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Differential chemokine response of fibroblast subtypes to complement C1q

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This paper is dedicated to the memory of its third author, William "Bill" Ammons, who suddenly passed away in Seattle in August 2006.

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Background and Objective: The pathogenesis of periodontitis includes an inappropriate activation of the classical complement cascade (C′) with accumulation of inflammatory C′ products in fluids and tissues. Our hypothesis is that *in vivo* the C′ product, C1q, may act as a regulatory component of the innate immune response of distinct matrix fibroblasts to the inflammatory environment. This study analyzed the C1q induction of pro-inflammatory cytokine secretion in fibroblast subtypes derived from distinct periodontal tissues, and identified a mechanism of the cell response.

Material and Methods: Primary human gingival fibroblast, periodontal ligament fibroblast, and granulation tissue fibroblast cultures were treated for 24 h with C1q. Protein arrays assessed the secretory profile of constitutive and C1q-inducible pro-inflammatory cytokines, and enzyme-linked immunosorbent assays were used to quantify the kinetics of each inducible cytokine.

Results: Granulation tissue fibroblast cultures were unresponsive to C1q challenge. In contrast, periodontal ligament fibroblasts responded with a release of monocyte chemoattractant protein (MCP)-1, interleukin-6, interleukin-8, and macrophage inflammatory protein (MIP)-1β higher than the basal level by 8.2-, 7.0-, 3.8-, and 7.2-fold, respectively. Human gingival fibroblast cultures increased secretion of these chemokines by 5.2-, 4.5-, 3.0-, and 9.8-fold, respectively. Inhibitor studies revealed that C1q-inducible release of chemokines by the human gingival fibroblast and periodontal ligament cultures was contingent upon p38 mitogen-activated protein kinase activity.

Conclusion: The ability of C1q to stimulate secretion of pro-inflammatory chemokines depends upon which specific fibroblast subtype is involved. Targeting C1q-activated intracellular signaling pathways may be an effective means to inhibit the production of chemokines that promote inflammatory cell infiltration into gingival and periodontal ligament tissues.

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Chronic inflammatory responses of the oral connective tissue to periodontal pathogens may result in pocket formation, loss of periodontal attachment, and resorption of alveolar bone. The clinical outcome is highly influenced by the host's local immune responses (1).

Several studies have reported that gingival fluids and inflamed gingival tissues from individuals with periodontitis contain high amounts of complement products (2–5). The complement system of innate immunity consists of a series of

plasma- and cell-expressed proteins that, when activated, can be extremely effective in destroying pathogens, but also can be equally damaging to the infected tissues. Activation of the classical complement cascade (C′) produces the biologic effects of inflammation, including increased vascular permeability, release of destructive enzymes, and induction of pro-inflammatory cytokines (6). The C′ product, C1q, is a circulatory protein that persists in tissue lesions because it binds to virtually every extracellular matrix component by virtue of its strong positive charge (pI > 9) and it interacts with receptors expressed on all nucleated cells (7). The high immunoglobulin (Ig)G and IgM levels, typical of refractory periodontitis, could enhance the availability of C1q in diseased tissues, because these antibodies are excellent activators of C′. In addition to functioning in antimicrobial defense, C1q participates in the cytokine expression of monocytes/macrophages, dendritic cells and endothelial cell populations (8–10).

Previous studies have revealed that C1q binding to cultured human gingival fibroblasts abrogates mitogen-induced proliferation of the cells through mechanisms that involve activation of the intracellular p38 mitogen-activated protein kinase (MAPK) stress pathway (11). This finding strongly suggested that the persistence of C1q in the extracellular matrix of periodontal lesions may exacerbate ongoing destruction of oral soft connective tissues.

Fibroblasts are the principal cell type of the soft periodontium. In health, the role of the cells is to maintain tissue integrity and homeostasis. Resident fibroblasts become an important participant in the periodontal lesion by serving as immunocompetent cells. They increase the production of cytokines that amplify and/or perpetuate the inflammatory response to clear the infection (12,13). An emerging concept is that this capability may be ascribable to specific subtypes rather than to the general fibroblast population (14–16).

The objective of this study was to identify cytokine mediators of periodontal tissue inflammation induced by C1q stimulation of resident fibroblasts, and to determine whether the response of subtypes isolated from gingival, periodontal ligament, and granulation tissues differed. Based on results that defined the C1q-induced secretion of several chemokines, further studies investigated whether activation of the p38 MAPK pathway could be a common mediator of their expression.

Material and methods

Cell cultures

Primary fibroblast cultures were established from human oral biopsies by means of the explant technique (17). Human gingival fibroblasts were cultured from interproximal gingival papillae harvested between the maxillary premolars of five donors with no systemic disease. The donors exhibited clinically and radiographically normal periodontal tissues; moreover, they had undergone dental prophylaxis and 3 wk of intensive oral hygiene before the gingival harvests. Granulation tissue fibroblast cultures were established from tissues taken (during open flap surgery) from five patients with moderate to severe chronic periodontitis according to the classification of the American Academy of Periodontology (18). Periodontal ligament cultures were grown from the middle third of the root of healthy third molars removed from five donors in the course of orthodontic treatment. Informed consent from donors and patients was obtained in compliance with the requirements of the Institutional Review Board of the University of Washington. The fibroblastic nature of the cells was verified by immunostaining with a battery of antibodies to vimentin, α-smooth muscle actin, epithelial keratin, and neurofilament protein (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) (17). To date, there is no exclusive phenotypic marker for periodontal ligament cells. Nevertheless, periodontal ligament cultures were immunostained for the presence of higher alkaline phosphatase activity compared with human gingival fibroblasts (19). Hoechst fluorescence staining (ICN Biomedicals, Aurora, OH, USA) determined that cultures were free of mycoplasma infection. Cells were used at early passages between transfers 4 and 8. Cultures were switched to serum-free medium overnight before treatment with C1q and control factors.

C1q stimulation

The cultures (1.5×10^6 cells per 100×15-mm dish) were stimulated for 6 h (early response) or for 24 h (late response), at 37°C in an atmosphere of 5% CO_2 and 95% air, with 30 μg/ml purified human C1q (CompTech, Tyler, TX, USA) in serum-free fibroblast growth medium (Cambrex Bioscience, Walkersville, MD, USA). The *Limulus* assay (BioWhittaker, Walkersville, MD, USA) excluded contamination of the C1q preparations with bacterial lipopolysaccharide. Reagents, culture media, and plasticware used in the experiments were endotoxin free. Positive controls consisted of cultures stimulated with 2 ng/ml interleukin-1β (R & D Systems, Minneapolis, MN, USA). Additional positive controls included cultures stimulated with 10 μg/ml collagenous fragments of C1q (cC1q) that specifically mediate pro-inflammatory cytokine production in endothelial cells (9). On a molar basis, cC1q fragments are more reactive than native C1q (17). Negative controls consisted of cells stimulated with 30 μg/ml human serum albumin, whereas cultures not incubated with any other protein provided baseline levels.

In the inhibition experiments, cells were pre-incubated for 45 min with 50 μg/ml pyridinyl imidazole SB203580 (Calbiochem, San Diego, CA, USA) before the addition of native C1q, control factors or no proteins to the culture media. At the end of incubation, fibroblast suspensions obtained by standard trypsinization procedure of the monolayers were assessed for total cell number and viability using the Trypan Blue exclusion method.

Human cytokine antibody protein array

After 6 or 24 h of incubation, cell-free supernatants were recovered by centrifugation at 800 *g* and total protein content was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Aliquots of the conditioned media containing a similar amount of total protein were analyzed using the Human Cytokine Array V (RayBiotech Inc., Norcross, GA, USA). Briefly, array membranes were incubated with each conditioned medium for 2 h, washed, reacted with biotin-conjugated anticytokine mix, washed again, and then developed with horseradish peroxidase-conjugated streptavidin. Arrays were visualized using the enhanced chemiluminescence reaction (Amersham Corp., Arlington Heights, IL, USA) on X-Omat AR film (Eastman Kodak Co., Rochester, NY, USA).

Quantitative enzyme-linked immunosorbent assay (ELISA) analysis

Quantitative ELISA immunoassays used serial dilutions of the conditioned media to measure secreted monocyte chemoattractant protein-1 (MCP-1), interleukin-6, interleukin-8, macrophage inflammatory protein-1β (MIP-1β) (Pierce/Endogen), and tumor necrosis factor-α (TNF-α) (R & D Systems), according to the manufacturers' instructions.

Statistical analyses

Data are expressed as the mean ± standard deviation (SD) of duplicate measurements and are representative of three independent experiments carried out in each subtype. The Student's *t*-test for independent samples ($\alpha \leq 0.05$) compared the native C1q-treated cultures with the respective controls.

Results

Cell viability was not affected by treatment of the subtypes, either with native C1q, or with positive and negative controls, as measured by Trypan Blue exclusion tests (data not shown).

C1q-enhanced secretion of pro-inflammatory chemokines MCP-1, interleukin-6, interleukin-8 and MIP-1β by the human gingival fibroblast and periodontal ligament subtypes

Qualitative determinations An initial screen, using a human antibody-based protein array system of the most prevalent cytokines, identified the mediators simultaneously released into the culture media during 6 or 24 h of treatment of the monolayers with C1q. In order of intensity, the most highly secreted cytokines at baseline level were MCP-1, interleukin-6, and interleukin-8. Their expression markedly increased in the periodontal ligament and human gingival fibroblasts after 6 h of treatment, with either native C1q or with cC1q fragments. After 24 h of treatment, a notable increase in MIP-1β secretion also became evident.

In contrast, the baseline cytokine profile of the granulation tissue fibroblast subtype did not change significantly after 6 or 24 h of C1q challenge (Fig. 1).

Quantitative determination In all subtypes, baseline values of pro-inflammatory cytokines remained unchanged with negative control human serum albumin. The subtypes, however, revealed a considerable variation in their response to native C1q/cC1q fragments, as shown by the kinetics of the chemokine released into the culture media (Fig. 2).

After 24 h of treatment with C1q, the cellular secretion of MCP-1, interleukin-6, interleukin-8 and MIP-1β was amplified by 8.2-, 7.0-, 3.8-, and 7.2-fold, respectively, over basal values, in periodontal ligament cultures.

Similarly the secretion of MCP-1, interleukin-6 and interleukin-8 was increased by 5.0-, 4.2, and 3-fold, respectively, and the secretion of MIP-1β by 9.8-fold, over baseline levels, in C1q-stimulated human gingival fibroblasts.

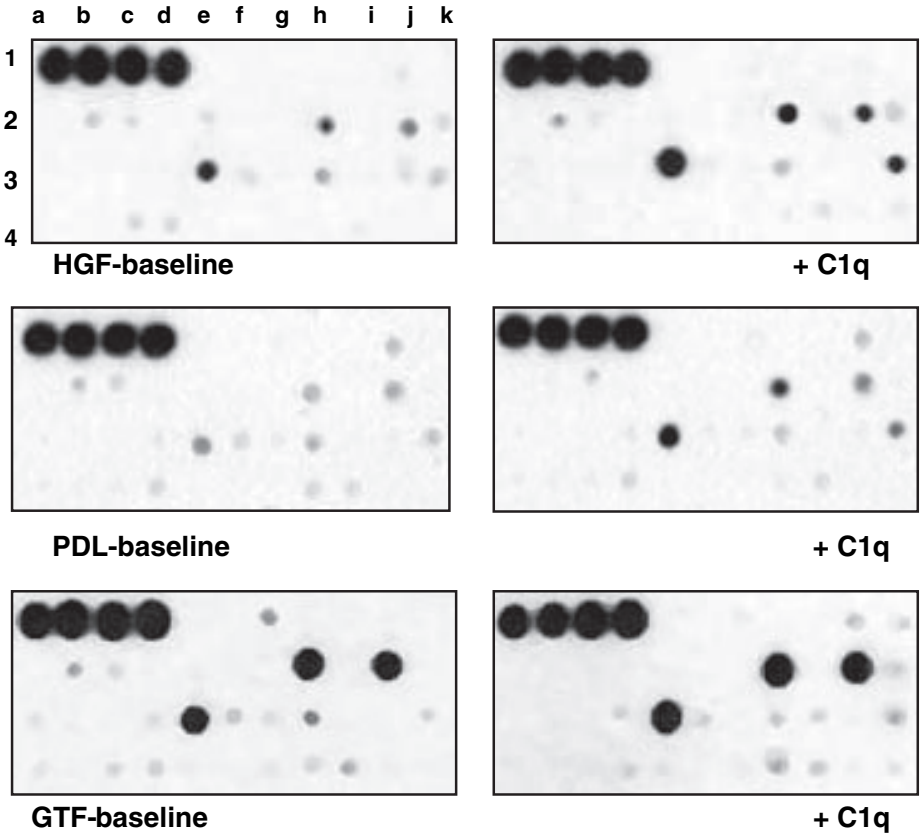
Unstimulated granulation tissue fibroblasts secreted the highest amount of MCP-1, interleukin-6 and interleukin-8, confirming a report that these cells express higher levels of genes and proteins for pro-inflammatory cytokines than healthy human gingival fibroblasts (20). However, the magnitude of the chemokine release was unaffected by C1q treatment.

In response to positive control interleukin-1β, chemokine release of periodontal ligament, human gingival fibroblast and granulation tissue fibroblast cultures was increased by approximately 4-, 6- and 1.8-fold, respectively, over baseline (data not shown).

Because of a report that the antibody-based array technology might lack adequate sensitivity to measure low levels of TNF-α in complex biological fluids (21), we also used the ELISA procedure to assess each cell-free supernatant for the presence of this cytokine. After 24 h of incubation with native C1q, no increase in secreted TNF-α over the baseline levels of 5.4–7.2 pg/ml were detectable in the culture media of any subtype (data not shown).

Specific inhibition of the p38 stress-pathway prevented C1q-induced cytokine release

The mechanism by which C1q enhances chemokine secretion by human gingival fibroblasts and periodontal ligament fibroblasts was further investigated. Based on an observation that the binding of either native C1q or cC1q to gingival fibroblasts activates the p38 MAPK (8), we reasoned that activation of this stress pathway in the periodontal subtypes could result in the production of pro-inflammatory cytokines and chemokines through post-transcriptional mechanisms, as demonstrated in other cellular systems (22). Therefore, we targeted the p38 pathway by incubating human gingival fibroblast and periodontal ligament cultures with specific inhibitor, SB203580, prior to challenge with C1q. The SB203580 inhibitor alone did not reduce constitutive chemokine production, but it did reduce the stimulatory effect of C1q in both subtypes by $\approx 50\%$ (Fig. 3).



	a	b	c	d	e	f	g	h	i	j	k
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA 78	GCSF	GM-CSF	GRO	GROα
2	I309	IL1α	IL1β	IL2	IL3	IL4	IL5	IL6	IL7	IL8	IL10
3	IL12	IL13	IL15	IFNγ	MCP1	MCP2	MCP3	MSCF	MDC	MIG	MIP-1β
4	MIP-1δ	Rantes	SCF	SDF1	TARC	TGFβ	TNFα	TNFβ	EGF	IGF1	Ang

Fig. 1. Cytokine protein arrays of the C′ product (C1q)-stimulated human gingival fibroblast (HGF), periodontal ligament fibroblast (PDL) and granulation tissue fibroblast (GTF) subtypes. Cytokine protein arrays using the conditioned media of subtypes yielded a semiquantitative assessment of the changes in cytokine secretion of monolayers treated for 24 h with 30 μg/ml native C1q (+C1q) compared with untreated cultures (baseline levels). Increases in monocyte chemoattractant protein-1 (MCP-1), interleukin-6, interleukin-8, and macrophage inflammatory protein-1β (MIP-1β) are evident in the human gingival fibroblast and periodontal ligament arrays after C1q stimulation. The lower panel shows the map of the antibodies to specific cytokines and positive and negative controls that were fixed onto the array membranes. ENA78: epithelial-cell-derived neutrophil activating protein-78; GCSF: granulocyte macrophage-colony stimulating factor; GRO: growth related oncogenic factor; I-309: inflammatory CC-chemokine peptide 309; IL: interleukin; IFNγ: interferon-gamma; MCSF: macrophage colony stimulating factor; MDC: macrophage derived chemokine; MIG: monokine induced by interferon gamma; Rantes: regulated upon activation normal T-cell expressed and secreted chemokine; SMC: stem cell factor 1; SDF1: stromal cell-derived factor 1; TARC: thymus activation-regulated chemokine; TGFβ: transforming growth factor-beta; TNF: tumor necrosis factor; EGF: epidermal growth factor; IGF1: insulin-like growth-factor 1; Ang: angiotensin.

Discussion

This study shows that cellular challenge by C1q increased significantly the simultaneous release of pro-inflammatory chemokines from normal human gingival fibroblasts and periodontal ligament fibroblasts, but not from granulation tissue fibroblasts.

The enhanced secretion of MCP-1, interleukin-6, and interleukin-8 was an

early response of the human gingival fibroblast and periodontal ligament subtypes to C1q because it was detectable after only 6 h of stimulation. Measurement of the chemokines at this early time-point verified the direct involvement of C1q signaling in the cell response by reducing the participation of excessive autocrine effects in their secretion. Autocrine effects, however, may be relevant *in vivo*; thus, we also quantified the chemokine secretion 24 h after C1q challenge. Whereas kinetics analysis showed a modest increase in the MCP-1, interleukin-6 and interleukin-8 levels compared with the 6-h incubation time, the late response of the cells included an additional significant secretion of MIP-1β (Figs 1 and 2). TNF-α up-regulation was described in macrophages stimulated with native C1q (10); however, neither the protein array system nor the ELISA assay detected considerable amounts of this cytokine in the culture media of stimulated and unstimulated fibroblast subtypes.

C1q-treated human gingival fibroblast and periodontal ligament fibroblast subtypes induced a higher increase in MCP-1, interleukin-6 and MIP1-β secretion compared with interleukin-8, which could favor tissue accumulation of activated macrophages and lymphocytes instead of neutrophils (23–25). These inflammatory cells play different roles in the pathogenesis of periodontal disease. Neutrophil infiltration is a hallmark of acute inflammation, but chronic inflammation is

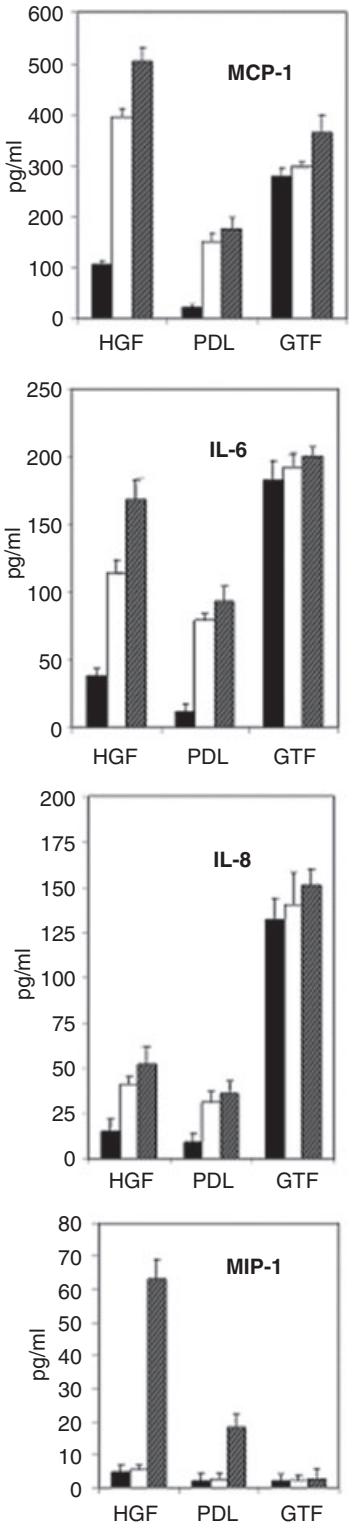


Fig. 2. Time course of the chemokine secretion by cultured human gingival fibroblast (HGF), periodontal ligament fibroblast (PDL), and granulation tissue fibroblast (GTF) subtypes incubated with 30 μg/ml of the native C′ product (C1q) for 0 h (solid bars), 6 h (open bars) and 24 h (solid bars). Quantification of levels of monocyte chemoattractant protein-1 (MCP-1), interleukin-6, interleukin-8, and macrophage inflammatory protein-1β (MIP-1β) in the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA), as described in the Material and methods. *A significant difference ($\alpha = 0.05$) was observed between untreated and C1q-treated cultures.

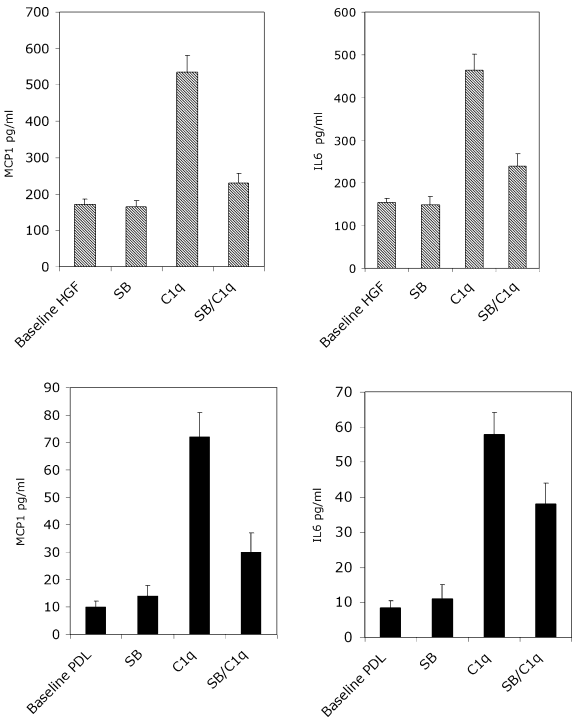


Fig. 3. The effect of inhibition of the C′ product (C1q)-stimulated p38 mitogen activated protein kinase (MAPK) pathway on the interleukin-6 and monocyte chemoattractant protein-1 (MCP-1) secretion of human gingival fibroblast (HGF) and periodontal ligament (PDL) subtypes. Cultures were pretreated with 50 μg/ml SB203580 (SB) and then stimulated with 30 μg/ml C1q, as described in the Material and methods. Data are expressed as the mean ± standard deviation for triplicate cultures. *A significant difference ($\alpha = 0.05$) was observed between untreated and inhibitor-treated cultures stimulated with C1q.

histologically associated with the presence of mononuclear cells. It is possible that C1q-stimulated human gingival fibroblast and periodontal ligament fibroblast subtypes, by virtue of their preferential chemokine release, might participate in the transition from an acute to a chronic inflammatory reaction in the periodontium.

The observation that periodontal ligament fibroblasts secreted lower amounts of the chemokines than human gingival fibroblasts might be dictated by the enclosed space of the dental socket, which is more conducive to targeted tissue interactions than the open oral cavity, where the effective level of the mediators would be diluted by saliva. Comparison of the subtypes for their relative chemokine increase (i.e. treated vs. non-treated), showed that periodontal ligament fibroblasts exhibited the highest reactivity to C1q, suggesting that this tissue may be especially vulnerable to C′ induction of pro-inflammatory chemoattractants.

By contrast, granulation tissue fibroblasts challenged with C1q did not modify significantly their constitutive release of chemokines. C1q is a chimeric molecule containing a globular lectin region (gC1q) contiguous to a collagenous domain (cC1q). Human gingival fibroblasts and periodontal fibroblasts bind cC1q through calreticulin receptors; however, granulation tissue fibroblasts express a different class of higher affinity receptors that engage the gC1q region (17). Modulation of the immune function of cells is mostly a function of the cC1q region (7–9); thus, the intrinsic inability of granulation tissue fibroblasts to interact with this domain may explain the subtype failure of increasing the chemokine release when the native molecule was added to their culture medium. On the other hand, granulation tissue fibroblast binding to the gC1q region of the inactive C1q–$C1r_2s_2$ complex C1 initiates the C′ cascade, generating free C1q at the cell surface (17). By this mechanism, *in vivo*, resident granulation tissue fibroblast populations might target fibroblasts of adjacent healthy gingival and periodontal ligament tissues for enhanced chemokine production. This possibility, coupled with the constitutively high levels of MCP-1, interleukin-6, and interleukin-8, released by the granulation tissue fibroblast cells, supports the recognized major role for this subtype in the amplification and persistence of inflammatory responses.

Stimulation of the periodontal ligament and human gingival fibroblast subtypes with positive-control interleukin-1β paralleled the magnitude of the C1q action on the chemokine release, suggesting that ligation of the cC1qR and the interleukin-1β receptor may activate similar signaling pathways. Interleukin-1β stimulation of the granulation tissue fibroblast subtype, however, enhanced only modestly (≈ 1.8-fold) the cells' release of constitutive MC1, interleukin-6 and interleukin-8. A likely explanation for this observation might be that granulation tissue fibroblasts are already programmed to express at baseline level their full potential for pro-inflammatory cytokine production, and thus the subtype appears to be less responsive to further immunoregulatory stimulation by inflammatory environmental factors.

To date, documentation of a differential tissue accumulation of C1q in the progression of periodontal diseases is not available. This information, and the elucidation of the potential effects of C1q deposits on cells of the periodontium other than the fibroblasts, would advance our understanding of the contribution of an inappropriate C′ activation to periodontal tissue damage. For instance, immobilized C1q acts as a chemoattractant for mast cells, dendritic cells and leucocytes, and it is a potent mediator of extracellular superoxide (O_2^-) production and degranulation of neutrophils (26,27). Gingival keratinocytes bind C1q (28), and this interaction could augment the cells' constitutive secretion of interleukin-8, thus promoting neutrophil infiltration and the initiation of periodontal disease. C1q cross-linking of gC1qR on the surface of human-activated monocytes/macrophages and dendritic cells suppresses interleukin-12 production by the cells, leading to inhibition of T helper 1 cell immunity, which is crucial for clearing pathogenic infections (8).

Active regulation of gingival fibroblast function has been proposed as a strategy for the prevention and treatment of periodontal diseases (29). In an effort to bolster therapeutic approaches, we explored *in vitro* the possibility of limiting the destructive effects of C1q on healthy connective tissues by inhibiting the induction of inflammatory cytokines. *In vivo*, blockade of the p38 MAPK by pyridinyl imidazoles was reported to exert anti-inflammatory effects, both in animal models of rheumatoid arthritis (30) and in phase I clinical trials (31). In the present study, the SB203580 compound largely prevented release of MCP-1, interleukin-6, and interleukin-8 in both human gingival fibroblast and periodontal ligament cultures during their early response to C1q (Fig. 3). We propose that pharmacological control of intracellular C1q signaling in target tissues would be more beneficial to the periodontal patient than therapeutics aimed at inhibiting the activation of C′ (32). Impairing the protective function of C′ may bring about hyporesponsiveness, which will fail to clear the infection.

In conclusion, the *in vitro* data presented here suggest that C1q, as a transient component of the periodontal damaged extracellular matrix, could amplify the inflammatory response by promoting migration of inflammatory infiltrates selectively into healthy gingival and periodontal ligament tissues, and that the presence of granulation tissue may exacerbate this damaging effect.

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