to bone loss (27).

Although neither DEL-1[E1–2]–Fc nor DEL-1[E1–3]–Fc inhibited in vitro osteoclastogenesis in mice, the latter construct inhibited bone loss in vivo. The E3 repeat is therefore required for some useful activity that is indirectly related to the regulation of osteoclastogenesis. Indeed, we found that the E3 repeat, but not the discoidin I–like domains, is involved in the regulation of neutrophil transmigration. Mechanisms whereby recruited neutrophils could contribute to osteoclastogenesis and bone loss include their ability to express membrane-bound RANKL (28) and release collagenase, which is involved in the initiation of bone resorption.

A recent study questioned the reliability of mice as models of human inflammatory diseases, where gene expression profiling of C57BL/6 mice and humans during endotoxemia revealed poor correlation between the human genes and mouse orthologs, at least for this condition (29). It was therefore important that we demonstrated that Del–1–Fc and DEL–1–Fc could inhibit periodontitis in NHPs—a close model of human disease. DEL-1 is highly homologous among different species (human DEL-1 displays 96% and 99% amino acid sequence identity with its mouse and cynomolgus counterparts, respectively), suggesting that it performs similar functions across species. Furthermore, the expression of DEL-1 under inflammatory conditions appears to be regulated in a similar manner across species. DEL-1 expression is diminished in mice and in patients with periodontitis (5, 30), as well as in the spinal cords of mice with experimental autoimmune encephalitis (the rodent model of MS) and brain tissues from patients with chronic-active MS lesions (but not chronic-inactive) (7). In addition to periodontitis and MS, deficiency or reduced levels of DEL-1 have been associated with acute lung inflammation (6), bleomycin-induced lung fibrosis (10), salivary gland inflammation (31), and systemic inflammation-related adrenal gland dysfunction (32) in mice. Thus, because DEL-1 is an endogenous molecule that is diminished in inflammatory disorders, repleting its levels could therapeutically restore tissue homeostasis.

Approved therapies involving endogenous molecule administration and replacement have increased in recent years. Although replacement of hormones, such as insulin and estrogens, may bear acute or chronic side effects, likely associated with their remote endocrine actions, there are many examples of endogenous molecule replacement, such as digestive enzyme replacement therapy or replacement of α1-antitrypsin in patients with α1-antitrypsin deficiency, which are well tolerated (33, 34). In addition, protein replacement therapies have a higher probability of regulatory approval compared with biologics or small molecules (35). Therefore, local administration to reinitiate the levels of DEL-1 in the periodontium, as performed in this study, is likely to involve only minor if any safety issues. Nevertheless, despite the similarities of human and NHP periodontitis, implementing a DEL-1–based approach in humans is a formidable challenge given the chronicity of the disease. At present, the required frequency of DEL-1 administration to maintain a therapeutic effect is uncertain. If DEL-1 can be formulated in a sustained-release, local delivery system, this would greatly facilitate translation. If a DEL-1–based drug passes preclinical toxicology and safety studies and is proven efficacious in early-phase clinical trials for periodontitis (that is, reducing periodontal inflammation and CAL), further translation would require pharmacokinetic studies to not only identify optimal dosing but also address whether the suggested local administration results in solely local drug availability or there is any systemic spillover. Subsequent long-term multicenter clinical trials could investigate the potential of the DEL-1–based drug to prevent periodontal bone loss compared to the current standard of care (scaling and root planing), whereas in very severe cases of the disease, DEL-1 could be combined with scaling and root planing and compared to periodontal surgery, in an effort to reduce the need for a surgical approach.

In conclusion, we have identified a hitherto unknown homeostatic function of DEL-1, namely, its capacity to directly regulate the differentiation and function of osteoclasts, which requires interactions with different integrins than the one involved in neutrophil transmigration and associated inflammation, LFA-1. Being a functionally versatile molecule that regulates critical upstream and downstream events that lead to inflammation and bone loss, DEL-1 appears to be a promising therapeutic for the treatment of disorders associated with inflammatory bone loss, including periodontitis, rheumatoid arthritis, and ankylosing spondylitis.

MATERIALS AND METHODS

Study design

The objective of this study was to understand the mechanisms whereby DEL-1 regulates osteoclastogenesis and test its capacity to block inflammatory bone loss in an NHP model. All animal procedures were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committees (IACUCs) of the University of Pennsylvania (mouse and NHP studies) and the Covance Research Products (NHP study only), where the NHP work was performed. Oral tissues and fluids and host cells were obtained for gene expression analysis and (immuno)histological and functional studies. Bone loss measurements and immunohistological analysis were performed in a blinded fashion; the conduct of the experiments and assessment of other outcomes were not blinded to the study personnel. Preclinical studies, including intervention experiments with DEL-1–Fc and Fc control, were performed in randomly assigned mice and in NHPs under a split-mouth design. Therefore, each NHP served as its own control, which allowed paired t test analysis and the potential to obtain statistical significant differences with n = 3, according to earlier results using the same model (36). In vivo experiments in mice involved at least five mice per group (determined by GraphPad StatMate power analysis for P = 0.05 and a power of 0.80). No animals (mice or NHPs) were withdrawn from the studies according to predetermined criteria in the IACUC protocols or were excluded from any of the analyses. All in vitro and in vivo experiments were performed two or more times for verification, except for the in vivo NHP study, which was performed once.

Animals

Mice. Mice were maintained in individually ventilated cages and provided sterile food and water ad libitum under specific pathogen-free conditions. They were used for experiments when they were 8 to 10 weeks old. The generation of C57BL/6–Edil3KO (DEL-1KO) mice...
has been described (6). Wild-type C57BL/6J mice were purchased from the Jackson Laboratory.

NHPs. Adult female cynomolgus monkeys (Macaca fascicularis) (3 to 7 years old; 4 to 7 kg) were purchased from an approved vendor from stocks that are bred in captivity and were used after a 7-day acclimation period. The animals were socially housed in steel cages elevated off the floor in a controlled environment with a temperature of 64° to 84°F and a light/dark cycle of 12:12 hours. Environmental enrichment was provided through daily handling by animal care technicians, environmental enrichment items, visual contact with other animals, and appropriate background music. Each animal was offered a measured amount of an approved feed mixture, and fresh, potable drinking water was available ad libitum. The animals were not euthanized at the completion of the study.

Ligature-induced periodontitis in mice

A 5-0 silk ligature was tied around the maxillary left second molar, and the mice were euthanized 5 days after placement of the ligatures (37). The contralateral molar tooth in each mouse was left unligated (baseline control for bone loss measurements). Periodontal bone loss was assessed morphometrically in defleshed maxillae using a dissecting microscope (40×) fitted with a video image measurement system (Nikon Instruments). Specifically, the CEJ-ABC distance was measured on six predetermined points on the ligated second molar and the affected adjacent regions (37). Bone loss was calculated by subtracting the six-site total CEJ-ABC distance for the ligated side of each mouse from the six-site total CEJ-ABC distance of the contralateral unligated side. Negative values (in mm) indicated bone loss relative to the baseline (unligated control). In intervention experiments, DEL-1–Fc and mutants thereof, or the LFA-1 small-molecule antagonist LFA878 (Novartis), were microinjected into the palatal gingiva of the ligated second maxillary molar (5, 38).

Clinical examinations, periodontitis, and sample collection in NHPs

Three adult cynomolgus monkeys were used for local (intragingival) administration of DEL-1–Fc or Fc control. All treatments and clinical examinations were performed on anesthetized animals. Experimental periodontitis was induced by tying size 2.0 silk ligatures around posterior teeth (maxillary second premolars and first molars). Ligatures were placed on both halves of the maxilla for a split-mouth experimental design: one side was treated with DEL-1–Fc and the other with Fc control.

Clinical examinations with periodontal probe and diagnosis were performed according to the criteria of the American Academy of Periodontology for human periodontitis (39). CAL, probing pocket depth (PPD), and bleeding on probing (BOP) were measured at six sites: mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual aspects of each of the premolar and molar maxillary teeth. Gingival index (GI) was assessed at four sites (mesio-buccal, mid-buccal, disto-buccal, and mid-lingual). GI and BOP are measures of periodontal inflammation, whereas CAL and PPD assess tissue destruction. Another index used, the tooth mobility index is often associated with bone loss (39). At the beginning of the study, the gingival margins in all animals were at the CEJ, and hence, the CAL readings equaled PPD (PPD therefore was not shown in Results). GCF was collected using PerioPaper strips (Oraflow) placed between the gums and the teeth, in the mesio-buccal sulcus of each ligated posterior tooth, for 30 s. At baseline and at the completion of the study (6 weeks), standardized bitewing dental x-ray images were taken using high-speed dental x-ray films to evaluate bone loss, as described in the Supplementary Methods.

Therapeutic treatments were performed two times per week, starting 3 days after study initiation. With a 30-gauge short needle, DEL-1–Fc was injected into the interdental papillae from the first premolar to the second molar (three sites; 50 μg per site in a volume of 50 μl) on one side of the mouth. On the contralateral side (control), an equal amount and volume of Fc control was injected in a similar manner. At the completion of the study, the ligatures were removed, and biopsies of gingiva and bone were removed en bloc corresponding to the first molar tooth at the ligated sides.

Osteoclastogenesis

RANKL-induced osteoclastogenesis was performed according to standard protocols using mouse bone marrow–derived monocyte/macrophage precursor cells (40), mouse RAW264.7 precursor cells (41), or human CD14+ monocytes (19), as described in the Supplementary Methods.

Statistical analysis

Data were evaluated by ANOVA and the Dunnett’s multiple-comparison test using the InStat program (GraphPad). Where appropriate (comparison of two groups only), two-tailed paired or unpaired t tests were performed. P < 0.05 was the level of significance. For clarity of presentation, data that do not involve measurements in established units, such as resorption area, mRNA expression, and luciferase activity, were expressed and presented as “relative to” corresponding controls; this approach did not affect the results of the statistical analysis.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/7/307/307ra155/DC1

Methods

Fig. S1. Linear structure and functions of the DEL-1 protein.
Fig. S2. Inhibition of DEL-1 expression by siRNA treatment.
Fig. S3. Integrin expression is inhibited by siRNA treatment.
Fig. S4. DEL-1 up-regulates BCL6 in human osteoclast precursors undergoing RANKL-induced differentiation.
Fig. S5. DEL-1 deficiency enhances osteoclastogenesis.
Fig. S6. Features of DEL-1 involved in regulation of osteoclastogenesis and resorption pit formation.
Fig. S7. The RGD motif of DEL-1 is involved in its ability to inhibit the resorptive activity of mature osteoclasts.

REFERENCES AND NOTES

integins: Role in leukocyte recruitment and as therapeutic targets in inflammatory disease. 30. J. Shin, K. B. Hosur, K. Pyaram, R. Jotwani, S. Liang, T. Chavakis, G. Hajishengallis, Expression

Acknowledgments: We thank B. Wang for comments and H. Li for advice with statistical analysis. Funding: Supported by grants from NIH/National Institute of Dental and Craniofacial Research/ National Institute of Allergy and Infectious Diseases (DE015254, DE021685, DE024716, and AI068730) to G.H., the Deutsche Forschungsgemeinschaft (CH279/5-1 and SFB-TR127 Project-A3), the Excellence Initiative by the German Federal and State Governments (Institutional Strategy, measure “support the best of Technische Universität Dresden”, and the European Research Council (ENDOMRIT) to T.C. Author contributions: J.S. established functional assays, performed experiments and statistical analysis, and interpreted data. T.M. performed animal studies, knockdown experiments, statistical analysis, and data interpretation. T.A. and E.H. performed animal experiments and host response analyses. K.H. and K.P. engineered molecular constructs and purified proteins. T.M. performed experiments and interpreted data. T.C. designed experiments, interpreted data, and co-edited the paper. G.H. conceived, designed, and supervised experiments, interpreted data, and co-edited the paper. G.H. conceived, designed, and supervised experiments, interpreted data, and co-edited the paper. T.C. designed experiments, interpreted data, and co-edited the paper.

Submitted 16 February 2015 Accepted 12 August 2015 Published 30 September 2015 10.1126/scitranslmed.aac5380

DEL-1 restrains osteoclastogenesis and inhibits inflammatory bone loss in nonhuman primates

Jieun Shin, Tomoki Maekawa, Toshiharu Abe, Evlambia Hajishengallis, Kavita Hosur, Kalyani Pyaram, Ioannis Mitroulis, Triantafyllos Chavakis and George Hajishengallis

DOI: 10.1126/scitranslmed.aac5380

DELivering new therapies for chronic inflammation

Chronic inflammation is prevalent in nearly half of adult teeth and gums in the U.S. population, and this so-called periodontitis can increase a patient’s risk of developing other inflammatory diseases in the heart (atherosclerosis) and joints (rheumatoid arthritis). Shin et al. capitalized on the natural anti-inflammatory activity of the protein DEL-1, finding that it not only blocked excessive immune cell infiltration into the periodontium but also had innate anti-osteoclastogenic activity; that is, it stopped bone loss by interrupting the signaling pathways to osteoclasts, the bone-resorbing cells. In vitro, in human and mice osteoclast precursor cells, DEL-1 prevented osteoclast differentiation by inhibiting NFATc1 activity. In vivo, in mouse and nonhuman primate models of periodontitis, giving DEL-1 locally reduced inflammation and tissue destruction, thus halting any tissue loss. The mechanism appears to be two-pronged: working “upstream” in disease signaling pathways to prevent inflammatory cell recruitment to the teeth and gums, as well as acting “downstream” to stop osteoclastogenesis. With data in a monkey model that represents the human disease, anatomy, and immune system closely, it is likely that DEL-1–based therapeutics could translate soon once safety of this endogenous molecule is confirmed.