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Treponema denticola chymotrypsin-like proteinase may contribute to orodigestive carcinogenesis through immunomodulation

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Background: Periodontal pathogens have been linked to oral and gastrointestinal (orodigestive) carcinogenesis. However, the exact mechanisms remain unknown. *Treponema denticola* (*Td*) is associated with severe periodontitis, a chronic inflammatory disease leading to tooth loss. The anaerobic spirochete *Td* is an invasive bacteria due to its major virulence factor chymotrypsin-like proteinase. Here we aimed to investigate the presence of *Td* chymotrypsin-like proteinase (*Td*-CTLP) in major orodigestive tumours and to elucidate potential mechanisms for *Td* to contribute to carcinogenesis.

Methods: The presence of *Td*-CTLP within orodigestive tumour tissues was examined using immunohistochemistry. Oral, tonsillar, and oesophageal squamous cell carcinomas, alongside gastric, pancreatic, and colon adenocarcinomas were stained with a *Td*-CTLP-specific antibody. Gingival tissue from periodontitis patients served as positive controls. SDS–PAGE and immunoblot were used to analyse the immumodulatory activity of *Td*-CTLP *in vitro*.

Results: *Td*-CTLP was present in majority of orodigestive tumour samples. *Td*-CTLP was found to convert pro MMP-8 and -9 into their active forms. In addition, *Td*-CTLP was able to degrade the proteinase inhibitors TIMP-1, TIMP-2, and α-1-antichymotrypsin, as well as complement C1q.

Conclusions: Because of its presence within tumours and regulatory activity on proteins critical for the regulation of tumour microenvironment and inflammation, the *Td*-CTLP may contribute to orodigestive carcinogenesis.

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Over the past few decades, a paradigm shift has been made in understanding the key role of the microbiome in health and disease. Discovery of the causative role of *Helicobacter pylori* in gastric cancer has been one of the driving factors in this area. It has been estimated that 20% of all cancers have a microbial aetiology (Parkin, 2006). Accumulating evidence has shown that microbes have a more potent role in gastrointestinal (GI) carcinogenesis than previously thought. Various members of the oral microbiota have been reported to be linked to cancers of the GI tract (Fitzpatrick & Katz, 2010; Ahn *et al*, 2012a, b).

In the oral microbiome, the so-called periodontopathogens, including *Fusobacterium nucleatum* (*Fn*), *Porphyromonas gingivalis* (*Pg*), *Treponema denticola* (*Td*), *Aggregatibacter actinomycetemcomitans* (*Aa*), and *Tannerella forsythia* (*Tf*) have been receiving increasing interest in the context of cancer aetiology. Periodontal microbes have a major role in the pathogenesis of periodontitis, chronic inflammatory disease that causes destruction of the tooth-supporting tissue and ultimately tooth loss. Novel epidemiological studies have linked periodontal pathogens to pancreatic and oral cancers (Ahn *et al*, 2012a; Michaud, 2013). Also, it has been reported that *Fn* can promote colorectal carcinogenesis (Rubinstein *et al*, 2013). Both *in vitro* and *in vivo* studies have suggested that *Fn* and *Pg* participate in oral carcinogenesis (Groeger *et al*, 2011; Gallimidi *et al*, 2015). Oral bacteria may contribute to carcinogenesis via different mechanisms such as inhibition of apoptosis, activation of cell proliferation, promotion of cellular invasion, induction of chronic inflammation, and production of carcinogens (Gallimidi *et al*, 2015). Bacterial pathogens have developed various mechanisms that can promote their ability to colonise the GI tract: strong adhesive properties; immune evasion; disruption of epithelial integrity; and intracellular survival (Ribet & Cossart, 2015).

Within the oral microbiome, *Td* is the most well-characterised and frequently isolated spirochete (Dashper *et al*, 2011). In addition to its association with periodontal disease, *Td* has been reported to contribute to the formation of dental abscesses and root canal infections. It is a highly invasive and motile organism, and an obligate anaerobe. One of the key virulence factors of *Td* is a cell surface-bound chymotrypsin-like proteinase (CTLP), also known as dentilisin, which has a plethora of functions. It can degrade multiple host proteins, hydrolyse bioactive peptides, contribute to *Td* penetration into the epithelium, and enhance integration of *Td* into biofilm communities (Grenier *et al*, 1990). In addition, *Td*-CTLP can modulate immunity and inflammation, and drive apoptosis in various cell types (Uitto *et al*, 1995; Cogoni *et al*, 2012; Fenno, 2012). However, results reporting the association of *Td* with malignancies are very limited and only one study has reported the presence of *Td* in oesophageal tumours (Narikiyo *et al*, 2004).

Host-derived matrix metalloproteinases (MMPs) have a critical role in inflammatory processes and are capable of degrading almost all extracellular proteins. MMPs are secreted by inflammatory cells, in concert with endothelial, epithelial, and smooth muscle cells. They facilitate leukocyte navigation through the extracellular matrix (ECM) and modify immune responses by processing non-matrix bioactive substrates (Salminen *et al*, 2015). The key regulators of MMP activity are tissue inhibitors of MMPs (TIMPs). There is a broad evidence highlighting the importance of MMPs and TIMPs in the regulation of tumour microenvironment and in the metastasis of GI cancers (Jiang *et al*, 2002; Verma *et al*, 2014).

In addition, other protease inhibitors are linked to carcinogenesis. Alpha-1-antichymotrypsin (α-1-AC) is an acute-phase protein belonging to the serpin superfamily of protease inhibitors with activity against various proteases such as neutrophil elastase and cathepsin G. It has critical functions in tissue homeostasis and its deficiency leads to chronic inflammation, which is associated with several types of cancer, including gastric and colorectal cancers (Kittas *et al*, 1982; Karashima *et al*, 1990; Sun & Yang, 2004). The complement system has been known as the pivotal arm of the innate immunity. In addition, being able to activate of the classical pathway, complement C1q is known to be expressed in several cancer tissues, including colorectal and pancreatic cancers (Bulla *et al*, 2016).

In this study, we examined the presence of *Td*-CTLP in major oral and GI (orodigestive) tumours *in vivo* using immunohistochemistry. In addition, we also investigated the ability of *Td*-CTLP to modulate immunomodulatory proteins, including MMPs, TIMPs, α-1-AC, and C1q *in vitro*. The association of *Td*-CTLP with orodigestive tumours has not been studied previously. We hypothesised that CTLP is present in GI tract tumour tissue and that it contributes to tumour progression is due its activity against various immunomodulatory proteins critical for the regulation of tumour microenvironment and inflammation.

MATERIALS AND METHODS

In vivo evaluation of the presence of *Td*-CTLP in cancer tissues

Antibody preparation. The CTLP antibody was prepared accordingly (Grenier *et al*, 1990). The animal experiments were approved by the experimentation ethics committee of Université Laval (license no. 94-190). Briefly, the purified CTLP (15 μg) was injected intramuscularly into a New Zealand White rabbit with complete Freund adjuvant. Subsequent intramuscular injections, without adjuvant, were done on days 8, 14, 22, 36, and 50. The rabbit was bled via the marginal ear vein on day 57. The purified immunoglobulin G (IgG) fraction was prepared by passing the antiserum through a column of protein A-Sepharose CL 4B (Sigma Chemical Co., St Louis, MO, USA). The sample was exhaustively washed on the column with 0.1 M borate–0.5 M NaCl buffer (pH 8.4). Immunoglobulin G was then eluted with 0.1 M glycine–0.5 M NaCl buffer (pH 2.5), followed by dialysis against 50 mM phosphate-buffered saline (pH 7.2). The anti-CTLP IgG fraction did not react with *Treponema vincentii*, an oral spirochete closely related to *Td*, in immunofluorescence and ELISA assays indicating high specificity of the antibody.

Immunohistochemical analysis. A total of 149 orodigestive tumour tissue samples were obtained from the archives of Department of Pathology, Helsinki University Hospital. The study design complied with the Declaration of Helsinki and was approved by the Ethical Committee of Helsinki University Hospital. Tissue samples comprised squamous cell carcinomas (SCCs) of tongue ($n=29$), tonsil ($n=25$), oesophagus ($n=3$), and adenocarcinoma of stomach ($n=32$), pancreas ($n=6$), and colon ($n=54$). All samples were formalin-fixed and paraffin-embedded. Gingival tissue sample from patients with periodontitis verified positive for *Td* by PCR were included as positive control (Marttila *et al*, 2014). Mammae ductal carcinoma ($n=1$), SCC of the lung ($n=1$), and various types of thyroid tumour tissues ($n=28$) were used for comparison as non-orodigestive tumour tissues.

Tissue sections were prepared according to standard protocol (Marttila *et al*, 2014). Antigens were retrieved in citrate buffer using microwaves (MicroMED T T Mega Histoprocessing Labstation; Milestone Srl, Sorisole, Italy). Immunohistochemical stainings were performed using primary rabbit polyclonal antibody (1:1500 dilution) against *Td*-CTLP. The tissue sections were then incubated with 1:200 dilution of corresponding biotinylated anti-rabbit secondary antibody from the kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) and further incubated with avidin–biotin–peroxidase complex. 3-amino-9-ethylcarbazole with 0.03% hydrogen peroxidase was used to reveal peroxidase-binding site and counterstained with Mayer's haematoxylin. Negative

control stainings were performed with non-immune species-specific Rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and by omitting the primary antibody. The samples were examined and images were taken with Leica DM6000 light microscope connected to a digital camera (DFC420 and DFC365FX; Leica Microsystems, Wetzlar, Germany).

In vitro analysis of *Td*-CTLP activity

Purification of Td-CTLP and proMMPs. The purification of *Td*-CTLP was done as previously described (Uitto *et al,* 1988; Grenier *et al,* 1990). The CTLP was isolated from a sonicated cell extract of *Td* 35405. The purity of the protease was verified by silver staining and proteolytic activity detection on SDS–PAGE, which showed only a single band with an apparent molecular mass of 95 kDa. The protease showed a high specificity for the synthetic chromogenic peptide succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide, was activated by reducing agents, and was inhibited by phenylmethylsulfonyl fluoride and sulfhydryl group reagents, which was consistent with the characteristics previously.

The purification of proMMP-8 and proMMP-9 was done as previously described (Sorsa *et al*, 1992, 1997). Briefly, supernatant from stimulated human neutrophils was subjected to gel filtration ion-exchange chromatography followed by affinity chromatography on heat-denatured type I collagen (gelatin) coupled to Sepharose for proMMP-9 and Cibacron-Blue Sepharose for proMMP-8. The purified proMMP-8 and proMMP-9 were latent and displayed no enzymatic activities.

Degradation of immunomodulatory components by Td-CTLP. Native soluble type I and type II collagen (for MMP-8 and *Td*-CTLP) as well as gelatin-zymography (for MMP-9 and *Td*-CTLP) degradation assays were performed as previously described (Sorsa *et al*, 1992, 1997). Native soluble 1.5 μM type I and II collagens were purified and characterised by CNBr-cleavage analysis as previously described (Sorsa *et al,* 1988). A unit of 400 ng of 75 kDa proMMP-8, 400 ng of 92 kDa proMMP-9, 400 ng of 28 kDa human recombinant TIMP-1 (R&D System, Oxon, UK), 400 ng of 21 kDa human recombinant TIMP-2 (R&D System), 400 ng α1-AC (Sigma-Aldrich, St Louis, MO, USA), and 400 ng C1q (Abcam, Cambridge, UK) were incubated with 80 ng of purified *Td*-CTLP for various time periods extending from 0 to 60 min at 37 °C. As positive control, proMMP-8 and -9 were also incubated for 60 min (37 °C) with 1 mM organomercurial activator aminophenylmercuric acetate (APMA; Sigma-Aldrich) and 25 ng human tumour-associated trypsin-2. Human tumour-associated trypsin-2 was purified from serum-free conditioned medium of COLO-205 human colon adenocarcinoma cells (American Type Culture Collection, CCL-222, Rockville, MD, USA) (Sorsa *et al*, 1997). After the incubations, the samples were removed, boiled for 5 min in Laemmli's sample buffer and run on SDS–PAGE (Coomassie Brilliant Blue staining) and western immunoblotted to analyse the conversions and fragmentations. Gelatin-zymography and sample preparations for it were performed as described previously (Sorsa *et al,* 1997).

RESULTS

Localisation of *Td*-CTLP in orodigestive cancer tissues *in vivo*. To study the presence of *Td*-CTLP in malignant tumours, various cancer specimens were stained by immunohistochemistry. *Td*-CTLP was detected in all types of orodigestive cancer tissues, including SCCs of tongue, tonsil, and oesophagus (Figure 1A–C). The treponemal proteinase was also detected in adenocarcinomas of the stomach, pancreas, and colon (Figure 1D–F). However, it was not detected in every sample as described by the number of positive samples in Figure 1. Immunoreactivity for *Td*-CTLP was mainly detected in granular deposits in epithelial cell cytoplasm within tumours. In addition, it was detected in some normal tonsillar epithelial cells. Some mucus secretory cells such as goblet cells, acinar cells, and ductal cells contained *Td*-CTLP as well. Mononuclear inflammatory cells also showed weak positivity in some specimens of all cancer types (data not shown).

As expected, the staining results on *Td*-positive gingival tissue affected by periodontitis showed positivity for *Td*-CTLP and negativity in non-orodigestive tumours, including breast and lung cancer tissue (data not shown). For thyroid, limit and weak expression was found in one follicular thyroid adenoma and one follicular thyroid carcinoma, but negative in the rest, including papillary thyroid carcinoma, medullary thyroid carcinoma, and hyperplastic thyroid tissue (data not shown). Negative controls, including stainings with IgG antibody and without primary antibody showed no positivity (data not shown).

Activity of *Td*-CTLP *in vitro*. To elucidate mechanisms in which way the *Td*-CTLP may affect tumour growth, some key proteins connected to the regulation of the proteinase activities of the tumours were incubated in the presence of the *Td*-CTLP (Figures 2–4). *Treponema denticola*-CTLP converted time-dependently the 75 kDa proMMP-8 to 60 kDa active forms of MMP-8 within 20–60 min as revealed by SDS–PAGE analysis (Figure 2A). The conversion of 92 kDa proMMP-9 to 80–82 kDa active form of MMP-9 by *Td*-CTLP also occurred in 20–60 min (Figure 2B). Corresponding conversions of proMMP-8 and -9 to their active counterparts were also obtained by 1 mM APMA and human tumour-associated trypsin-2, used as positive controls (lane 2 in Figure 2A and B, respectively). Furthermore, *Td*-CTLP fragmented 28 kDa TIMP-1 and 21 kDa TIMP-2 to small peptides and lower-molecular-weight fragment as analysed by SDS–PAGE (Figure 2C and D, respectively). The proMMP conversions analysed by SDS–PAGE were further confirmed by western immunoblotting (Figure 3A and B). *Treponema denticola*-CTLP also efficiently fragmented 65 kDa α-1-AC (Figure 4A) and 68 kDa C1q (Figure 4B) to smaller peptides.

Td-CTLP did not degrade native type I and II collagens (Figure 5A, lanes 4 and 8, respectively). MMP-8 alone degraded to some extent type I and II collagens (Figure 5A, lanes 3 and 6, respectively). MMP-8 treated by *Td*-CTLP for 60 min prior incubation with type I and II collagen resulted to significantly enhanced degradation of both these collagens, preferably type II collagen (Figure 5A, lanes 3 and 7, respectively). Type I gelatin-zymographic analysis revealed that *Td*-CTLP is a gelatinolytic 80–95 kDa protease (Figure 5B, lane 1) and time-dependently converted gelatinolytic 92 kDa pro MMP-9 (Figure 5B, lane 2) to active 82 kDa form (Figure 5B, lanes 3–5, indicated by an arrow).

DISCUSSION

In this study, we showed for the first time that the CTLP, a key virulence factor specific to the oral spirochete *Treponema denticola (Td)*, is present in several orodigestive tumours. In addition, our *in vitro* experiments provide evidence that *Td*-CTLP shows immunomodulatory activity that can have a crucial role in promoting and regulating carcinogenesis. *Td* is known to be a key pathogen in periodontitis, a chronic inflammatory disease of the tooth supporting connective tissue caused by the dysbiotic microbiome. Previous studies regarding *Td*-CTLP have focused on its potential role in the pathogenesis of periodontitis. However, the role of the treponemal proteinase in carcinogenesis has not been considered before. Recent studies have suggested a link between periodontitis and oral cancer (Gholizadeh *et al*, 2016). The current knowledge on the role of *Td* in chronic inflammation, and its

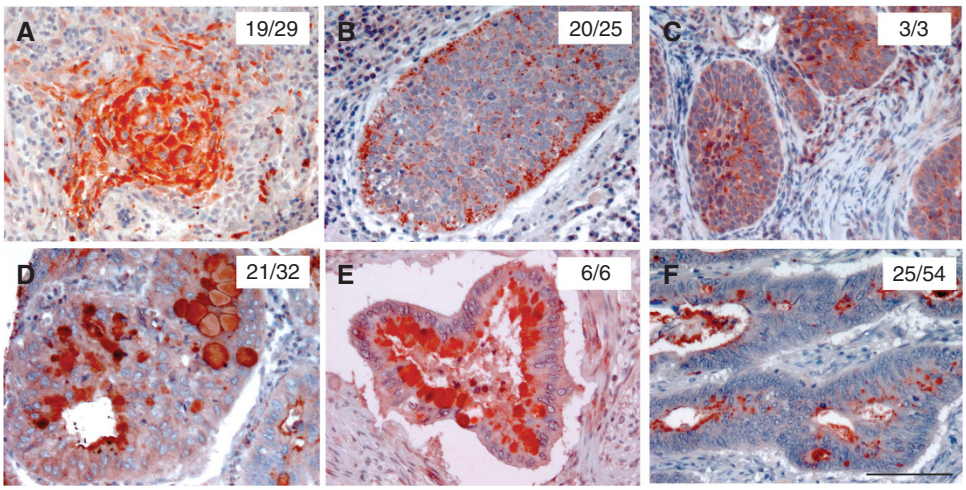

Figure 1. The presence of *Td*-CTLP in orodigestive tumour tissues. Immunohistochemical stainings of the *Td*-CTLP in tongue (**A**), tonsillar (**B**), oesophageal (**C**), gastric (**D**), pancreatic (**E**), and colon cancer (**F**) tissues. Number of positive tissue samples per total sample size for each tumour type shown. 3-amino-9-ethylcarbazole (AEC) was used as chromogen (red). All red-stained areas on each tissue section indicate specific detection of *Td*-CTLP. Scale bar is 100 μm, relevant for all panels.

highly invasive and immunomodulatory nature via its powerful proteolytic machinery, urged us to investigate whether this spirochete with the aid of its proteolytic enzyme has a part in the aetiology of cancer.

Currently, there is very limited data available about the presence of *Td* in malignant tumours. Narikiyo *et al*, using culture independent-methods, reported frequent presence of *Td* together with oral streptococci within oesophageal tumours obtained from several centres globally. *Td* was more frequently isolated from tumour than non-tumour sites. In our study, *Td*-CTLP was also present in oesophageal tumour samples, and interestingly in the majority of other orodigestive tumours as well.

Td can be isolated from the oral cavity of healthy individuals, but in disease, the number of *Td* has been shown to increase substantially alongside other periodontal pathogens (Dashper *et al*, 2011). The presence of *Td*-CTLP within orodigestive tumours implicates that mucosal exposure along the GI tract to *Td* is due to ingestion of the spirochete with food and saliva. In our study, this implication is partly contradicted by the discovery of *Td*-CTLP in pancreatic cancer. This can be explained by the fact that the pancreas is connected to the bowel by its duct. It is also possible that bacteria reach the pancreas via blood circulation. To accompany the latter, *Td* has been detected in carotid and aortic atheromatous plaques in patients with chronic periodontitis (Cavrini *et al*, 2005).

We have previously investigated the localisation of *Td* and its CTLP in oral gingival tissues affected by periodontitis (Marttila *et al*, 2014). The localisation of *Td*-CTLP correlated well with the presence of *Td* bacteria, and the CTLP was detected in all layers from mucosal biofilm all the way through the epithelium to the connective tissue, thus indicating the invasiveness of this organism. Similar to our previous study, *Td*-CTLP was mainly localised intracellularly within tumour cells in the current study. *Td*-CTLP is known to facilitate binding of the spirochete to other bacterial counterparts in polymicrobial biofilms and *Td* has been found to coincide in the tissues with *Pg,* a periodontal pathogen that has been associated with various types of orodigestive malignancies such as colorectal, pancreatic, and oral cancers (Nagy *et al*, 1998; Ahn *et al*, 2012a; Michaud *et al*, 2013).

In cancer progression and proliferation proteolytic activity is a crucial factor, mainly carried out by MMPs. *In vitro*, *Td*-CTLP converted proMMP-8 and -9 to their active forms as demonstrated by western blots and type I and II collagen and gelatin-zymographic MMP-activity assays. In fact, the *Td*-CTLP-activated MMP-8 degraded preferentially native soluble type II collagen relative to type I collagen, which is according to recorded substrate specificity of MMP-8 (Hasty *et al*, 1987; Sorsa *et al*, 1988). The MMPs regulate tumour cell tissue invasion and extravasation, angiogenesis and inflammation, and thus have a major impact on tumour microenvironment (Kessenbrock *et al*, 2010). Elevated levels of MMP-9 have been associated with poor prognosis and metastasis in tongue, oesophageal, gastric, pancreatic, and colorectal cancers (Zeng *et al*, 2013; Aparna *et al*, 2015; Jakubowska *et al*, 2016). In periodontitis, the presence of *Td* is associated with increased levels of MMP-8 and -9 (Yakob *et al*, 2013). *Td* is known to trigger MMP-8 and -9 expression and secretion by inflammatory cells *in vitro* (Ding *et al*, 1996; Choi *et al*, 2003). Interestingly, there has been controversy over the effect of MMP-8 in cancer. Matrix metalloproteinase-8 is overexpressed in oropharyngeal cancers and moreover its expression is associated with poor prognosis in ovarian and bladder cancer (Moilanen *et al*, 2002; Stadlmann *et al*, 2003; Kader *et al*, 2006). In addition, MMP-8 overexpression also exhibits tumour- and metastasis-suppressing roles and associates with improved survival in tongue and breast cancers (Gutierrez-Fernandez *et al*, 2008; Korpi *et al*, 2008). In addition to ECM modulation, MMP-8 and -9 show potent immunomodulatory activity in tumour tissues (Kessenbrock *et al*, 2010).

Tissue inhibitors of MMPs act on tumour status primarily through inhibition of MMPs. In addition, MMP-independent effects of TIMPs on cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis, and synaptic plasticity have been reported (Brew & Nagase, 2010). TIMP-1 and -2 are widely expressed (Jackson *et al*, 2016). TIMP-1 can promote tumour progression, angiogenesis, inhibit apoptosis, and it is associated with poor survival. TIMP-2 is primarily known for its inhibitory activity on MMP-2, but it also has MMP-independent activities. In the current study, TIMP-1 and -2 were degraded by *Td*-CTLP to inactive forms. Thus, by acting on TIMPs, *Td*-CTLP could exert effect on MMP activity and inhibit MMP-independent activities of TIMPs within the tumour site contributing carcinogenesis.

In our study, we examined two other inflammation related factors, acute phase protein α-1-AC and complement component C1q. *Td*-CTLP degraded both proteins. Expression of α-1-AC has been reported in pancreatic and gastric cancers (Allgayer *et al*, 1998; Koomen *et al*, 2005). A recent report shows that C1q is

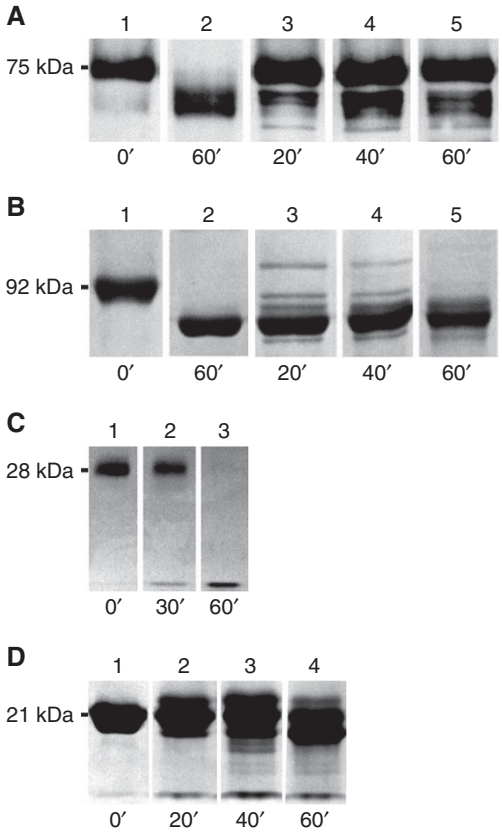

Figure 2. SDS–PAGE of fragmentation of pro MMP-8 and -9 and TIMP-1 and -2 by *Td*-CTLP. (**A**) Lane 1: proMMP-8 incubated with buffer; lane 2: proMMP-8 + APMA (organomercurial MMP-8 activator) indicates that proMMP-8 was completely activated by APMA after 60 min; lanes 3–5: *Td*-CTLP activated proMMP-8 during 20, 40, and 60 min incubations. (**B**) Lane 1: proMMP-9 incubated with buffer; lane 2: proMMP-9 + APMA (organomercurial MMP-8 activator) indicates that proMMP-9 was completely activated by APMA after 60 min; lanes 3–5: *Td*-CTLP activated proMMP-9 during 20, 40, and 60 min incubations. (**C**) Lane 1: TIMP-1 incubated with buffer; lanes 2 and 3: TIMP-1 incubated with *Td*-CTLP indicates that TIMP-1 was degraded to smaller fragments after 60 min. (**D**) Lane 1: TIMP-2 incubated with buffer; lanes 2–4: TIMP-2 incubated with *Td*-CTLP indicates that TIMP-2 was degraded to smaller fragments after 60 min.

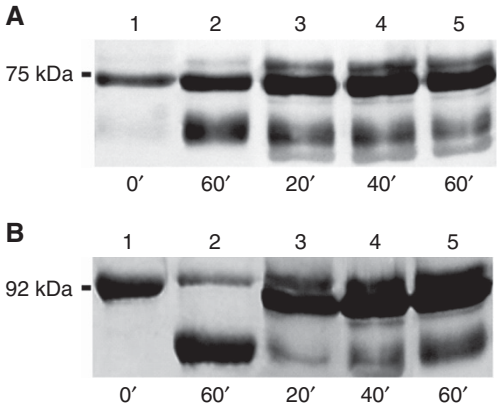

Figure 3. Western immunoblot analysis of conversion of pro MMP-8 and -9 by *Td*-CTLP. (**A**) Lane 1: pro MMP-8 incubated with buffer; lane 2: pro MMP-8 incubated with tumour-associated trypsin-2; lanes 3–5: pro MMP-8 incubated with *Td*-CTLP for 20, 40, and 60 min. (**B**) Lane 1: pro MMP-9 incubated with buffer; lane 2: pro MMP-9 incubated with tumour-associated trypsin-2; lanes 3–5: pro MMP-9 incubated with *Td*-CTLP for 20, 40, and 60 min, respectively. *Treponema denticola*-CTLP and tumour-associated trypsin-2 converted pro MMP-8 and -9 to their active forms.

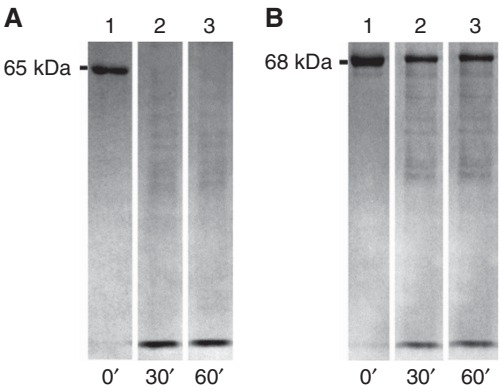

Figure 4. SDS–PAGE of fragmentation of immunomodulatory proteins by *Td*-CTLP. (**A**) Lane 1: α-1-AC incubated with buffer; lanes 2 and 3: α-1-AC incubated with *Td*-CTLP indicated that *Td*-CTLP completely degraded α-1-AC in 30 and 60 min. (**B**) Lane 1: C1q incubated with buffer; lanes 2 and 3: C1q incubated with *Td*-CTLP indicated that *Td*-CTLP degraded C1q in 30 and 60 min incubations.

present in GI cancers and that locally synthesised C1q can promote cancer cell adhesion, migration, and proliferation *in vivo* (Bulla *et al*, 2016).

The strength of the study is the use of a highly specific polyclonal antibody against *Td*-CTLP. *In vivo* localisation of the enzyme in various orodigestive tumours provide a potential insight into its biological effects. The lack of functional analysis of CTLP in tumours is a limitation of the study. In addition, the proteins investigated *in vitro* were not stained alongside CTLP in tumour sites, but as discussed here plentiful of reports highlight their presence and role within orodigestive tumours. The enzyme–substrate ratios used *in vitro* were based on our previous studies with microbial proteases and MMPs, and should not be regarded to be physiologically or pathologically relevant (Uitto *et al*, 1988; Sorsa *et al*, 1992). There are no data available regarding the level of such microbial proteases in human body fluids and tissues, and therefore these ratios are practically impossible to estimate. These data thus represent a potential existence of such microbial–protease–MMP tissue destructive cascade.

Our data open a new perspective into the possible role of periodontal pathogens as aetiologic factors in orodigestive carcinogenesis. Pathogenic oral bacteria may indeed be translocated into the GI tumours and modulate their growth. Further studies are warranted to provide deeper understanding about the mechanisms of *Td* and its virulence factor CTLP in carcinogenesis.

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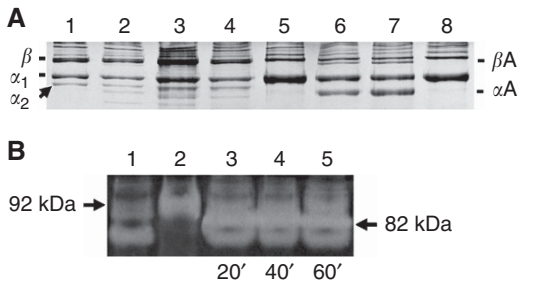

Figure 5. (**A**) SDS–PAGE (10%; Coomassie Brilliant Blue staining) analysis of the effects of *Td*-CTLP on native soluble type I and II collagen degradation by MMP-8. Lane 1: 1.5 μM type I collagen; lane 2: type I collagen incubated with pro MMP-8; lane 3: type I collagen incubated with *Td*-CTLP-treated MMP-8; lane 4: type I collagen incubated with *Td*-CTLP; lane 5: type II collagen; lane 6: type II collagen incubated with MMP-8; lane 7: type II collagen incubated with *Td*-CTLP. α_1, α_2, and β_1 indicate intact soluble native type I and II collagen monomers and dimers and βA and αA indicate their characteristic $\frac{3}{4}$-cleavage products resulting from collagenase activity. (**B**) Type I gelatin-zymographic analysis of the effects of *Td*-CTLP on human 92 kDa proMMP-9. Gelatinolytic 80–95 kDa *Td*-CTLP (lane 1) converts time-dependently (0–60 min) 92 kDa pro MMP-9 (lane 2) to 82 kDa gelatinolytically active form of MMP-9 (lanes 3–5, indicated by an arrow).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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