The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-α

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Expression of the chitinase 3-like protein HC-gp39 (human cartilage glycoprotein 39) is associated with conditions of increased matrix turnover and tissue remodelling. High levels of this protein have been found in sera and synovial fluids of patients with inflammatory and degenerative arthritis. In order to assess the role of HC-gp39 in matrix degradation induced by inflammatory cytokines, we have examined its effect on the responses of connective tissue cells to TNF-α (tumour necrosis factor-α) and IL-1 (interleukin-1) with respect to activation of signalling pathways and production of MMPs (matrix metalloproteases) and chemokines. Stimulation of human skin fibroblasts or articular chondrocytes with IL-1 or TNF-α in the presence of HC-gp39 resulted in a marked reduction of both p38 mitogen-activated protein kinase and stress-activated protein kinase/Jun N-terminal kinase phosphorylation, whereas nuclear translocation of nuclear factor κB proceeded unimpeded. HC-gp39 suppressed the cytokine-induced secretion of MMP1, MMP3 and MMP13, as well as secretion of the chemokine IL-8. The suppressive effects of HC-gp39 were dependent on phosphoinositide 3-kinase activity, and treatment of cells with HC-gp39 resulted in AKT-mediated serine/threonine phosphorylation of apoptosis signal-regulating kinase 1. This process could therefore be responsible for the down-regulation of cytokine signalling by HC-gp39. These results suggest a physiological role for HC-gp39 in limiting the catabolic effects of inflammatory cytokines.

Key words: chondrocyte, cytokine, fibroblast, human cartilage glycoprotein 3, inflammation, matrix metalloprotease secretion.

INTRODUCTION

Human cartilage glycoprotein 39 (HC-gp39, also known as YKL-40 or Chi3L-1) belongs to the family of chi-lectins (chitinase-like lectins), which are found in vertebrates, as well as invertebrates [1–5]. The members of this protein family are structurally related to the family 18 glycohydrolases, which includes a broad range of prokaryotic and eukaryotic chitinases, glycohydrolases which cleave the β-1,4 linkage between adjacent *N*-acetyl glucosamine residues of the chitin polymer. A unique feature of chi-lectins is the lack of enzymic activity, despite the retention and conservation of the substrate-binding cleft of the chitinases [6–8]. The lack of enzymic activity is attributed to the substitution of the catalytically essential glutamic acid residue in the active site of the glycohydrolases with a neutral or hydrophobic amino acid. In spite of the abundance of molecular and structural information on these proteins, evidence for a physiological function has only recently emerged.

On the basis of reported tissue distribution, a role of HC-gp39 in tissue remodelling has been proposed [1,9]. Prominent sites of HC-gp39 production are degenerate articular cartilage and inflamed or hyperplastic synovium [1,10–12], fibrotic liver tissue [13] and gliomas, where a correlation of HC-gp39 production with malignancy has been reported [14]. The presence of HC-gp39 at sites of inflammation has been described in several studies [15,16], and the use of serum levels of this protein as a disease marker for the progression of joint erosion has been proposed [17]. Cintin et al. [18] have recently reported that serum levels of HC-gp39 correlate negatively with survival in patients with colorectal carcinomas; a similar correlation has been suggested for patients with breast carcinomas [19]. Very little is known about the distribution of HC-gp39 in normal tissues or about its role in embryonic or fetal development. One major site of synthesis is the involuting mammary gland upon cessation of lactation [20,21], however, the role of HC-gp39 in this process has not been elucidated.

We have recently reported that purified HC-gp39 stimulates the growth of connective tissue cells in concentration ranges similar to those effective for IGF-1 (insulin-like growth factor-1) [22]. This mitogenic activity is mediated by signalling through the MAPK (mitogen-activated protein kinase) and the PI3K (phosphoinositide 3-kinase) signalling pathways. The guinea-pig orthologue stimulates synthesis of the cartilage matrix component, aggrecan, in chondrocytes, in addition to cell growth [23], suggesting that this chi-lectin acts generally to promote anabolic events in connective tissues.

Normal connective tissue turnover is governed by balanced anabolic and catabolic processes. An imbalance through either aspect leads to increased production or loss of connective tissue. The tissue fibrosis observed in cirrhosis of the liver, or the synovial hyperplasia which invariably accompanies the progression of osteoarthritis, are examples of the former, while the progressive joint erosion, which is the hallmark of both inflammatory and degenerative arthritis, is an example of the latter. It is now well recognized that pathological loss of extracellular matrix, particularly that of cartilage in inflammatory and degenerative arthritis, is driven to a large extent by the inflammatory cytokines TNF-α (tumour necrosis factor-α) and IL-1 (interleukin-1),

Abbreviations used: ADAMTS, a disintegrin and metalloprotease with thrombospondin motif(s); ASK1, apoptosis signal-regulating kinase 1; chi-lectin, chitinase-like lectin; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ERK, extracellular-signal-regulated kinase; FCS, fetal calf serum; HC-gp39, human cartilage glycoprotein 39; IGF-1, insulin-like growth factor-1; IκB, inhibitory κB; IL, interleukin; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MMP, matrix metalloprotease; NF-κB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; SAPK, stress-activated protein kinase; TNF, tumour necrosis factor.

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leading to increased production of members of the MMP (matrix metalloprotease) and ADAMTS [a disintegrin and metalloprotease with thrombospondin motif(s)] families, as well as a number of chemokines. Degradation of the major cartilage matrix components type II collagen and aggrecan are thought to be due mainly to the action of the collagenases MMP1 and MMP13 and the aggrecanase ADAMTS-4 [24]. The signalling pathways utilized by TNF-α and IL-1 to increase production of MMPs and chemokines are well characterized; they include the MAPKs p38 MAPK kinase and SAPK/JNK (stress-activated protein kinase/Jun N-terminal kinase), as well as translocation of NF-κB (nuclear factor κB). To determine whether HC-gp39 would counteract the catabolic effects of TNF-α or IL-1, we analysed its effects on the activation of these major signalling pathways, as well as on the production of MMP1 and MMP3 and the chemokine IL-8. The results reported here support the hypothesis that HC-gp39 can dampen the cellular response to these inflammatory mediators.

Parts of this work have been reported previously at the 65th Annual Meeting of the American College of Rheumatology, October 2002 [24a].

EXPERIMENTAL

Materials

HC-gp39 was purified from chondrocyte-conditioned culture medium as described previously [1]. Analysis of the purified preparation by SDS/PAGE and Coomassie Brilliant Blue staining showed the presence of only one band in the 40 kDa region. The biological activity of the purified material was assessed by its capacity to activate AKT/protein kinase B in skin fibroblasts [22]. Purified HC gp-39 was stored at $-20\,^{\circ}C$ in aliquots to avoid denaturation of the protein through repeated freeze–thawing. Recombinant human IL-1β and recombinant human TNF-α were purchased from R & D Systems (Minneapolis, MN, U.S.A.). The PI3K inhibitor LY294002 and MEK-1/2 (MAPK/ERK kinase-1/2) inhibitor U0126 were obtained from New England Biolabs (Montreal, QC, Canada). ECL (enhanced chemiluminescence) Western-blotting detection reagents and Hybond ECL membranes were purchased from Amersham Biosciences (Baie d'Urfe, QC, Canada). Tissue culture supplements and media were purchased from Gibco BRL (Burlington, ON, Canada). FCS (fetal calf serum) was purchased from Biofluid (Rockville, MD, U.S.A.).

Antibodies

Anti-(phospho-p38 MAPK) (Thr^{180}/Tyr^{182}), anti-(phospho-SAPK/JNK) (Thr^{183}/Tyr^{185}), anti-(human p38 MAPK), anti-(human JNK) and anti-(phospho-AKT) (Ser/Thr) substrate antibodies, immobilized on agarose beads, as well as horseradish-peroxidase anti-rabbit immunoglobulin conjugates were purchased from New England Biolabs. Rabbit anti-(human ASK1) (apoptosis signal-regulating kinase 1; H300) and anti-(human IκB-α) (inhibitory κB-α) (C-21), goat anti-(human MMP3) (C-19), and the mouse monoclonal anti-(NF-κB p65) (F-6) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal anti-(human MMP1) and rabbit anti-(human IL-8) sera were obtained from R & D Systems. The mouse monoclanal antibody against human MMP13 (AB-1) was supplied by Oncogene (San Diego, CA, U.S.A.). Goat anti-mouse IgG coupled to horseradish peroxidase was purchased from Bio-Rad (Mississauga, ON, Canada). Alexa Fluor®488 F(ab′)2 fragment of goat anti-rabbit IgG (H + L) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Cell culture

Human fibroblasts prepared from skin biopsies of an adult male were kindly provided by Dr P. J. Roughley (Genetics Unit, Shriners Hospital for Children, Montreal, Canada). These were collected as part of a clinical study by Dr Roughley for which ethical approval had been obtained. The human chondrocytes used in the present study were derived from articular chondrocyte stocks frozen at passage level 3. The cells were prepared by collagenase digestion of cartilage harvested from the femoral chondyle and tibial plateau of a 2-year-old donor at autopsy, as described previously [25]. The collection of cartilage samples at autopsy had been approved by the chief pathologist for cases where next-of-kin had given permission for a complete autopsy. All cell types were routinely cultured as monolayers in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FCS, 2 mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin.

For analysis of signalling pathways, as well as MMP and IL-8 secretion, cells were seeded at 10^6 cells/well in 6-well cluster dishes and allowed to adhere in the presence of DMEM containing 10 % FCS for 48–72 h. The basal medium for all stimulation experiments was DMEM supplemented with 2 μg/ml BSA. Monolayers were washed 3 times with basal medium prior to stimulation with HC-gp39 or cytokines. Cells were stimulated in serum-free medium to avoid serum-induced activation of ERK1/2 and PI3K signalling pathways [26–28].

Analysis of signalling pathways

The levels of activated SAPK/JNK and p38 MAPK were determined by Western blot analysis using antibodies specific for the activated phosphorylated forms of the corresponding MAPKs. Following exposure to the agents under study, cells were lysed in 100 μl of SDS sample buffer (62.5 mM Tris/HCl, pH 6.8, 10 % glycerol, 2 % SDS, 5 % mercaptoethanol and 0.0013 % Bromophenol Blue). The samples were fractionated by SDS/PAGE on 10 % acrylamide gels, and transferred on to Hybond ECL membranes. Western blotting was performed following the manufacturer's instructions. Binding of primary antibodies was detected with horseradish-peroxidase-conjugated secondary antibodies and visualized using the ECL system.

The levels of corresponding total SAPK/JNK and p38 MAPK were determined in the same samples using pan-specific antibodies for these protein kinases. Where indicated, membranes were stripped and re-probed with the appropriate pan-specific antibody, with similar results. To determine optimal stimulation times, time-course studies were performed for activation of the signalling pathways by IL-1β and TNF-α. Phosphorylation of p38 MAPK was detectable within 5 min and remained elevated up to 6 h after addition of cytokines, with peak activation between 15 and 60 min. For all further analyses of p38 MAPK activation, cells were stimulated for 30 min. Phosphorylation of SAPK/JNK followed a narrower time course and differed for the two cytokines. The response to TNF-α reached a peak at 15 min, whereas the IL-1β response peaked at 45 min. These stimulation times were used for all further analyses of SAPK/JNK signalling. For analysis of the effect of HC-gp39 on the activation of these signalling pathways, the cells were pre-incubated for 60 min in the presence of either 100 ng/ml or various concentrations ranging from 25 to 1000 ng/ml HC-gp39 in basal medium, before addition of TNF-α or IL-1β. When selective kinase inhibitors were used, the cells were pre-incubated with either 25 μM LY294002 or 10 μM U0126 for 60 min, as recommended by the manufacturer, before addition of HC-gp39 30 min prior to addition of cytokines.

As removal of serum for extended time periods resulted in significant activation of p38 kinases in the cell culture system used in the present study, the cells were not serum-starved prior to the stimulation experiments.

Activation of the NF-κB pathway was analysed by immunocytochemistry to visualize the nuclear translocation of this transcription factor. Cells were seeded into 4-well chamber slides at 5×10^3 cells/well and allowed to adhere for 48 h in DMEM containing 10 % of FBS. The cells were then washed 3 times with 1 ml of serum-free DMEM and treated with either basal medium alone or 1 μg/ml HC-gp39 in basal medium for 60 min, followed by TNF-α (50 ng/ml) or IL-1β (5 ng/ml) for 15, 30 or 45 min. As in the above analyses, the presence of HC-gp39 was maintained during cytokine treatment of the cells. Cells were then washed 3 times with 1 ml of cold PBS and fixed with 1 % formalin in PBS for 10 min, followed by permeabilization in 1 % Triton X-100 in PBS for 30 min. The immunostaining for NF-κB p65 was performed following the manufacturer's instructions supplied with the antibody. IκB-α degradation was analysed in parallel experiments. Cells plated in 6-well culture dishes were treated with cytokines in the presence or absence of HC-gp39 as above for 20 min. The cells were lysed in SDS sample buffer and lysates were analysed for levels of IκB by SDS/PAGE and Western blotting.

AKT-mediated phosphorylation of ASK1 was analysed using an AKT substrate pull-down assay. The antibody used for these studies recognizes serine- or threonine-phosphorylated consensus motifs for AKT-mediated phosphorylation. Cells were stimulated with the appropriate agents and lysed in 200 μl of lysis buffer (20 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin) and cleared by centrifugation. Supernatants were incubated overnight with the immobilized anti-(phospho-AKT) (Ser/Thr) substrate antibody. Immunoprecipitates were washed three times with lysis buffer and solubilized in SDS sample buffer. The samples were resolved by SDS/PAGE (10 % gel) and immunoprecipitated ASK1 was detected by Western blotting using a specific antibody against ASK1. For immunoprecipitation with ASK1 antiserum, cell lysates were incubated overnight with primary antibody against ASK1, followed by adsorption to protein G–agarose. Bound protein was solubilized in SDS sample buffer and analysed by Western blotting, as above. Total ASK1 levels were also determined by Western blotting of cell lysates prior to immune precipitation.

Analysis of MMP and IL-8 production

Cells were seeded on to 6-well culture plates and allowed to adhere as described above. Pretreatment with kinase inhibitors and HC-gp39 followed the same procedure as utilized for analysis of signalling events; however, BSA was omitted from the basal medium for these experiments. Culture medium (2 ml/well) was harvested after 24 h of treatment with cytokines, dialysed against 0.1 M ammonium acetate, freeze-dried and redissolved in 100 μl of SDS sample buffer. The samples were separated by SDS/PAGE with 10 % (w/v) acrylamide gels (for MMPs) and 15 % (w/v) acrylamide gels (for IL-8), and analysed by Western blotting for IL-8, MMP1, MMP3 and MMP13, using the appropriate antibodies.

RESULTS

Elevated levels of HC-gp39 are found in pathological conditions, such as rheumatoid arthritis, with increased local production of inflammatory cytokines, particularly TNF-α and IL-1. Many of the cellular responses to these cytokines are mediated through activation of the SAPK/JNK and p38 MAPK pathways, as well as through the nuclear translocation of NF-κB. Since we had previously shown that HC-gp39 could activate MAPK-mediated signalling cascades in connective tissue cells [22], its effects on cytokine-induced signalling was therefore investigated.

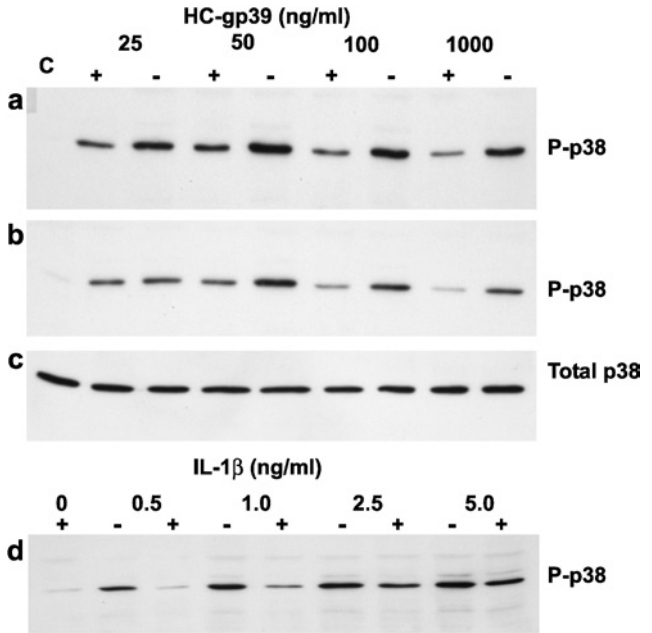


Figure 1 HC-gp39 reduces p38 kinase phosphorylation in response to cytokines

Skin fibroblasts were treated for 30 min with 2.5 ng/ml IL-1β (**a**) or 25 ng/ml TNF-α (**b** and **c**) in the absence (−) or presence (+) of HC-gp39 at concentrations from 25–1000 ng/ml. p38 phosphorylation was also analysed in cells in the absence (−) or presence (+) of 100 ng/ml of HC-gp39 and IL-1β at concentrations ranging from 0 to 5 ng/ml (**d**). Cell lysates were analysed by SDS/PAGE and Western blotting for phosphorylated p38 kinase (P-p38) (**a**, **b** and **d**) and total p38 kinase (**c**). Total p38 kinase is only shown for cells stimulated with TNF-α, the profile of total p38 kinase was similar for the cell extracts from cells stimulated with IL-1β.

HC-gp39 reduces activation of p38 and SAPK/JNK MAPKs by IL-1 and TNF-α

Both p38 MAPK and SAPK/JNK were activated in skin fibroblasts and chondrocytes following exposure of the cells to TNF-α or IL-1 in a dose- and time-dependent fashion. A significant reduction in this activation of p38 (Figure 1) and SAPK/JNK MAPKs (Figure 2) was observed when skin fibroblasts were stimulated with either IL-1 or TNF-α in the presence of HC-gp39 at concentrations ranging from 0.6 to 24 nM (25–1000 ng/ml, Figures 1a, 1b, 2a and 2b). The antibody used for analysis of SAPK/JNK activation recognizes the phosphorylated forms of all isoforms of this MAPK. There seems to be no preferential activation by either cytokine, nor is there a differential suppression of activation by HC-gp39.

In the presence of a constant amount of HC-gp39 (100 ng/ml or 2.4 nM) the suppressive effect of HC-gp39 on both signalling pathways was overcome by increased cytokine concentrations (Figures 1d and 2d for p38 and SAPK/JNK respectively), indicating that the extent of activation may be controlled by the balance in concentrations between HC-gp39 and the inflammatory cytokines. Addition of HC-gp39 alone to skin fibroblasts at concentrations of 5 to 1000 ng/ml did not result in phosphorylation

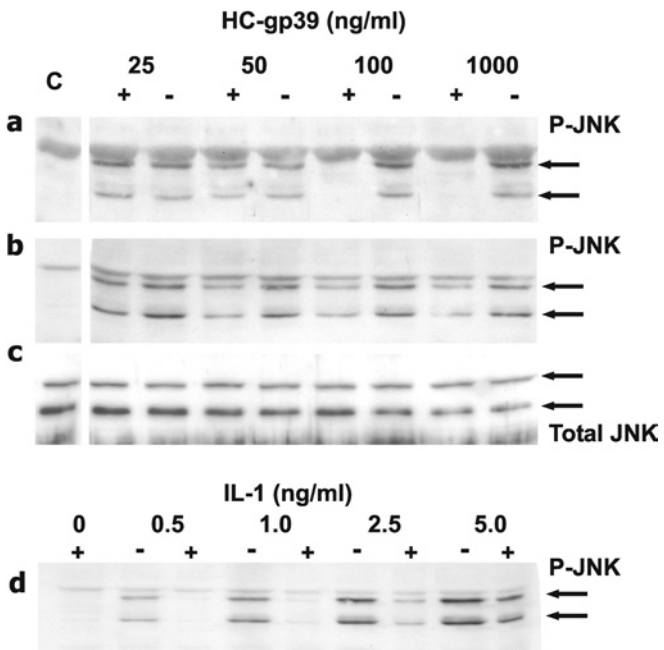


Figure 2 Inhibition of SAPK/JNK activation by HC-gp39

Skin fibroblasts were treated for 15 min with 2.5 ng/ml IL-1β (**a**) or 25 ng/ml TNF-α (**b** and **c**) in the absence (−) or presence (+) of HC-gp39 at concentrations from 25–1000 ng/ml. SAPK/JNK phosphorylation was also analysed in cells in the absence (−) or presence (+) of 100 ng/ml of HC-gp39 and IL-1β at concentrations ranging from 0 to 5 ng/ml (**d**). Cell lysates were analysed by SDS/PAGE and Western blotting for phosphorylated SAPK/JNK (P-JNK) (**a**, **b** and **d**) and total SAPK/JNK (**c**). The migration positions of the 46 and 55 kDa isoforms of JNK1/2 are indicated by arrows. Total SAPK/JNK is only shown for cells stimulated with TNF-α, the profile was similar for the cell extracts from cells stimulated with IL-1β.

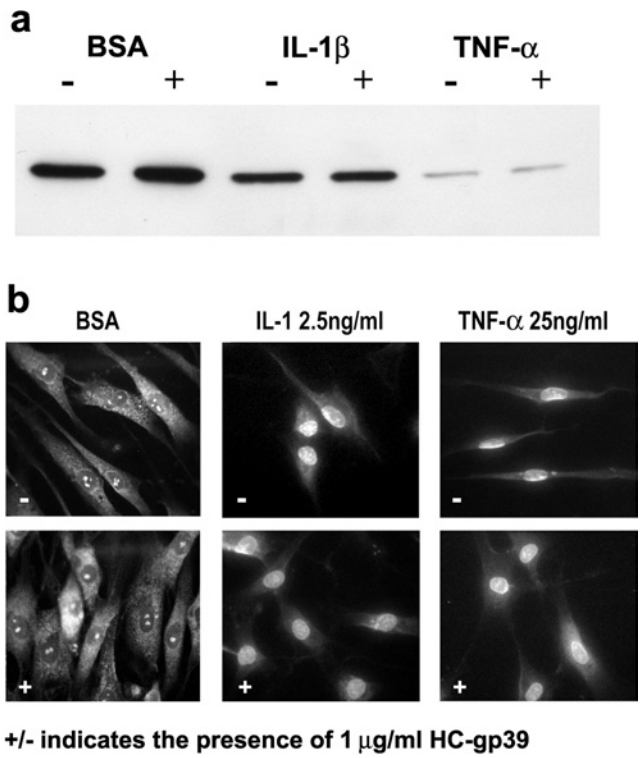


Figure 3 HC-gp39 does not affect NF-κB signalling

NF-κB signalling was analysed by determining levels of the inhibitor IκB in cell lysates following stimulation for 20 min with IL-1β or TNF-α in the presence (+) or absence (−) of HC-gp39 at 1 μg/ml (**a**). In addition, nuclear translocation of NF-κB following stimulation with IL-1β or TNF-α was analysed immunohistochemically (**b**). As in (**a**), cells were stimulated in the presence or absence of 1 μg/ml HC-gp39 and fixed after 15 min of incubation. Immunolocalization of NF-κB was performed on permeabilized cells, utilizing a primary specific antibody and a fluorophore-conjugated secondary antibody, as described in the Experimental section.

of either p38 MAPK or SAPK/JNK over a time period of up to 72 h. Similar negative results were observed in passaged human articular chondrocytes and synovial fibroblasts. The time course of p38 or SAPK/JNK activation was not shifted in the presence of HC-gp39, and total cellular kinase levels were not affected by treatment with either cytokine or HC-gp39. Suppression of TNF-α and IL-1 signalling by HC-gp39 was not unique to skin fibroblast, as this effect was also observed in human synovial fibroblasts and articular chondrocytes (results not shown).

NF-κB signalling is not affected by HC-gp39

Activation of NF-κB is required for many of the biological actions of TNF-α and IL-1. We therefore determined if HC-gp39 could also modulate the extent of signalling through this pathway. Treatment of skin fibroblasts with either IL-1 (5 ng/ml) or TNF-α (50 ng/ml) resulted in rapid degradation of I-κB (Figure 3a) and nuclear translocation of NF-κB (Figure 3b). Treatment of cells with HC-gp39 at concentrations of 5 to 1000 ng/ml did not activate either process. Pretreatment of the cells with HC-gp39 and stimulation with either cytokine in the presence of HC-gp39 did not affect IκB degradation and nuclear translocation of NF-κB (Figure 3). Thus it is not likely that HC-gp39 affects any cellular responses that are mediated through the NF-κB pathway.

HC-gp39 reduces IL-1- and TNF-α-stimulated production of MMPs and IL-8

The ultimate response of many connective tissue cells to TNF-α or IL-1, leading to the development of tissue pathology, is the increased production of MMPs and chemokines, which are responsible for the increased turnover and damage of the extracellular matrix associated with inflammatory conditions. MMP1 (or collagenase-1) and MMP3 (stromelysin) are both strongly induced in connective tissue cells by IL-1 and TNF-α, as is the prototypic chemokine, IL-8. Both the p38 and SAPK/JNK signalling cascades have been implicated as mediators for the increased production of these agents, suggesting that their induction could be affected by HC-gp39. Confluent skin fibroblast cultures were exposed to IL-1β (1 ng/ml) or TNF-α (10 ng/ml) in the presence or absence of 100 ng/ml HC-gp39. These concentrations resulted in strong induction of MMP1, MMP3 and IL-8 secretion (Figure 4). HC-gp39 alone did not affect either IL-8 or MMP3 production by skin fibroblasts, whereas MMP1 levels were increased marginally, compared with the cytokine response. However, in the presence of either IL-1 or TNF-α, a strong reduction in the production of both MMPs and IL-8 was observed. There was no increase in intracellular MMP1, MMP3 or IL-8 following cytokine exposure in the presence of HC-gp39, indicating that the secretion process was not affected. The reduced production of MMPs and IL-8 in the presence of HC-gp39 was accompanied by reduced levels of mRNA for these molecules, as determined by reverse-transcriptase PCR (results not shown), suggesting that this effect was due to either decreased transcriptional activity and/or increased turnover of mRNA.

When human articular chondrocytes in monolayer cultures were stimulated with either IL-1β or TNF-α in the presence of

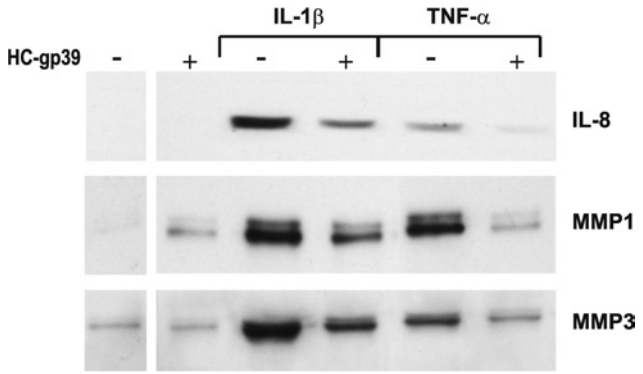


Figure 4 Reduction of cytokine-induced secretion of IL-8 and MMPs by HC-gp39

Skin fibroblasts were stimulated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) in the presence (+) or absence (−) of HC-gp39 (100 ng/ml), as described for Figure 1. Culture medium was collected after 24 h and analysed for IL-8, MMP1 or MMP3 levels by Western blotting, using specific antibodies as described in the text.

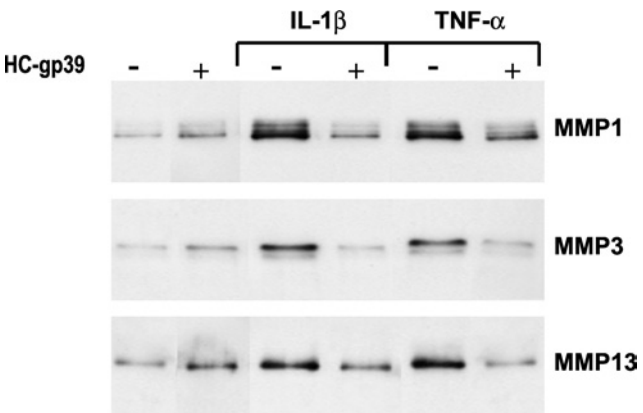


Figure 5 Effect of HC-gp39 on MMP production by human articular chondrocytes

Monolayers of human chondrocytes were stimulated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) in the absence (−) or presence (+) of 100 ng/ml HC-gp39. Culture medium was collected 24 h later and analysed for the presence of MMP1, MMP3 and MMP13, using specific antibodies and Western blotting, as for Figure 4.

HC-gp39, a similar reduction of MMP secretion was observed (Figure 5). In addition to MMP1 and MMP3, the accumulation of MMP13 in the culture medium was also down-regulated by HC-gp39.

Down-regulation of the cellular response to inflammatory cytokines by HC-gp39 depends on the PI3K pathway

We have shown previously that HC-gp39 induces PI3K-mediated phosphorylation of AKT very effectively [22]. Activation of AKT interferes with stress-induced signalling and promotes the survival of cells [29], thus the inhibitory effects of HC-gp39 on TNF-α/IL-1 signalling could be mediated through this pathway. As HC-gp39 also activates the ERK1/2–MAPK pathway, the inhibitors LY294002 (for PI3K) and U0126 (for MEK1/2) were used to determine the contribution of each pathway in the down-regulation of cytokine signalling through the p38 and JNK pathways by HC-gp39. Skin fibroblasts were stimulated with IL-1 or TNF-α in the absence or presence of HC-gp39 and the kinase inhibitors. Both p38 and SAPK/JNK activation were suppressed by HC-gp39, as above (Figure 6). This effect was abolished by inhibition of PI3K, but not MEK1/2. In the absence of HC-gp39, neither inhibitor affected cytokine-mediated activation of p38 kinase nor JNK at the concentrations and stimulation times used in the present study. Thus the modulation of cytokine signalling by HC-gp39 is mediated mainly through the PI3K-signalling cascade.

A similar analysis was performed for the production of the effector molecules, MMP1, MMP3 and IL-8 in response to IL-1 or TNF-α stimulation (Figure 7). Skin fibroblasts were cultured for 24 h in the presence of the cytokines and combinations of HC-gp39 and LY294002 (10 μM) or U0126 (5 μM). Lower inhibitor concentrations were used in these experiments to minimize effects on cell viability due to the longer exposure times. Although neither inhibitor affected the amount of MMP1, MMP3 or IL-8 produced in the presence of IL-1 or TNF-α, the suppressive effect of HC-gp39 was removed by the PI3K inhibitor LY294002, but unaffected by inhibition of ERK1/2-mediated pathways. Thus, as was observed for the activation of the stress-induced signalling pathways by the cytokines, these results implicate the involvement of the PI3K-signalling cascade in mediating the effects of HC-gp39.

Stimulation of ASK1 phosphorylation by HC-gp39

The activity of ASK1 is pivotal for the transmission of stress- and cytokine-induced cellular responses to the p38 and SAPK/JNK pathways, particularly in the induction of apoptosis by TNF-α. Thus regulation of its activity could provide a sensitive control mechanism for determining the cellular fate. Kim et al. [30] have shown that activation of AKT through the PI3K pathway can lead to inhibition of ASK1, and this may be a result of association between the two kinases and phosphorylation of ASK1 by AKT. We therefore investigated whether HC-gp39 would induce AKT-mediated phosphorylation of ASK1, using immunoprecipitation with an antibody recognizing serine/threonine-phosphorylated AKT substrates, followed by Western blotting with an antibody against ASK1. Treatment of skin fibroblasts with 100 ng/ml of HC-gp39 resulted in a transient phosphorylation of ASK1, which was maximal at 1 h and undetectable by 2 h (Figure 8a). The effect was dose dependant, as a response could be detected at 50 ng/ml of HC-gp39 (Figure 8b). While there was no effect on this process by the MEK1/2 inhibitor U0126, the PI3K inhibitor LY294002 abolished phosphorylation of ASK1 in the presence of HC-gp39, supporting the suggestion that HC-gp39 induces this process via PI3K-mediated activation of AKT. IL-1 and TNF-α stimulate tyrosine phosphorylation of ASK1, which is not recognized by the AKT substrate antibody (Figure 9). However, more importantly, the HC-gp39-induced phosphorylation of ASK1 does not appear to be affected by the presence of the cytokines at the concentrations used. These findings suggest that the suppressive effect of HC-gp39 on the cellular responses to IL-1 or TNF-α is mediated through inhibition of ASK1 signalling and reduced propagation of the inflammatory signals downstream from this point.

DISCUSSION

HC-gp39 belongs to a family of chitinase-3-like proteins with hitherto ill-defined functions *in vivo*. Similar to the imaginal disk growth factors in *Drosophila*, HC-gp39 stimulates growth in certain cell populations [22,23], although, unlike the imaginal disk growth factors [3], there is no requirement for insulin. The structural fold of these proteins suggests a lectin-like function

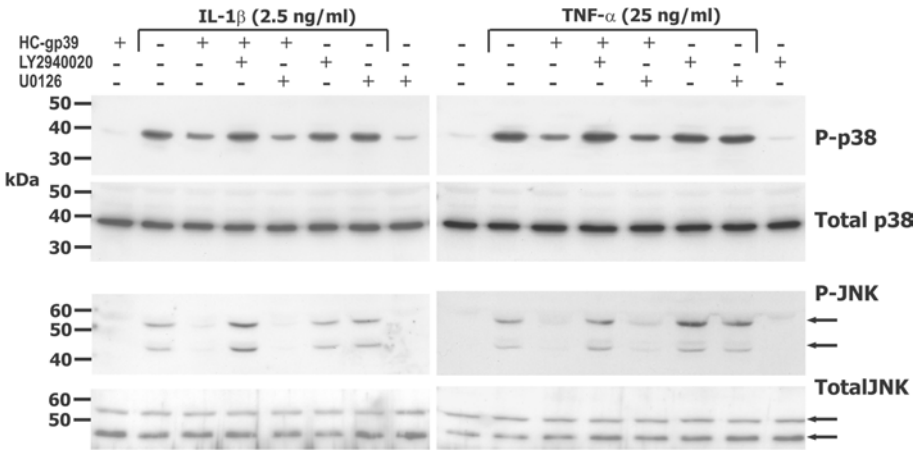


Figure 6 Suppression of cytokine signalling by HC-gp39 requires PI3K activity

To evaluate the contribution of MAPK and PI3K signalling on the effect of HC-gp39, skin fibroblasts were pre-treated for 60 min with the MEK-1/2 inhibitor U0126 or the PI3K inhibitor, LY294002, in addition to HC-gp39 (100 ng/ml), as indicated above the panels. Cells were then stimulated with cytokines, and p38 and JNK phosphorylation was analysed as described in the Experimental section. The arrows indicate the migration positions of the 46 and 55 kDa isoforms of JNK1/2. Phosphorylation of both p38 and SAPK/JNK in response to cytokines is restored to normal levels when PI3K is inhibited, but not when MEK-1/2 is inhibited. P-p38, phospho-p38; P-JNK, phospho-JNK.

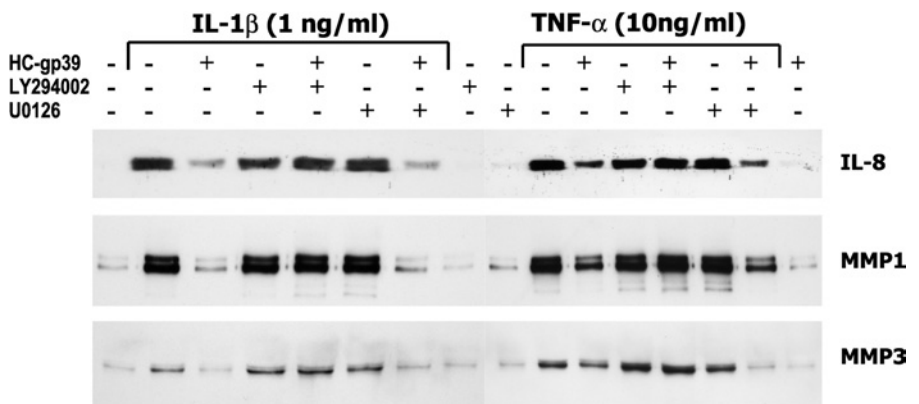


Figure 7 Suppression of the cytokine response by HC-gp39 requires PI3K activity

The contribution of MAPK and PI3K signalling to the reduction in the secretion of cytokine-induced effector molecules by HC-gp39 was analysed in skin fibroblasts treated as described for Figure 6, except that culture medium was collected after 24 h of stimulation with TNF-α (10 ng/ml) or IL-1 (1 ng/ml), and analysed for the presence of IL-8, MMP1 and MMP3 by Western blotting, as described in the Experimental section. The levels of all three effector molecules in the culture media return to normal in the presence of HC-gp39 when PI3K is inhibited by LY294002, but not in the presence of U0126 that inhibits MEK-1/2.

[31–33], although the physiological ligands are currently unknown, and thus the collective term chi-lectins has been proposed [8,31].

The results of the present study show that this protein can down-regulate the catabolic or degradative aspects of the inflammatory response by connective tissue cells and this may, in fact, be its major role in mammals. The sites of synthesis of HC-gp39 are consistent with such a role. Thus HC-gp39 is expressed in rheumatoid synovium [1,12], and, most prominently, as a late stage event in macrophage activation [12,13,34]. The production and role of both TNF-α and IL-1 in inflammation is well established, and a pivotal role has been ascribed to these cytokines in the chronicity of rheumatoid arthritis (as reviewed by Recklies and Poole [35] and many others). The levels of HC-gp39 reported in synovial fluid of patients with rheumatoid arthritis or osteoarthritis range from approx. 20 to 2000 ng/ml [36,37], which is well within the concentration range effective for modulation of cytokine-induced signalling pathways and production of effector molecules. Whether HC-gp39 interacts with other components of synovial fluid which affect its activity remains to be established.

The processes affected by HC-gp39, which were investigated in the present study, are those shared by the two cytokines, such as increased production of matrix-degrading enzymes and chemokines. Depending on the cell type, TNF-α may elicit different responses, ranging from induction and further amplification of inflammatory signals in cells of the immune system to apoptosis to increased catabolic activity, resulting in destruction of the extracellular matrix. The concomitant production of HC-gp39 in these circumstances may serve to limit the catabolic effects of TNF-α, without affecting other responses. Whether HC-gp39 also affects responses which are uniquely elicited by TNF-α, such as activation of monocytes and T-cell function or induction of apoptosis in responsive cells, remains to be established. However, cell survival in the presence of adverse stimuli, such as oxidative stress, has been linked to activation of AKT [29], as has the resistance of rheumatoid synovial fibroblasts to induction of

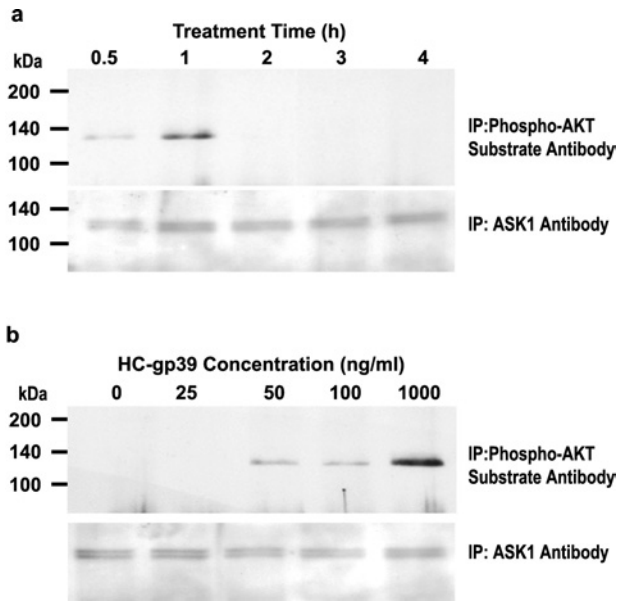


Figure 8 HC-gp39 induces AKT-mediated serine/threonine phosphorylation of ASK1

Skin fibroblasts were incubated in the presence of 100 ng/ml of HC-gp39 for various time periods (**a**) or for 45 min in the presence of HC-gp39 at the indicated concentrations (**b**). Cell lysates were immunoprecipitated with an antibody recognizing phospho-AKT substrates or with an antibody against ASK1, as indicated. Immunoprecipitates (IP) were separated by SDS/PAGE, and ASK1 was identified by Western blotting. Total ASK1 levels were also analysed by Western blot in the cell lysates prior to immunoprecipitation and found to be equivalent following treatment with HC-gp39 (results not shown).

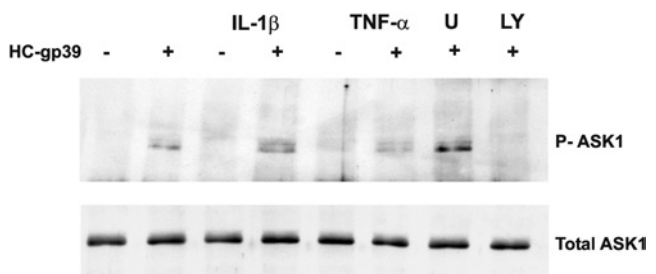
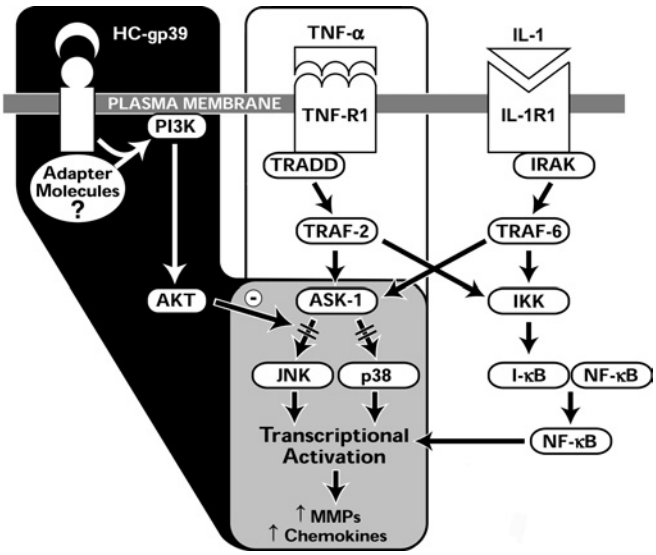


Figure 9 TNF-α or IL-1β do not affect the AKT-mediated serine/threonine phosphorylation of ASK1 in the presence of HC-gp39

Skin fibroblasts were incubated in the presence (+) or absence (−) of HC-gp39 (100 ng/ml) and either TNF-α or IL-1β, as indicated, for 45 min. Cells were also pre-treated for 60 min with U0126 (U) or LY294002 (LY) prior to stimulation with HC-gp39. Cell lysates were analysed for serine/threonine phosphorylated ASK1(P-ASK1), as described for Figure 8. Neither cytokine affected the pattern of HC-gp39-stimulated ASK1 phosphorylation. Total ASK1 levels in cell lysates prior to immunoprecipitation were analysed by Western blotting.

apoptosis by TNF-α [38], and thus it is likely that cell-survival may also be affected by the presence of HC-gp39.

Both the growth factor and the anti-catabolic activities of HC-gp39 utilize the PI3K-signalling pathways. Inhibition of the activity of this protein kinase abolishes the suppressive effects of HC-gp39, both at the levels of signal transduction, as measured by activation of p38 and SAP/JNK and at the level of the final outcome, namely production of MMPs and IL-8. PI3K-mediated activation of the threonine/serine PKB (AKT) is a prominent signalling response of all connective tissue cells examined (skin fibroblasts, articular chondrocytes, synovial fibroblasts) exposed



Scheme 1 Major signalling cascades mediating responses to TNF-α and IL-1 in connective tissue cells and the regulatory pathway utilized by HC-gp39

For the sake of clarity, only selected components of the signalling cascades are illustrated, and TNF-α signalling is represented by its interaction with its R1 receptor (TNF-R1). The activation of ASK1 following binding of TNF-α or IL-1 to their respective receptors involves several specific kinase-mediated steps and adapter molecules in addition to those shown here. HC-gp39 binding to a putative receptor leads to PI3K-mediated phosphorylation of AKT, which can down-regulate ASK1 activity by catalysing phosphorylation of Ser^{83} [30]. This in turn leads to decreased activation of JNK and p38, and finally decreased production of MMPs and chemokines. Activation of the NF-κB pathway is mediated at the level of TRAF-2 and TRAF-6, upstream of ASK1, and thus is not affected by HC-gp39. TRADD, TNF receptor 1-associated via death domain; TRAF, TNF receptor-associated factor; IRAK, IL-1-receptor-associated kinase.

to HC-gp39 [22], and thus any process which is regulated by this pathway could be modulated by HC-gp39. We thus propose that the anti-catabolic effect of HC-gp39 involves activation of AKT leading to the attenuation of ASK1-mediated signalling pathways, which are initiated by binding of cytokines to their cellular receptors. This is shown in Scheme 1.

The effectiveness of HC-gp39 to suppress TNF-α- or IL-1-induced MMP and IL-8 production suggests that in these cells and under the culture conditions used in the present study, the SAPK/JNK and p38 MAPK pathways are the predominant mediators of MMP and chemokine induction. Since nuclear translocation of NF-κB was unaffected in the presence of HC-gp39, cellular responses dependent mainly on this pathway should proceed unimpeded. Although evidence exists for predominant roles of either the NF-κB or the p38 and SAPK/JNK pathways in the regulation of cellular responses to the inflammatory cytokines IL-1 and TNF-α, it is becoming increasingly clear that there is significant cross-talk between the different signalling cascades. Thus while IL-8 production in response to cytokines was thought to be mainly controlled by the proximal NF-κB binding site and thus by signalling through the NF-κB cascade, more recent evidence suggests input from the more distal AP1 (activator protein 1) site and signalling through the SAPK/JNK and p38 pathways [39,40], the common point being activation of ASK1. Our results show that serine/threonine phosphorylation of ASK1 by AKT is stimulated by HC-gp39. It has been demonstrated that AKT-mediated phosphorylation of ASK1 leads to inhibition of its activity [30,41], and thus reduced activation of the p38 and SAPK/JNK pathways. The role of ASK1 in the transduction of TNF-α signalling and the activation of SAPK/JNK and p38 kinases is well established [42,43], and Brauchle et al.

[44] have shown that ASK1 is a strong activator of the MMP1 promoter. These observations support the suggestion that the inhibition of inflammatory signalling by HC-gp39 may occur through inhibition of ASK1.

Estève et al. [45] have recently reported a similar, AKT-mediated inhibition of MMP9 (gelatinase B) expression in C6 glioma cells. These authors show that platelet-derived growth factor counteracts induction of MMP9 by IL-1 or TNF-α in a PI3K-dependent manner, suggesting a mechanism similar to the one proposed for HC-gp39. However, in contrast with these studies, inhibition of PI3K did not increase MMP1 and MMP3 or IL-8 production in skin fibroblasts in response to either cytokine. It is interesting to note that a subgroup of human glioblastomas expressing extremely high levels of HC-gp39 expresses very low levels of a MMPs, including MMP9, at least at the message level [14]. The causal relationship between these two events remains to be established.

Whether HC-gp39 can down-regulate the expression of other classes of matrix-degrading enzymes remains to be established. The IL-1- and TNF-α-induced degradation of the major cartilage proteoglycan, aggrecan, is now thought to be due mainly to the action of members of the ADAMTS family, particularly ADAMTS-4 and ADAMTS-5 [24]. While ADAMTS-5 is expressed constitutively, there is still considerable controversy as to whether cytokine-induced expression of ADAMTS-4 activity is regulated transcriptionally [24] or at the level of activation of a constitutively produced proenzyme [46].

The cellular responses to HC-gp39 are similar to those elicited by IGF-1, and both proteins act synergistically with respect to their growth-stimulating activity [22]. IGF-1 has been reported to inhibit MMP induction and collagen degradation in chondrocytes treated with a combination of IL-1 and oncostatin M [47]. Although it is not known which signalling pathways are affected in this system, IGF-1 is a strong activator of PI3K [48–50] and could exert its protective effects in a similar fashion to HC-gp39.

The ability of HC-gp39 to initiate several signalling cascades in connective tissue cells suggests the existence of a specific receptor for this protein. The nature of this receptor remains to be elucidated. It is unlikely that HC-gp39 affects TNF-α or IL-1 signalling through direct interaction and inactivation of these molecules, since TNF-α-mediated activation of the NF-κB pathway proceeds unimpeded in the presence of HC-gp39. Similarly we have ruled out interaction of HC-gp39 with the IGF-1 signalling axis, as inhibition of cytokine signalling was still observed in the presence of a neutralizing antibody of the IGF-1 receptor.

The molecular structure of HC-gp39 has recently been determined and a high structural similarity with the human chitinase CHIT1 was observed [31], particularly in the active site region. In contrast to suggestions by Mohanty et al. [32], HC-gp39 was found to bind *N*-acetylglucosamine oligomers, although with much lower affinity than the nanomolar concentrations of the protein effective for stimulation of AKT phosphorylation. Although such carbohydrate structures are not present in vertebrates, the largest one being the chitobiose of the linkage region of N-linked oligosaccharides, this indicates that HC-gp39 contains an effective ligand-binding site, and that the physiological ligand may very well be an oligosaccharide. A lectin-like function has been reported for the murine family member Chi3L3 (YM-1 or eosinophil chemotactic factor), which binds with relatively high affinity to glucosamine oligomers [8,51]. However, the physiological relevance of this is unclear, as these structures are not known to exist in vertebrates, and a not-yet-identified oligosaccharide ligand is postulated. The concentrations of Chi3L3 required to stimulate eosinophil chemotaxis [4] are about 2 orders of magnitude higher than those required for stimulation of cell growth [22] or for inhibition of cytokine responses by HC-gp39, as demonstrated in the present study. Whether this particular biological activity of Chi3L3 is indeed its main physiological function and whether this depends on its ligand-binding capacity, remains to be established.

The capacity of HC-gp39 to down-regulate cytokine-induced MMP and chemokine production by connective tissue cells suggests that this protein plays an important role in regulating the inflammatory response of tissues. The expression of HC-gp39 in association with normal tissue turnover, such as involution of the mammary gland [20,21], indicates that it may play a protective role in this situation, limiting the turnover of the mammary gland extracellular matrix. The existence of signalling pathways responsive to HC-gp39 suggest that the binding specificity of this protein could be exploited in the development of therapeutic agents limiting the excessive matrix degradation characteristic of inflammatory and degenerative joint diseases.

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