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Peri-implantitis fibroblasts respond to host immune factor C1q

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Background and Objective: Current therapies for peri-implantitis apply the same clinical protocols as those used for the treatment of periodontitis; however, outcomes remain unpredictable. We hypothesized that resident fibroblasts of the peri-implantitis stroma and periodontitis stroma differ in their phenotype and response to host immune factors. Fibroblasts are highly heterogeneous and comprise discrete subtypes with the potential of modulating inflammatory activities. The aim of the present study was to characterize the expression of receptors for complement C1q of innate immunity on human peri-implantitis fibroblasts and investigate effects of C1q on the proinflammatory properties of the cells.

Material and methods: Fibroblasts were cultured from gingival tissues exhibiting peri-implantitis and periodontitis, and from healthy gingivae as a control. Expression of C1q receptors for the collagen (cC1qR) and globular domains (gC1qR) of the protein was determined by flow cytofluorometric analysis (FITC) of specific antibodies bound to the surface of the cells. Secretion of C1q-inducible proinflammatory mediators was quantified after 24 h incubation using array-based ELISAs.

Results: The percentage of fibroblasts FITC-positive for cC1qR was 67, 75 and 12% in peri-implantitis, healthy and periodontitis cultures, respectively, whereas the percentage of gC1qR FITC-positive fibroblasts was 5, 3 and 59%, respectively. The C1q interactions with peri-implantitis and healthy fibroblasts increased secretion of the chemokines interleukin-6 and interleukin-8 twofold, and monocyte chemoattractant protein-1 fourfold over baseline values, whereas periodontitis fibroblasts were unresponsive. Complement C1q increased levels of vascular endothelial growth factor sevenfold and transforming growth factor-β1 12-fold over baseline values in peri-implantitis cultures, only.

Conclusions: Peri-implantitis fibroblasts differ from periodontitis fibroblasts in phenotypic expression of cC1qR and function, and from healthy fibroblasts in proinflammatory, angiogenic and fibrogenic function. Peri-implantitis fibroblasts may represent a novel subtype.

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The use of dental implants is standard treatment for the rehabilitation of edentulous areas. The success of implant therapy depends on osseointegration. Unfortunately, between 28 and 56% of implants that have been in function at least 4–5 years show marginal bone loss and increased probing depth regardless of the system types because of peri-implantitis and risk variables favoring the occurrence of this complication (1,2). Peri-implantitis is a complex, multifactorial condition associated with the presence of inflammatory infiltrates between metal surface and supporting bone (3,4), high levels of chemotactic, angiogenic and matrix degradation products into the

lesion (5,6), and bone loss extending all the way around the most coronal surface of the implant (saucer; 7).

Infections of the implant-bearing soft and hard tissues initiate peri-implantitis, which shares with periodontitis a similar bacterial flora. Therapies that apply the clinical protocols for the treatment of periodontitis have not been consistently successful in re-establishing the bone and soft tissue levels nor in eradicating disease progression; therefore, the affected implants remain at risk of failure (8). More effective clinical approaches could be supported by critical research on the biological processes underlining the inadequacy of the host's tissues to maintain osseointegration.

A characteristic feature of many chronic periodontal diseases is the persistence in the soft connective tissue and predilection for certain sites of inflammatory reactions induced by host-related immune factors. While the involvement of such factors in periodontitis has been studied extensively (9,10), knowledge of their role in the progression of peri-implantitis remains limited to an association with interleukin (IL)-1 positive genotype (11), and aberrant human β-defensin-1 and -2 function (12).

The complement system is an important humoral component of innate immunity and directs the induction and differentiation of adaptive immune responses. It consists of a series of activated plasma- and cell-expressed proteins that, in addition to providing a key effector mechanism for the elimination of microbial pathogens, interacts with specific surface receptors of the host's cells to promote processes essential for the resolution of inflammation (13). Component C1q of the classical complement cascade is a circulatory protein that persists in tissues because it binds to ubiquitous receptors of nucleated cells and to virtually every extracellular matrix substance by virtue of its strong positive charge (pI > 9). Human studies indicate that C1q is released at much higher levels in diseased crevicular fluid and gingival tissues than in health (14). Structurally, C1q consists of collagen (cC1q) and globular (gC1q) regions, which elicit an array of intracellular signals upon interacting with specific receptors of distinct periodontal fibroblast populations (15). Fibroblasts comprise discreet subsets with diverse properties and functions (16,17). Certain subsets contribute to disease persistence through their production of cytokines and chemokines, and thus play a key role in orchestrating the site specificity of inflammation (18,19). The binding of the C1q collagen region to fibroblasts cultured from healthy ligament and gingiva triggers the release of proinflammatory chemokines, which enhance humoral responses of the innate immunity to clear infections. In contrast, granulation tissue fibroblasts cultured from chronically inflamed periodontium do not respond to cC1q stimulation, because most of the cells instead bind the globular C1q regions (20). Interaction of gC1q with cells elicits suppression of the innate immunity by abrogating IL-12 production (21).

The role of complement in peri-implantitis has not been explored. The hypothesis of the present study was that interaction of the host's inflammatory factor C1q with resident peri-implantitis fibroblasts might exacerbate progression of the disease. To test this hypothesis, granulation tissue fibroblasts were cultured from severely inflamed peri-implantitis lesions and analyzed for expression of surface receptors for the collagen or the globular regions of C1q. Effects of the C1q interaction on the cellular secretion of proinflammatory and angiogenic products were determined. Knowledge would clarify immune functions of the stroma of peri-implantitis lesions and may provide the basis for novel therapeutic approaches.

Material and methods

Surgical protocol for peri-implantitis

Ten biopsy samples were harvested from partially edentulous patients with failing implants located in all jaw positions and associated with severe tissue inflammation. The residual number of teeth varied between 18 and 26. These cases were surgically treated with an access flap in order to debride or remove the implants. Patients with mild to moderate peri-implantitis were excluded from the study because they were treated nonsurgically. Participation was restricted to patients without systemic diseases, with no smoking habit and whose implants had been in function at least for 4 years. The surgical procedure consisted of an incision that was extended mesially and distally to the implant in order to obtain adequate access. After reflecting the muco-periosteal flap, the inflamed granulation tissue was removed with a Molt curette. When implants were clinically immobilized in the bone, they were removed with a trephine bur (Biomet 3i, Palm Beach Gardens, FL, USA), and the granulation tissue surrounding the failed implant was collected.

Fibroblast cultures

Peri-implantitis granulation tissue fibroblasts (GFIs) were established from explants by means of the outgrowth method (6). Isolated cells were seeded at 10,000–40,000 cells per well in LabTek chambers. Following fixation in cold acetone, the permeabilized monolayers were reacted for 20 min at room temperature with a battery of fluorescein isothiocyanate (FITC)-conjugated IgG monoclonal mouse antibodies (mAbs) raised against the following cell markers: vimentin, α-smooth muscle actin, epithelial pankeratins and CD34 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Stress actin filaments were labeled with 1.5 U of FITC–phalloidin (Molecular Probes, Eugene, OR, USA). Incubation of the cells with 2 mg/mL nonimmune mouse IgG for 5 min at room temperature before adding the specific antibodies blocked nonspecific binding. Periodontitis granulation tissue fibroblasts (GFPs) were derived from inflamed connective tissue taken during open flap surgery from ten patients with moderate to severe chronic periodontitis. Normal gingival fibroblasts (HFs) were isolated from clinically and radiographically healthy biopsies of ten donors without systemic diseases, after they

had completed 3 wk of oral hygiene and prophylaxis treatment. The phenotypic characterization of GFPs and HFs by immunostaining was reported previously (20). The Institutional Review Board of the University of Washington authorized the study. All the subjects granted written informed consent.

The primary cultures were maintained in chemically defined fibroblast growth medium (FGM; Cambrex Bioscience, Walkersville, MD, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Laurenceville, GA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Grand Island, NY, USA). Cultures were free of mycoplasma infection, as determined by Hoechst fluorescence staining (ICN Biomedicals, Aurora, OH, USA). The Limulus assay (BioWittaker, Walkersville, MD, USA) verified absence of contamination by endotoxins in all materials used in the laboratory. The monolayers were disrupted by treatment with 0.05% trypsin–EDTA (Invitrogen) and subcultured at a 1:2 split ratio, which yields one population doubling in human diploid fibroblast mass cultures (22). In order to avoid genotypic and/or phenotypic variations associated with long-term propagation, subtypes were analyzed between the fourth and sixth passages when they had reached approximately a 10-population doubling level.

Flow cytofluorimetric analysis of C1q receptors

Monolayers were detached with nonenzymatic dissociation buffers (Sigma, St Louis, MO, USA) to preserve the integrity of surface receptors (R). Single cell suspensions in serum-free FGM containing 5% nonfat milk were reacted for 1 h on ice with each of the following primary monospecific antibodies: 10 μg/mL chicken IgY polyclonal Ab (pAb) to cC1qR (GenWays Biotech, San Diego, CA, USA), or 20 μg/mL mouse IgG1 mAb to gC1qR (Covance Inc., Princeton, NJ, USA). Previous studies in our laboratory and others on titration of the cC1qR–pAb and mAb–gC1qR determined that concentrations were optimal for saturating the C1q surface receptors of the fibroblasts, and that there was not any significant cross-reactivity between these specific antibodies recognizing the cC1qR or the gC1qR domains (23,24). Antibodies for negative controls consisted of nonimmune 10 μg/mL chicken IgY or 20 μg/mL mouse IgG1 (Jackson Immune Research Laboratories, West Grove, PA, USA). The reacted suspensions were stained with 10 μg/mL secondary FITC-conjugated goat anti-chicken (H + L) or rabbit anti-mouse (H + L) $F(ab^1)_2$ fragments (Molecular Probes), thus eliminating nonspecific binding through the Fc receptors present on the cells. Immunostained cell suspensions were fixed in 4% paraformaldehide in saline for 10 min at room temperature, washed and resuspended in 0.2 mL saline buffer. Flow cytometry analysis was performed using an Influx Cytometer (Cytopeia, Seattle, WA, USA) with 488 nm excitation. After gating on Forward and Light Angle Scatter to remove debris, emission was collected at 525–530 nm for FITC. A threshold of FITC intensity was set such as that autofluorescent and negative cells were 98% below this level. Positive immunostaining was recorded by the software of the instrument as the percentage of cells above this threshold (Fig. 2).

Complement C1q stimulation

Fibroblast suspensions were plated at a concentration of 1.5×10^6 cells per 100 mm × 15 mm dish in FGM medium with 10% fetal bovine serum and allowed to attach overnight. Monolayers were washed with saline to remove serum components, and 5 mL serum-free FGM was added containing physiological amounts (30 μg/mL) of purified human C1q (CompTech, Tyler, TX, USA). Cultures stimulated with 30 μg/mL human serum albumin (Sigma) served as negative controls, whereas cultures not stimulated with any other protein provided baseline levels. Cultures were incubated for 24 h at 37°C in an atmosphere of air enriched with 5% CO_2. At the end of incubation, the culture supernatants were collected and centrifuged at 4°C for 5 min at 1000 *g* to remove cellular debris, and the total protein content of each supernatant was measured (Pierce BCA protein assay, Rockford, IL, USA).

Array-based quantitative ELISA analysis

Aliquots of the supernatants, each containing 0.5 mg/mL total proteins, were analyzed by mini-array ELISA (Endogen SearchLight, Woburn, MA, USA), a technology that allows for the simultaneous quantitation of multiple factors in single samples (6).

Descriptive statistical analysis

Data are the means ± SD of duplicate measurements and are representative of three independent experiments carried out in each subtype. Student's unpaired t-test ($p \geq 0.05$) compared peri-implantitis cells with healthy gingival control cells and fibroblasts isolated from chronically inflamed periodontitis sites.

Results

Immunophenotypic analysis of the stromal cells isolated from the granulation tissue of peri-implantitis lesions (GFIs)

Immunostained cultures from peri-implantitis tissues were evaluated for their fibroblastic nature. The cells tested positive for the markers of mesenchimal lineage vimentin and stress fibers of actin (Fig. 1A,B) and negative for pan-keratin, a marker of epithelial cells (Fig. 1D), and CD34, a marker of endothelial cells (data not shown), thus excluding contamination with these cell types. Interestingly, a few of the GFI cells tested positive for α- smooth muscle actin, a structural feature typical of myofibroblasts (Fig. 1C). This finding supports evidence that inflammatory environments, like that of advanced peri-implantitis lesions, favor differentiation of fibroblasts into stromal myofibroblasts, which are a key cell type for the repair of connective tissue (25). The GFI phenotype remained stable through 21–25 passages *in vitro* (6,20).

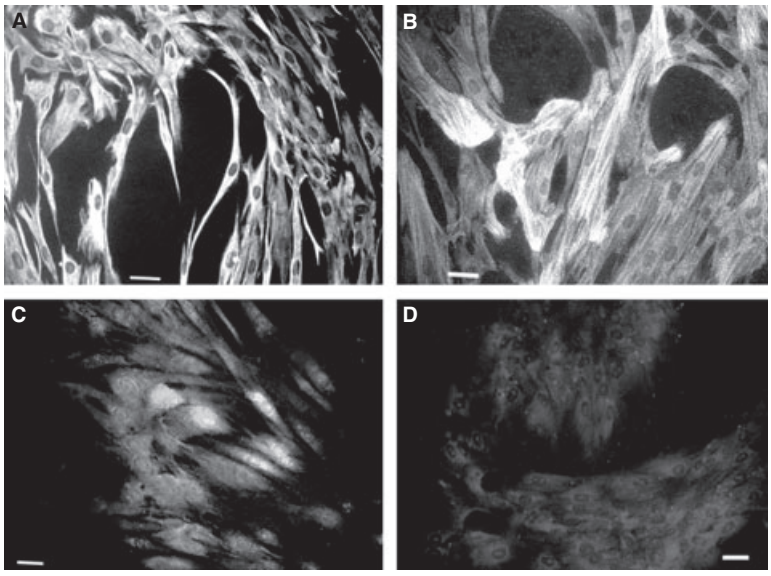

Fig. 1. Histological verification of the fibroblast-like phenotype of the peri-implantitis granulation tissue fibroblasts (GFIs). Monolayers at the second or third passage in culture were fixed, permeabilized and immunostained as described in the Methods section. All cells tested positive for the mesenchymal markers vimentin (A) and stress actin (B), and negative for the epithelial marker pan-keratin (D). A few cells tested positive for α-smooth muscle actin, a marker of myofibroblasts (C). Stained cells were viewed at 450–490 nm with an Eclipse E400 inverted microscope (Nikon, Melville, NY, USA) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Scale bar 10 μm.

Granulation tissue peri-implantitis fibroblasts (GFIs) express surface receptors (R) for the collagen region of C1q (cC1q)

Conventional flow cytofluorimetric analysis of GFIs immunostained with specific antibodies to cC1qR and gC1qR showed that the cells predominantly expressed binding sites for the collagen domain of C1q and did not significantly react with antibody to the globular region of the complement protein. Positive FITC–cC1qR fibroblasts were approximately 67% of the GFI population (Fig. 2A), whereas positive FITC–gC1qR cells were only 12% of the GFI population (Fig. 2B). As previously reported (16), healthy cultures contained approximately 75% positive FITC–cC1qR and 9% positive FITC–gC1qR fibroblasts, whereas granulation tissue periodontitis fibroblast cultures contained approximately 12% positive FITC–cC1qR and 59% positive FITC–gC1qR cells.

In all experiments, the FITC fluorescence of the primary isotype antibodies was used as the negative control, and ranged between 1.67 and 2.05% of the main peak fluorescence of the HF and GFP cell populations used as a positive control.

Complement C1q enhances proinflammatory, angiogenic and fibrogenic activities of GFIs

Amounts of proinflammatory, angiogenic and fibrogenic mediators measured in the culture medium of the fibroblast populations were normalized to a value of 100 μg total protein.

Chemokine secretion— Quantitative array-based ELISAs confirmed that baseline levels of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in the GFIs were approximately fourfold higher (146 ± 20, 192 ± 15 and 465 ± 50 pg/mL, respectively) than in cultures from healthy gingiva (35 ± 8, 56 ± 6 and 102 ± 10 pg/mL, respectively), and were very similar to those of fibroblasts cultured from chronically inflamed periodontal tissues (162 ± 13, 214 ± 25 and 398 ± 30 pg/mL, respectively (6).

Figure 3 shows that upon a 24 h stimulation with purified C1q, secretion of the proinflammatory chemokines remained unchanged in the periodontitis fibroblast cultures. In contrast, C1q stimulation of the GFIs increased secretion of IL-6 and IL-8 approximately twofold (295 ± 18 and 400 ± 32 pg/mL, respectively) and MCP-1 fourfold (1638 ± 121 pg/mL) over the baseline secretion of unstimulated cells. A similar pattern of a C1q-induced twofold increase of IL-6 and IL-8 (62 ± 7 and 115 ± 15 pg/mL, respectively) and a fourfold increase of MCP-1 (456 ± 45 pg/mL) over baseline values was observed in the healthy fibroblast cultures.

Vascular endothelial growth factor secretion— The GFI baseline levels of vascular endothelial growth factor (VEGF; 3384 ± 187 pg/mL) were approximately sixfold and twofold higher than in HFs (550 ± 35 pg/mL) and GFPs (1130 ± 97 pg/mL), respectively, as previously reported (6). Treatment with C1q increased VEGF secretion of the GFI sevenfold (27,142 ± 205 pg/mL) over baseline values, but the complement protein did not have any effect on the VEGF released by the healthy and periodontitis fibroblast cultures (Fig. 4A).

Transforming growth factor-β_1 (TGF-β_1) secretion— Baseline levels of transforming growth factor (TGF)-β_1 were similar in GFIs and healthy fibroblasts, at approximately 850 pg/mL, and fourfold lower than levels of this mediator in the periodontitis cultures (3415 ± 280 pg/mL), as previously reported (6). Stimulation with C1q increased TGF-β1 secretion 12-fold over baseline values (10,756 ± 98 pg/mL) in the GFI culture, only (Fig. 4B).

These values remained stable through the 12th passage in culture. In all the populations, baseline levels of any of the above-mentioned mediators remained unchanged after 24 h treatment with the negative control human serum albumin.

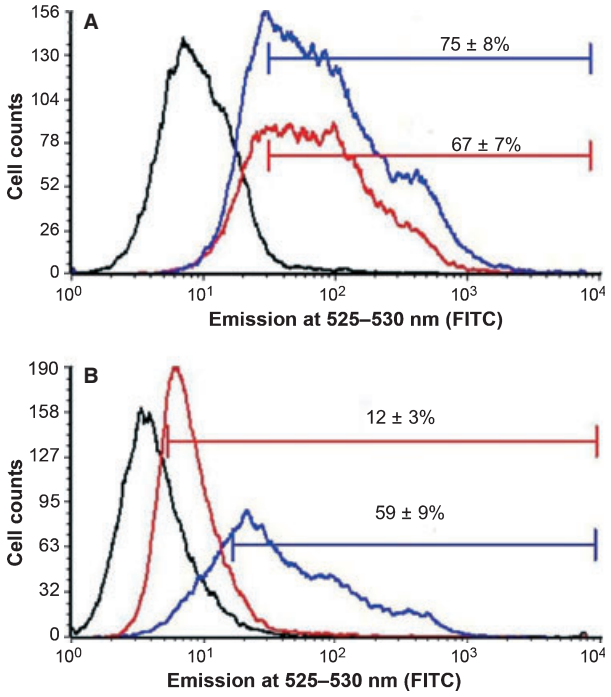

Fig. 2. Flow cytofluorimetric analysis of C1q receptors expressed by GFIs. Single cell suspensions were reacted with primary specific antibody to cC1qR (A) or gC1qR (B), and then immunostained with FITC-conjugated secondary antibodies, as described in the Methods section. The red profiles represent the reactivity of the GFIs, whereas the black profiles represent the cells' reactivity to the nonimmune isotype antibodies that were used as negative controls. Blue profiles represent the distribution of cC1qR (A) and gC1qR (B) on healthy fibroblast and periodontitis cultures, respectively, which were used as positive controls. Values of fluorescence intensity (log scale) are shown on the *x*-axis and relative cell number on the *y*-axis. Dead cells with debris, and aggregates, as identified by their low forward scatter properties and by a doublet discrimination setting, were excluded from the histograms. Results are representative of three different experiments.

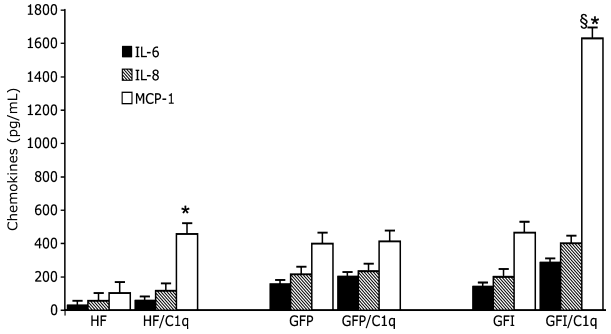

Fig. 3. Complement C1q enhances production of interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in peri-implantitis (GFIs) and healthy fibroblasts (HFs), but not in periodontitis fibroblasts (GFPs). The GFI, HF and GFP cultures were incubated for 24 h in serum-free medium containing 30 μg/mL purified human C1q. The conditioned supernatants were analyzed for the secretion of chemokines with an array-based quantitative ELISA, as described in the Methods section. Data are the means of serial dilutions obtained from 15 different cell lines. Differences between C1q-stimulated GFIs and HFs (*) and between C1q-stimulated GFIs and GFPs (§) were statistically significant ($p \leq 0.05$; Student's unpaired *t*-test).

Discussion

Until recently, fibroblasts were thought to be inert structural cellular components of tissues. It is now recognized that the cells actively participate in the immune response and regulate the quality, quantity and survival of inflammatory infiltrates by locally secreting cytokines in response to substances present in the microenvironment (19). Furthermore, fibroblasts isolated from different sites exhibit different functional properties. Previous work on human periodontal fibroblasts identified two functionally different subsets based on their phenotypic expression of receptors for the collagen or globular regions of C1q (6). The present study shows that the predominant population of granulation tissue fibroblasts isolated from peri-implantitis lesions exhibit receptors for cC1q, in contrast to fibroblasts derived from chronically inflamed periodontitis sites, which bind the gC1q region (Fig. 2).

As interactions of the cC1q and gC1q regions with their cell receptors elicit distinct humoral responses that either enhance or inhibit the innate immunity (26), we examined whether C1q might modulate differently the proinflammatory, angiogenic and fibrogenic properties of the cultured peri-implantitis and periodontitis fibroblasts.

The activated cC1qR phenotype promotes secretion of proinflammatory cytokines in a wide variety of cell types, including human periodontal fibroblasts (15). Although the percentages of cC1qR-positive cells were very similar in both the GFI and control HF cultures, 67 and 75%, respectively, baseline levels of the chemokines were significantly higher in the GFI than in the HF cultures and were of the same order of magnitude as in the GFP cultures (Fig. 3). This observation suggests that inflammation can irreversibly alter gene expression profile of a fibroblast. For example, upon stimulation with inflammatory mediators, the gene expression of a subset of synovial fibroblasts (peripheral) can be modified to resemble that of the opposite subset (lymphoid) (27).

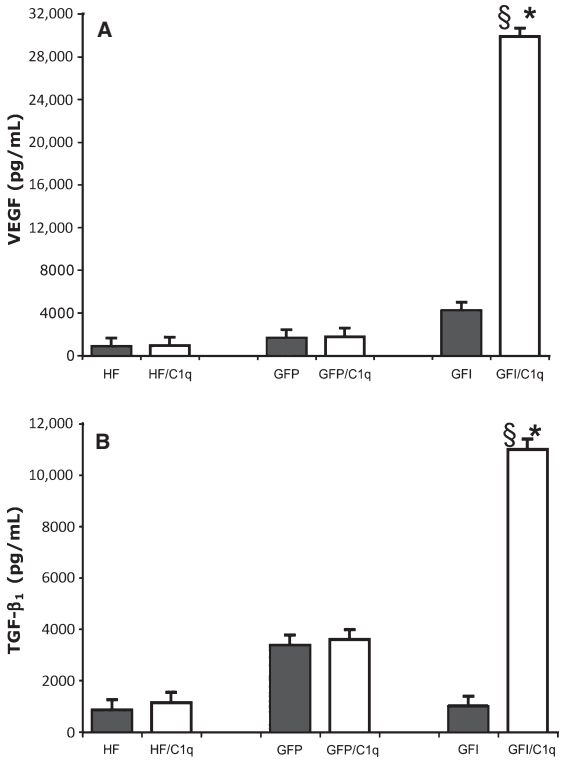

Fig. 4. Complement C1q enhances production of vascular endothelial growth factor (VEGF) and transforming growth factor-β_1 (TGF-β_1) in peri-implantitis cultures only. The GFI, HF, and GFP cultures in serum-free medium were incubated for 24 h with 30 μg/mL C1q. An array-based quantitative ELISA was used to analyze the conditioned supernatants for levels of VEGF (A) and TGF-β_1 (B), as described in the Methods section. Data are the means of the values of serial dilutions obtained from 15 different cell lines. Differences between C1q-stimulated GFIs and HFs (*) and between C1q-stimulated GFIs and GFPs (§) were statistically significant ($p \leq 0.05$; Student's unpaired *t*-test).

Mediators IL-6 and IL-8 promote periodontal tissue infiltration of lymphocytes and neutrophils, respectively, and MCP-1 is a potent chemoattractant for monocytes and macrophages (28). Stimulation with C1q led to a moderate, twofold, increase in the secretion of IL-6 and IL-8 in both GFI and HF cultures, whereas it led to a fourfold increase in MCP-1 levels, suggesting that influx of phagocytic cells might be especially favored by the interaction of matrix-associated C1q protein with the cC1qR of the periodontal stroma.

The presence of a pronounced erythema is characteristic of soft connective tissue affected by peri-implantitis. Previous studies have indicated that peri-implantitis fibroblasts exhibit enhanced constitutive expression of VEGF and virtual absence of its main inhibitor, angiopoietin-1 (6). Vascular endothelial growth factor is considered to be the most essential factor for the differentiation of the vascular system. Upon C1q stimulation, a sevenfold increase over baseline of VEGF occurred in the GFI cultures only (Fig. 4A). This finding is in contrast with the report that, in mice, activation of the alternative complement pathway, but not the classical or lectin pathways, is essential for neovascularization (29). Our data suggest that the cC1qR of GFIs might have the ability to activate multiple, synthetic signaling pathways, including those leading to enhanced formation of new blood vessels. Support for the concept that the activated cC1qR of GFIs might contribute to neovascularization in peri-implantitis, and thus perpetuate extravasation of inflammatory infiltrates into the lesion, is provided by the observation that only in this C1q-treated subtype did the level of secreted TGF-β1 increase 12-fold over baseline (Fig. 4B). The family of TGF-β members consists of multifunctional proteins that have a pivotal role as signaling components in angiogenesis, as well as in the process of fibrogenesis (30).

We reported that constitutive levels of TGF-β1, which were similar to those of normal gingiva, were much lower in GFIs than in periodontitis cultures, and suggested that an inherent lack of fibrogenic activity may represent a biological marker to distinguish peri-implantitis from periodontitis (6). However, the present observation that C1q stimulation dramatically increased the TGF-β1 level of GFIs suggests that *in vivo* the activated cells may acquire the ability to provide the necessary matrix for the survival of infiltrates in the lesion.

Host immune factors remain activated long after infections have subsided and determine the severity and persistence of inflammatory reactions of oral tissue (31). It is likely that *in vivo* C1q, as a transient component of the extracellular matrix, selectively promotes the migration and survival of inflammatory infiltrates into sites of osseous-integrated implants. This novel information may advance our understanding of the complex and multifactorial biological factors implicated in peri-implantitis to enable effective therapeutic approaches in man. Adult human fibroblasts are potent immunoregulators; therefore, targeting specific subsets is likely to become an important strategy for the treatment of oral diseases.

Acknowledgements

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