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Cutting Edge: Proliferating Fibroblasts Respond to Collagenous C1q with Phosphorylation of p38 Mitogen-Activated Protein Kinase and Apoptotic Features

Sandra Bordin2 and Douglas Whitfield

Interactions of C1q collagen tails with human fibroblasts induce G1 mitotic arrest. The hypothesis tested in this study is that the antiproliferative effect of C1q tails is mediated through activation of stress responsive pathway(s). Upon C1q treatment, proliferating fibroblasts were examined by immunoblotting with a panel of Abs to the mitogen-activated protein kinase (MAPK) superfamily. The cells selectively increased phosphorylation of p38 MAPK, upstream dual activator MAPK kinase 3/6, and downstream transcription factors activating transcription factor 2, ETS domain transcription factor 1, and C/EBP homologous protein in a time-dependent manner. Phosphorylations were mediated, in part, by ligation of surface C1q tail-binding calreticulin. These events correlated with the appearance of apoptotic nuclei and DNA fragmentation in the cultures, which increased with a time response curve. The apoptotic features were linked to p38 activities because the selective inhibitor SB203580 prevented both phosphorylation of the pathway and DNA fragmentation. Hence, p38 activation might provide a molecular basis for linking mitotic arrest and apoptosis of fibroblasts by C1q tails. The Journal of Immunology, 2003, 170: 667–671.

 Fibroblasts are essential for maintaining the integrity of connective tissues. Substances of the local microenvironment contribute either positively or negatively to their survival. Fibroblasts encounter activated complement components selectively at inflammatory sites following enhanced vascular permeability and local complement synthesis. Excessive production or inappropriate activation of complement cascade causes disorderly fibroblast behavior, which leads to the irreversible destruction of functional connective tissue (1, 2). The molecular events involved in the susceptibility of fibroblasts to direct damages by specific complement components are poorly defined.

C1q, the initiator of classical complement cascade, exerts a detrimental effect upon regeneration of cultured normal human fibroblasts (HF)3 (3). Structurally, C1q consists of discrete collagen tails and globular heads (4), which elicit an array of intracellular signals upon binding to distinct fibroblast subtypes (5–6). Functionally, the binding of C1q tails to HF blocks the cycling fraction in G1 phase and suppresses DNA synthesis (3), whereas the binding of C1q globular heads does not hamper HF proliferation (7).

C1q is related to the superfamily of “soluble defense collagens,” which influence cell responses to stress (8). The ubiquitous receptor for C1q tails calreticulin (CRT), also known as the receptor for collagen tails of C1q (cC1qR) (9), is a stress protein (10). Taken together, the observations suggested that, in connective lesions, C1q may function as sensor and executor of environmental stress by signaling HF with a regenerative potential to abort cell division until conditions return to normal.

To test this hypothesis, we examined activation of stress responsive mitogen-activated protein kinase (MAPK) pathways in proliferating HF stimulated either with concentration of C1q tails or intact molecules that induce G1 arrest, and with equivalent amount of C1q globular heads. The three main families of MAPK are the extracellular signal response kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 kinase. Generally, ERKs mediate signals promoting cell proliferation, differentiation, or survival, whereas JNK and p38 MAPKs are involved in cell responses to stress (11).

In a previous study, HF stimulated with intact C1q did not activate ERKs (3). In this study, we show that, instead, the cells selectively activated the p38 pathway in a time-dependent manner in response to either C1q tails or the intact molecule, but not in response to C1q globular heads.

3 Abbreviations used in this paper: HF, cultured normal human fibroblast; ATF2, activating transcription factor 2; cC1qR, receptor for collagen tails of C1q; gC1qR, receptor for globular heads of C1q; ERK, ETS domain transcription factor 1; CHOP, C/EBP homologous protein; CRT, calreticulin; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; ERK, extracellular signal response kinase; JNK, c-Jun N-terminal kinase; PKA-I, type I protein kinase A.
We questioned the potential role of cell surface structures as mediators of the C1q-induced p38 activation. Several candidate receptors for C1q have been proposed. Some of these structures were shown by later work to be unlikely to act as surface C1qR, as they either were absent from the cell surface (12) or simply did not bind to C1q (13).

In this study, we report that the engagement of surface CRT participated in the signaling of C1q tails. This event was paralleled by a significant increase in morphologic and biochemical features characteristic of apoptosis. The pharmacological inhibitor SB203580 provided further insights into the link between p38 activation and apoptotic features of the cells.

Materials and Methods

Cell lines

Primary HF were grown from healthy human gingival biopsies of five donors (aged 22–34 years) with institutional approval and informed consent (3). Life span of the lines was between passages 23 and 30. In this study, HF were recovered between passages 7 and 13, and maintained in serum-free growth medium (fibroblast growth medium-2; Clonetics, San Diego, CA).

Reagents

Purified human C1q was from Advanced Research Technology (San Diego CA). C1q tails and globular heads were prepared by peptin and collagenase digestion, respectively, of intact molecule (14). The following reagents were purchased from indicated sources: culture medium and supplements (Clonetics); Hoechst 33258 (Molecular Probes, Eugene, OR); electrophoresis buffers, precasted minigels, and transfer membranes (Bio-Rad, Hercules, CA); immunoblotting reagents (Cell Signaling Technology, Beverly, MA); SB203580 (Calbiochem, San Diego, CA); Limulus assay (BioWhittaker, Walkersville, MD); and Cell Death Detection ELISA PLUS (Roche, Indianapolis, IN). Substances not otherwise specified were from Sigma-Aldrich (St. Louis, MO). Protein content was determined by the BCA assay (Pierce, Rockford, IL).

Immunoblotting

Proliferating HF were stimulated with 30 μg/ml C1q globular heads or intact molecule, or with 10 μg/ml C1q tails, and vehicle (0.25% BSA) at 37°C in 5% CO₂. Cell extracts (20–30 μg of each protein sample) were fractionated by SDS-PAGE and electrotransferred onto nitrocellulose membrane (5). The membranes were probed with primary Ab to phosphorylated forms of p38 MAPK (Thr180/Tyr182), MAPK kinase (MKK)3/6 (Ser185/195), activating transcription factor 2 (ATF2; Thr62/77), ETS domain transcription factor 1 (Elk1; Ser389), and C/EBP homologous protein (CHOP) (Santa Cruz Biotechnology, Santa Cruz, CA). Bound Ab was detected with HRP-conjugated anti-rabbit IgG and visualized with ECL. Immunoreactive bands were quantified with the NIH Image software program.

DNA staining of nuclei

HF were treated as described above. Cultures incubated with 1 μM staurosporin or 200 mM sodium azide served as positive controls for apoptosis and necrosis, respectively. After selected time intervals, the cells were fixed for 10 min in 4% paraformaldehyde and stained for 15 s with Hoechst 33258 (2.5 μg/ml in PBS). Stained nuclei were viewed at 450–490 nm with an Eclipse E800 inverted microscope (Nikon, Melville, NY) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Quantification of DNA fragmentation

HF were treated with C1q tails and intact molecule for selected time intervals. The cytosolic fractions (13,000 g of supernatant) served as the Ag source in a sandwich ELISA using primary anti-histone Ab and secondary anti-DNA Ab conjugated to HRP. Enrichment of oligonucleosomes into the cytoplasm was calculated using the formula: enrichment factor = Aλ/0 of sample (dying and dead cells)/Aλ/0 of control (cells without treatment).

Flow cytometry

Monolayers were detached with nonenzymatic dissociation buffers to preserve the integrity of surface proteins. Single-cell suspensions were incubated for 1 h on ice with each of the following primary Abs (10 μg/ml HBSS): rabbit polyclonal to C1qR (68-kDa C1q binding protein; a gift of Dr. B. Ghebrehiwet (State University of New York, Stony Brook, NY); chicken polyclonal CTR (anti-N terminus; Affinity BioReagents, Golden, CO); mouse monoclonal anti-C1R (anti-CD35) and R139 (anti-126-kDa C1qRp binding protein; BD PharMingen, San Jose, CA); and anti-gC1qR (33-kDa C1q binding protein; mAb 60.11 and mAb 74.5.2; Co Vance, Richmond, CA). Control Ab consisted of irrelevant mouse isotypes IgG1k and IgG2bc (BD PharMingen), and nonimmune rabbit IgG and chicken IgY (Jackson Immunoresearch Laboratories, West Grove, PA). Cells were stained for 30 min on ice, in the dark, with Alexa Fluor 488-conjugated goat secondary Ab (1:200; Molecular Probes). Suspensions were analyzed on a FACScan using PC Lysis software (BD Biosciences, Mountain View, CA).

Blocking of cell surface cC1qR/CRT

Monolayers were preincubated for 15 min at 37°C with rabbit polyclonal to C1qR or chicken polyclonal to CRT (anti-N terminus). Nonimmune rabbit IgG or chicken IgY served as negative controls. Then, 10 μg/ml C1q tails were added to the culture medium. After 1–2 h incubation, cell extracts were examined for p38 activity.

Statistical analyses

Data are the mean ± SD of three to five independent experiments. Student’s t test was used to determine statistical significance (p < 0.05). The paired Student’s t test and the linear correlation coefficient Pearson’s (r) were used to analyze change in protein phosphorylation.

Results

C1q tails, or intact C1q, phosphorylated p38 pathway of proliferating HF

First, we measured relative quantities of phosphorylated p38 MAPK and JNK at baseline and selected time points after stimulation with 30 μg/ml intact C1q or globular heads, and with 10 μg/ml C1q tails by immunoblotting with specific Ab. Phosphorylation of p38 MAPK was maximal between 1 and 2 h (~2-fold increase; p < 0.05, n = 5) and declined below baseline after 3-h stimulation with intact C1q or tails (Fig. 1A). Instead, the kinase did not phosphorylate in response to C1q globular heads (p > 0.05, n = 5) (data not shown). Under the same conditions, phosphorylation of JNK was detected only in the lines of two donors.

![Figure 1](http://www.jimmunol.org/) Activation of fibroblast p38 MAPK, MKK3/6, and ATF-2 by C1q tails. Monolayers were stimulated with either C1q tails (10 μg/ml) or intact molecule (30 μg/ml) or intact molecule (30 μg/ml) Activity was determined by Western blots of cell extracts using Ab to phosphorylated forms of p38 MAPK (A), MKK3/6 (C), or ATF-2 (B). Immunoreactive bands were quantified by densitometry. Results are representative of four independent experiments. *, p < 0.05.
This was not due to absence of the kinase in the lines of remaining donors, because inactive JNK was detectable in all tested cultures by immunoblotting with nonphosphorylated specific Ab (data not shown). Basal phosphorylation of untreated controls was rather elevated, very likely reflecting cell activity in response to the growth factors contained in the medium (15). The Limulus assay ensured that p38 activity of the cells was not due to accidental LPS contamination in reagents or culture medium (endotoxin < 5 pg/ml).

Second, we determined that all lines phosphorylated the upstream activator of p38, MKK3/6 kinase (p < 0.05, n = 5). MKK3/6 activity was also maximal between 1- and 2-h stimulation (Fig. 1C) (16). Instead, no phosphorylation of MKK4/7, the upstream activator of JNK, was detected.

Third, we assessed that the lines phosphorylated nuclear transcription factor ATF2 (~3-fold increase; p < 0.05, n = 5) (Fig. 1B), which binds critical regions in the promoter of genes regulating cell growth (17). A similar pattern was observed during phosphorylation of Elk1 and CHOP (data not shown). Phosphorylation of c-Jun, which is activated via ERK and/or JNK, was not detected, conﬁrming that HF respond to C1q with selective activation of p38 pathway.

Assessment of apoptosis: nuclear morphology and DNA fragmentation

Upon 18- to 24-h stimulation with intact molecule or C1q tails, 40–60% of nuclei exhibited shrinkage, intense staining, and disintegration into blebs (Fig. 2, B and C). Such fragmented and condensed morphology generally correlates with the induction of apoptosis (18). The nuclei of untreated HF were uniform in size, shape, and staining (Fig. 2A). Similarly, the nuclei of cells incubated with C1q gloubular heads under the same experimental conditions did not show any alteration (data not shown). Parallel ELISAs determined that levels of chromatin fragmentation in cytosol of cells stimulated with intact molecule and C1q tails increased 3- to 5-fold (p < 0.01, n = 5) compared with untreated control (Fig. 3). The cytoplasmic enrichment in histone-bound DNA fragment is a feature of cells undergoing apoptosis (19). Together, the morphological and biochemical changes of the cultures responding to C1q tails and intact molecule suggested that the cells were withstanding stress.

Specific inhibition of p38 MAPK prevented C1q-associated apoptosis

SB203580, an inhibitor of p38 MAPK but not of MKK3/6 (20), prevented C1q-induced phosphorylation of both p38 and ATF2 (IC50 = 0.5 and 15 μM, respectively) (Fig. 4). Inhibition of p38 activity by such low concentrations virtually excluded nonspecific effects of the compound. Cell viability, as determined by the trypan blue stain, demonstrated that the inhibitor did not have cytotoxic effects. In parallel experiments, SB203580 reverted DNA fragmentation (Fig. 3). The data suggested that p38 activity was involved in the induction of apoptotic features.

Surface C1qR/CRT mediates, in part, phosphorylation of p38 MAPK

The extent to which surface binding proteins for C1q tails mediated p38 activation was determined in cultures that had been preincubated with specific Ab to CRT or cC1qR, CR1 (21), C1qRp (22), and nonimmune Ig. Immunoblotting of cell extracts revealed that only Ab to cC1qR and CRT, molecules known to be identical
controls treated with species and isotype matched nonimmune Ig.

Discussion

The study shows that C1q specifically activated p38 stress pathway of HF by virtue of its collagen-like tails. Isolated tails were more effective than intact molecule in inducing p38 activity. Previous work showed that the tails also overmatched intact C1q for HF binding, G1 mitotic arrest, and inhibition of DNA synthesis (3). In contrast, C1q globular heads did not activate p38 pathway.

The dramatic decline in p38 MAPK phosphorylation after 3-h stimulation, even in the presence of C1q, was the critical element determining that kinase activity had occurred in the cultures (24). The slow kinetics of p38 activation suggested that the C1q effect might be indirect. Cytosols of HF stimulated with C1q display high levels of type I protein kinase A (PKA-I) (3). Given that in several systems p38 MAPK is a downstream target of the cAMP/PKA-I pathway, in cultures of HF, C1q-induced PKA-I activity may be a prerequisite for p38 signaling (25, 26).

The phosphorylation of the p38 activator MKK3/6 supported the conclusion that C1q tails specifically induce p38 signaling. MKK3/6 participates in the negative regulation of cell cycle progression of transformed murine fibroblasts (27). Therefore, it will be of interest to determine the involvement of the kinase in the HF growth arrest by C1q (3).

Downstream, p38 activates multiple transcription factors. In this study, we show C1q-inducible phosphorylation of ATF2 and report phosphorylation of Elk1 and CHOP. The findings suggested that interaction of HF with C1q might be a dynamic process regulated at the transcriptional level and dependent on new gene expression.

HF responded to C1q tails and intact molecule with nuclear morphology (Fig. 2) and DNA fragmentation (Fig. 3) typical of apoptotic cells. We investigated involvement of p38 MAPK in the HF features because the kinase may operate both upstream and downstream of caspase activity in the apoptotic response (11). Blocking the kinase with inhibitor SB203580 abolished DNA fragmentation in a dose-dependent manner (Fig. 4). We concluded that sustained phosphorylation of p38 by C1q might enhance sensitivity of HF to intracellular apoptotic signals. Present findings on HF differ from the observation that C1q tails do not enhance apoptosis of quiescent rat glomerular cells (28). The discrepancy may be related to the complexity of complement-mediated apoptosis, which depends on many variables, including cell type, intensity of the stimulus, and composition of the microenvironment (29–31).

Participation of HF surface structures in p38 activity was assessed in function-blocking experiments using Ab to the cellular binding proteins for C1q that have been characterized to date. Ab to both CRT and cC1qR prevented p38 phosphorylation by ~40%, whereas Ab to the alternative C1qR was inactive. If p38 activation were mediated only by CRT, the treatment of the cells with specific Ab would have blocked phosphorylation completely. Ab binding to HF may have been weak. Given that CRT lacks a transmembrane domain, we favor, instead, an alternative explanation that functional HF receptors for C1q tails consist of complexes of immunologically distinct proteins, some of which possess membrane-spanning regions (4, 8, 24). For instance, surface CRT signals on macrophages through engagement of CD91 (32), and on endothelial cells through association with both receptor for globular heads of C1q (gC1qR)/p33 and β3 integrin (33). Integrins participate in CRT-mediated adhesion of HF to substrate-bound C1q tails (14), and β3 integrin mediates p38 MAPK activation of epithelial cells (34). Together, the observations support speculation of a cooperative role for β3 integrin also in transducing C1q signals for p38 activity in HF. Concerning participation in this event of receptor for globular domains of C1q, Table I shows that the lines used in this study were not immunoreactive with gC1qR/p33 Ab. These data, together with observation that C1q globular heads did not phosphorylate p38 MAPK, indicated that, on normal HF, partner molecules other than p31qR/p33 signal with CRT/cC1qR to activate p38 pathway.

In conclusion, we have presented data that C1q tails participate in p38 stress pathway activation and induction of apoptotic features of specific fibroblast populations with a proliferative potential. This mechanism may provide an important target for the treatment of complement-related damage in connective tissue.

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In conclusion, we have presented data that C1q tails participate in p38 stress pathway activation and induction of apoptotic features of specific fibroblast populations with a proliferative potential. This mechanism may provide an important target for the treatment of complement-related damage in connective tissue.


