The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways

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Human cartilage glycoprotein 39 (HC-gp39) is a glycoprotein secreted by articular chondrocytes, synoviocytes and macrophages. Increased levels of HC-gp39 have been demonstrated in synovial fluids of patients with rheumatoid or osteoarthritis. The increased secretion of HC-gp39 under physiological and pathological conditions with elevated connective-tissue turnover suggests its involvement in the homoeostasis of these tissues. We report here that HC-gp39 promotes the growth of human synovial cells as well as skin and fetal lung fibroblasts. A dose-dependent growth stimulation was observed when each of the fibroblastic cell lines was exposed to HC-gp39 in a concentration range from 0.1 to 2 nM, which is similar to the effective dose of the well-characterized mitogen, insulin-like growth factor-1. At suboptimal concentrations, the two growth factors work in a synergistic fashion. The use of selective inhibitors of the mitogen-activated protein kinase and the protein kinase B (AKT) signalling pathways indicates that both are involved in mediating the mitogenic response to HC-gp39. Phosphorylation of both extracellular signal-regulated kinases 1/2 and AKT occurred in a dose- and time-dependent fashion upon addition of HC-gp39. Activation of these signalling pathways could also be demonstrated in human chondrocytes. Thus HC-gp39 initiates a signalling cascade in connective-tissue cells which leads to increased cell proliferation, suggesting that this protein could play a major role in the pathological conditions leading to tissue fibrosis.

Key words: chondrocyte, growth factor, mitogen-activated protein kinase (MAP kinase), mitogenesis, synovial cell.

INTRODUCTION

Human cartilage glycoprotein 39 (HC-gp39) is secreted by a variety of mammalian cells. Based on structural homology, it belongs to the glycohydrolase family 18, which comprises a large number of bacterial and eukaryotic chitinases. In mammals, five members of this protein family have been described [1–4]. Two of these, mammalian chitinase-1, or chitotriosidase, and the recently identified acidic mammalian chitinase [5], are capable of hydrolysing the β 1,4 linkage between the adjacent *N*-acetylglucosamine residues of chitin oligomers or colloidal chitin [6]. None of the other mammalian proteins display any glycohydrolase activity.

Although a major secreted product of cultured human articular chondrocytes or cartilage explants, HC-gp39 is not generally synthesized by healthy chondrocytes *in vivo*. However, the presence of mRNA for HC-gp39 has been noted in osteoarthritic cartilage [1] and confirmed by hybridization *in situ* [7]. The presence of the protein was demonstrated by immunolocalization in chondrocytes of degenerate cartilage [8]. Johansen et al. reported increased levels of HC-gp39 in synovial fluids and sera from patients with both osteoarthritis [9] and rheumatoid arthritis [10], and a positive correlation between HC-gp39 levels and disease activity was noted [9]. HC-gp39 levels tended to decrease with various treatment modes, suggesting that the accumulation of HC-gp39 is a result of processes related to the pathogenesis of joint degeneration and/or inflammation.

HC-gp39 secretion has also been demonstrated from a number of other cell types, such as smooth-muscle cells [11], synovial cells [12] and macrophages [13–15], where its synthesis precedes that of the closely related, enzymically active mammalian chitinase-1 [3]. In fact, it has been suggested that HC-gp39 secretion is a late-differentiation event of monocytes [13]. Increased circulating levels of HC-gp39 have also been reported in patients with breast cancer [16] and colorectal cancer [17] as well as in patients with cirrhosis of the liver [18]. In addition to these pathological conditions, expression of the murine and bovine equivalents of HC-gp39 has been observed in the early stages of mammary gland involution following cessation of lactation [19,20]. The expression pattern of HC-gp39 suggests that its production is related to situations where increased connective-tissue remodelling occurs, and that it may play a role in events associated with increased turnover of extracellular matrix.

A unifying feature of the mammalian chitinase-like proteins is their molecular structure, which consists of an essentially complete chitinase-3-like catalytic domain of approx. 40 kDa. However, with the exception of the mammalian chitinase-1 produced by macrophages [6,21], they do not possess glycohydrolase activity. This lack of activity can be explained by the non-conservative substitution of an essential glutamic acid residue in the active site of these proteins [22,23] with leucine (in the case of HC-gp39) or glutamine (in Chi3-L2 or eosinophil chemotactic factor). The remainder of the sequence, which forms the active-

Abbreviations used: bpV, potassium bisperoxo (1,10-phenanthroline)oxovanadate; HC-gp39, human cartilage glycoprotein 39; HFL, human fetal lung fibroblast; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase and ERK kinase; PI-3K, phosphoinositide 3-kinase; AKT, protein kinase B; IGF-1, insulin-like growth factor-1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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site cleft in the chitinases, is very highly conserved amongst all members of glycohydrolase family 18, suggesting that the overall structure is very similar in the enzymically active and inactive proteins. The existence of chitinase-3-like proteins containing substitutions of the catalytically essential glutamic acid residue in the active-site cleft is not restricted to mammals, as inspection of the genomic databases for the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the zebra fish *Danio rerio* reveals the presence of multiple putative chitinase-3-like proteins in these organisms.

Although HC-gp39 was identified in 1993, its function has until recently remained elusive. Kawamura et al. [24] showed that a family of growth factors for imaginal disc cells of the fruit fly belongs to the chitinase-3 like protein family, suggesting that the mammalian counterparts of these proteins might have similar mitogenic properties. This suggestion was indeed confirmed by the recently published observation that the guinea-pig homologue of HC-gp39 promotes proliferation in chondrocytes and synovial cells [25]. We had found that HC-gp39 stimulated DNA synthesis and proliferation of human connective-tissue cells at subnanomolar concentrations (reported at the 64th Annual Meeting of the American College of Rheumatology, Philadelphia, PA, U.S.A., in November 2000 [26]). The work described here extends these observations to demonstrate that HC-gp39 activates both extracellular signal-regulated kinase (ERK)- and protein kinase B (AKT)-mediated signalling cascades, which are associated with the control of mitogenesis.

EXPERIMENTAL

Materials

Recombinant human insulin-like growth factor-1 (IGF-1) was purchased from R&D Systems (Minneapolis, MN, U.S.A.), potassium bisperoxo (1,10-phenanthroline)oxovanadate (bpV) was from Sigma (St. Louis, MO, U.S.A.), [^{3}H]thymidine was from New England Biolabs (Montreal, QC, Canada) and fetal calf serum (FCS) was from Biomedia (Drummondville, QC, Canada). Other tissue-culture supplies were obtained from Flow Laboratories (Mississaga, ON, Canada). The dye Hoechst 3358 was supplied by Molecular Probes (Eugene, OR, U.S.A.). Rabbit antisera specific for the phosphorylated forms of the ERK1/ERK2 mitogen-activated protein (MAP) kinases and of AKT, as well as pan-specific antisera to these kinases and horseradish peroxidase anti-rabbit Ig conjugates, were obtained from New England Biolabs. Enhanced chemiluminescence reagents for visualization of bound Igs in Western blots were from Amersham Bioscience (Montreal, QC, Canada). The MAP kinase and ERK kinase (MEK) inhibitors PD98059 and U0126, and the phosphoinositide 3-kinase (PI-3K) inhibitor LY294002, were obtained from New England Biolabs.

HC-gp39 was purified from culture supernatants of human articular chondrocytes as described previously [1] and its purity was assessed by SDS/PAGE and staining with Coomassie Brilliant Blue. N-terminal sequencing of the purified preparation indicated the presence of only one protein, consistent with the sequence of HC-gp39. Protein content was determined using the Bradford assay [27]. Molar concentrations for the purified preparations were calculated based on a molecular mass of 42000 Da. Purified HC-gp39 was stored frozen at -20 °C in aliquots to avoid repeated freeze-thawing and denaturation of the protein. Recombinant human chitinase-1 (chitotriosidase) was prepared using the *Pichia pastoris* expression system (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions (J. Krupa and A. D. Recklies, unpublished work).

Cell culture

Human synovial cells were prepared from tissue obtained at joint arthroplasty from patients with osteoarthritis. The tissue was digested with trypsin and bacterial collagenase as described by Golds et al. [28] and cells were stored frozen following expansion. Human fibroblasts prepared from skin biopsies of an adult male were provided kindly by Dr P. J. Roughley (Genetics Unit, Shriners Hospital for Children, Montreal, QC, Canada). The human fetal lung fibroblast (HFL) cell line, HFL-1, was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Frozen stocks of this cell line were expanded through two or three passages before use.

Human chondrocytes were prepared from cartilage obtained at autopsy from a 2-year-old individual. Cells were isolated as described previously [29] and stored frozen after passage 1. For the work described here frozen chondrocytes were expanded and used between passage levels 3 and 5. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS and 100 μg/ml penicillin/100 units/ml streptomycin in an environment of 95 % air/5 % CO_2.

Assessment of mitogenic activity

Mitogenic activity was assessed by measurement of total DNA content of treated or untreated cultures as well as by determination of DNA-synthesis rates. Synovial cells or skin fibroblasts were seeded into 6-well tissue-culture plates at 50000 cells/well and allowed to adhere for 72 h in DMEM containing 10 % FCS. The cells were then serum-starved for 24 h and exposed to HC-gp39 at concentrations ranging from 0 to 100 ng/ml in unsupplemented DMEM. As a positive control the growth response to similar concentrations of IGF-1 was determined in parallel experiments. The cells were harvested after 4 days of exposure to mitogenic agents and DNA levels in cell lysates were measured by a fluorometric assay using the dye Hoechst 3358 [30].

Rates of DNA synthesis were analysed by [^{3}H]thymidine incorporation. Synovial cells were seeded in 24-well tissue-culture plates at a density of 15000 cells/well; skin fibroblasts and HFL-1 cells were seeded at 8000 cells/well. All cells were cultured for 72 h in DMEM supplemented with 10 % FCS, followed by a 24 h serum-starvation period in unsupplemented DMEM. IGF-1 or HC-gp39 was then added in DMEM at the concentrations indicated, followed by [^{3}H]thymidine (10 μCi/ml) 24 h later. Supplementation with BSA did not influence the magnitude of the responses to growth factors in this system, and thus their effects were analysed in the absence of BSA, unless indicated otherwise. Cultures were terminated after 48 h of exposure to growth factors. The cell layers were briefly washed, trypsinized and DNA was collected on glass-fibre filters using a cell harvester. [^{3}H]Thymidine content was determined by liquid scintillation spectroscopy using a Packard liquid scintillation counter.

To assess the effects of protein kinase inhibitors on the mitogenic response to HC-gp39 or IGF-1, the inhibitors were added at concentrations recommended by the supplier 2 h prior to the mitogen. Thymidine incorporation was determined as described above.

Analysis of signalling pathways

The involvement of the MAP kinase and PI-3K pathways in the mitogenic response to HC-gp39 was evaluated by analysis of phosphorylation of ERK1/ERK2 and AKT respectively, using

Western blotting and phosphorylation-specific antisera. Cells were seeded into 6-well tissue-culture plates as above in DMEM containing 10% FCS and allowed to grow to near-confluence. Skin fibroblasts, HFLs and synovial cells were serum-starved for 48 h, followed by exposure to growth factors at the concentrations indicated for time periods up to 6 h for synovial cells and 3 h for HFL-1 cells, skin fibroblasts and articular chondrocytes. The latter were exposed to serum-free DMEM for 3 days prior to stimulation with daily medium changes to remove endogenously produced HC-gp39. For dose–response curves, cells were incubated in the presence of various growth-factor concentrations for 1 h. Protein kinase inhibitors, where used, were added to the cell cultures 1 h prior to the addition of mitogens.

At the end of the incubation period, cell layers were washed twice in ice-cold PBS, lysed in SDS sample buffer and cell lysates were analysed by SDS/PAGE and Western blotting. Blots were exposed to the phosphorylation-specific antibody at dilutions recommended by the manufaturer and, following visualization, they were stripped by incubation in a 0.2 M Tris/glycine buffer, pH 2.8 (containing 0.1% SDS and 0.1% Tween 20) [31], and reprobed with a pan-specific antibody to determine total ERK1/ERK2 or AKT protein. In some cases separate blots were prepared for each analysis. Similar results were obtained.

RESULTS

In contrast to many other cell types, human articular chondrocytes in monolayer culture can be maintained in unsupplemented culture medium for about 2 weeks without any loss of viability. The lack of a requirement for growth-factor supplementation could be due to the presence of endogenously produced HC-gp39, since these cells have been shown to produce sizeable amounts of this protein *in vitro* [1,32]. As endogenous production of HC-gp39 subsides, survival of human chondrocytes could be further extended *in vitro* by supplementation with exogenous HC-gp39. These observations suggested that HC-gp39 could act as a growth and survival factor for chondrocytes and possibly other connective-tissue cells, since increased production has been associated with a variety of fibrotic conditions. To test this hypothesis further, proliferative responses of connective-tissue cells were studied. We chose skin and fetal lung fibroblasts, as these cells do not produce HC-gp39 under any culture conditions. In addition, two synovial cell lines that only produce very low endogenous levels of HC-gp39 were analysed.

Human skin fibroblasts exposed to HC-gp39 following 24 h of serum starvation showed improved survival compared with cells exposed to BSA or serum-free medium. In addition, 24–30 h after addition of HC-gp39 numerous mitotic and post-mitotic cells were present in the cultures, indicating that HC-gp39 was indeed mitogenic for these cells. To quantify this response, the DNA content of fibroblast cultures maintained for 4 days in the presence of HC-gp39 was determined in comparison with IGF-1, a well-characterized growth factor for connective-tissue cells. Results obtained with synovial fibroblasts are shown in Figure 1(a), demonstrating increased DNA levels in cultures maintained in the presence of HC-gp39 compared with cells maintained in unsupplemented medium or in medium supplemented with BSA. The effectiveness of HC-gp39 in stimulating increases in DNA levels was similar to that of IGF-1. Similar results were obtained with skin and lung fibroblasts.

The increased DNA content of the cell cultures could be due to either decreased cell death by apoptosis or necrosis as a consequence of serum withdrawal or to increased mitotic rates, or a combination of both. To determine whether HC-gp39 stimulated cell growth, DNA-synthesis rates were evaluated by

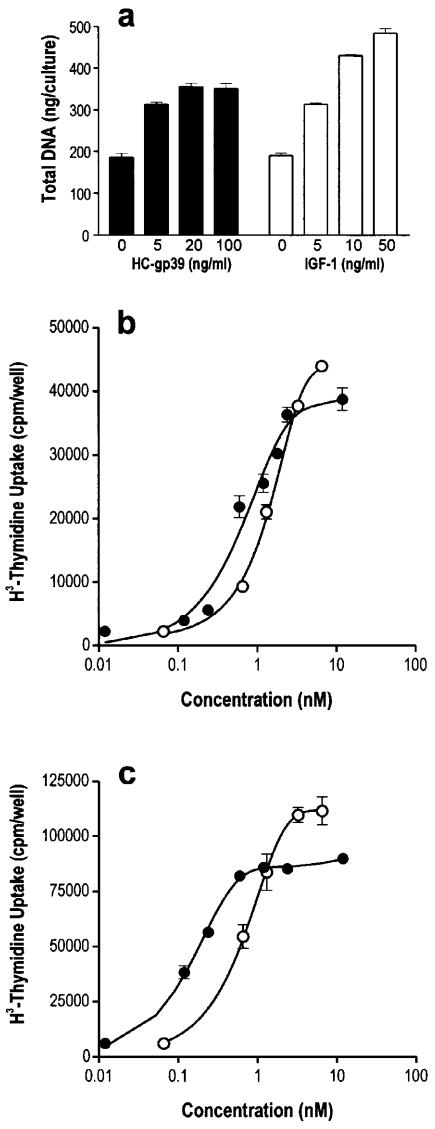

Figure 1 Stimulation of connective-tissue cell growth by HC-gp39

Exposure of connective-tissue cells to HC-gp39 leads to increased DNA content (**a**) and stimulation of DNA synthesis (**b** and **c**). Total DNA content of synovial cell cultures was determined after 4 days of exposure to purified HC-gp39 or IGF-1 at the concentrations indicated in (**a**). The mitogenic response of synovial cells (**b**) or skin fibroblasts (**c**) was determined by analysis of [^{3}H]thymidine incorporation 24 h after addition of the growth factors. Cells were labelled for 24 h, harvested and incorporated radioactivity was determined. ●, Response to HC-gp39; ○, response to IGF-1. The data shown are the means ± S.D. from triplicate cultures for each concentration. Error bars representing less than 5% variation are not shown.

incorporation of [^{3}H]thymidine into cellular DNA. A dose-dependent mitogenic effect was observed in human synovial cells (Figure 1b), as well as in skin fibroblasts (Figure 1c) treated with HC-gp39 at concentrations between 0.1 and 10 nM. The magnitude of the response was similar to that elicited by IGF-1, and HC-gp39 was effective in a similar molar concentration range.

To determine whether the observed mitogenic effect was specific for HC-gp39, or whether this was a property of other members of this protein family with high sequence similarity, the mitogenic activity of a recombinant preparation of human chitinase-1 was investigated. No response was observed in the

Table 1 Growth response of human connective-tissue cells to HC-gp39 and human chitinase

Growth-response values are given as a percentage of the level of [^{3}H]thymidine incorporation of cultures treated with FCS. The growth response of synovial cells and skin fibroblasts (both of which were derived from adult donors) as well as HFL-1 cells was evaluated using [^{3}H]thymidine incorporation as described in the text. All responses are given as a percentage of the level obtained with 10% FCS (19646 ± 924 c.p.m./well for synovial fibroblasts, 82877 ± 3871 c.p.m./well for skin fibroblasts and 118951 ± 10841 c.p.m./well for HFL-1 cells).

	Growth response (%)		
Culture additions	Synovial cells	Skin fibroblasts	HFLs
BSA (500 ng/ml)	2.6 ± 0.6	5.8 ± 1.1	18.9 ± 0.7
Human chitinase (2.5 nM)	3.5 ± 4.8	6.8 ± 0.3	18.3 ± 2.0
HC-gp39 (1.2 nM)	56.0 ± 9.3	103.6 ± 3.8	172.8 ± 4.1
Human chitinase (2.5 nM) + HC-gp39 (1.2 nM)	51.6 ± 2.5	104.4 ± 9.4	191.9 ± 4.8
IGF-1 (0.65 nM)	16.2 ± 1.5	65.8 ± 9.3	127.0 ± 12.3
Human chitinase (2.5 nM) + IGF-1 (0.65 nM)	18.8 ± 4.4	58.7 ± 9.8	122.4 ± 8.4
Human chitinase (2.5 nM) + FCS	100.9 ± 16.6	107.7 ± 3.8	99.1 ± 5.6

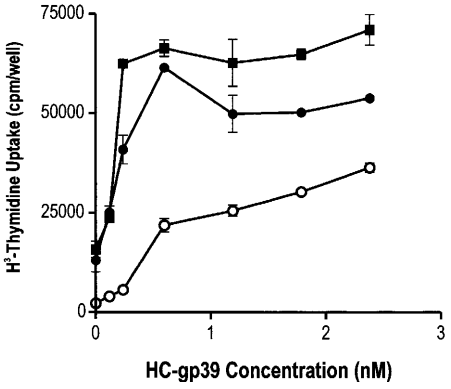

Figure 2 HC-gp39 and IGF-1 act synergistically in the induction of mitogenesis

Synovial fibroblasts were grown to semi-confluence and serum-starved for 24 h. Purified HC-gp39 was added to the cultures at the indicated concentrations in the absence (○) or presence (●) of 5 ng/ml human IGF-1, or in the presence of the phosphotyrosine phospatase inhibitor bpV (1 μM; ■). [^{3}H]Thymidine was added 24 h after the addition of growth factors, and incorporation of radioactivity was determined 24 h later. Data are shown as means ± S.D. from triplicate cultures for each condition.

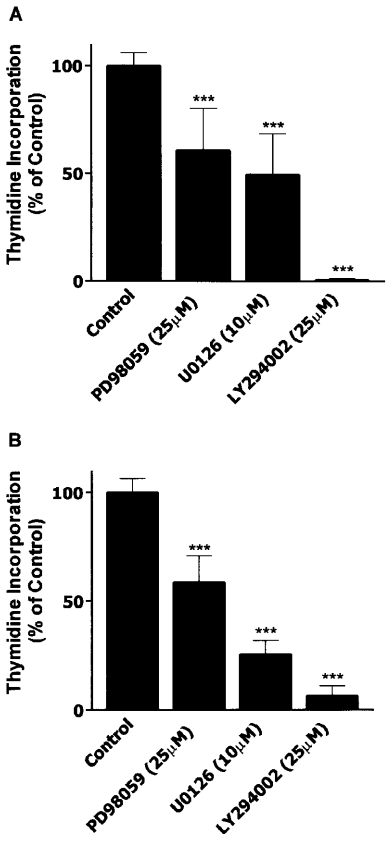

Figure 3 Activation of both ERK1/ERK2 and PI-3K signalling pathways is required for the mitogenic action of HC-gp39

Human fibroblasts were grown to semi-confluence and serum-starved for 24 h before addition of 100 ng/ml HC-gp39 (**a**) or 50 ng/ml IGF-1 (**b**). Inhibitors of ERK1/ERK2 activation (25 μM PD98059 or 10 μM U0126) or of PI-3K (25 μM LY294002) were added 1 h prior to the growth factors. Thymidine incorporation was determined between 24 and 48 h of the culture period as described for Figure 1.The data are means ± S.D. from triplicate culture wells of a representative experiment.

presence of this protein at concentrations up to 1 μg/ml (20 nM) in skin, fetal lung or synovial fibroblasts (Table 1), nor did this enzyme interfere with the mitogenic response of the cells to HC-gp39, IGF-1 or FCS. These observations therefore suggest that the mitogenic activity is a unique property of HC-gp39.

HC-gp39 and IGF-1 worked synergistically, enhancing the response of synovial cells at suboptimal concentrations of IGF-1 (Figure 2). Similar results were obtained with the phosphatase inhibitor, bpV. At low concentrations, this compound increased mitogenesis in all three cell lines tested, although at higher concentrations cytotoxicity was evident. As was observed for IGF-1, the response to HC-gp39 was greatly increased at the lower concentrations in the presence of 1 μM bpV.

Since the major cellular effect of bpV is due to its activity as a specific phosphotyrosine phosphatase inhibitor, the potentiating effect on HC-gp39 action suggested that a phosphorylation-dependent signalling pathway may be involved in the cellular mitogenic response. This possibility was investigated further using inhibitors of two pathways commonly associated with mitogenesis and cell survival, namely the MAP kinase pathway [33,34] and the PI-3K-mediated pathway [35]. Thymidine incorporation in response to HC-gp39 was reduced by the MAP kinase pathway inhibitors PD98059 and U0126 (Figure 3A). The PI-3K inhibitor, LY294002, proved to be most effective, reducing thymidine incorporation rates to background levels. IGF-1-stimulated mitogenesis was inhibited to a similar degree by the PI-3K inhibitor, but U0126, which is selective for both MEK1 and 2, was more effective than PD98059 (Figure 3B), whereas there was no statistically significant difference between these two inhibitors in the presence of HC-gp39.

Based on these observations the activation of the MAP kinase and PI-3K signalling pathways by HC-gp39 was analysed in comparison with IGF-1 in all three cell lines. A dose-dependent phosphorylation of ERK1 and ERK2 was observed for synovial cells (Figure 4a), HFLs (Figure 4b) and skin fibroblasts (results not shown). To investigate whether HC-gp39 would initiate a similar signalling pathway in articular chondrocytes, passaged cells were maintained in DMEM for 72 h with frequent medium changes to reduce endogenously produced HC-gp39 levels [1]. As

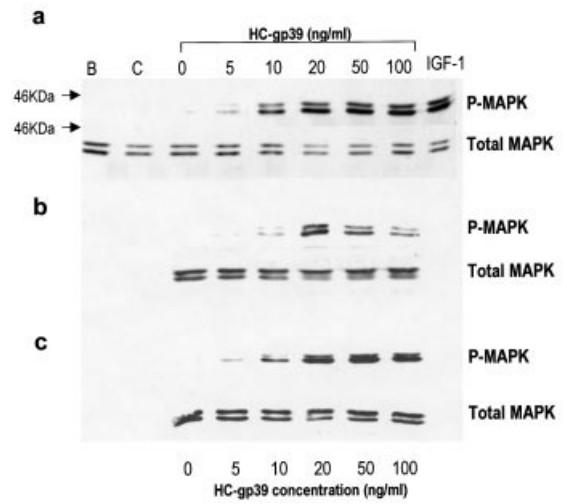

Figure 4 HC-gp39 induces a dose-dependent phosphorylation of ERK1/ERK2

Human synovial cells (**a**), fibroblasts (**b**) or human articular chondrocytes (**c**) were grown to near-confluence and serum-starved for 48 h followed by exposure to purified HC-gp39 at concentrations ranging from 0 to 100 ng/ml, BSA (100 ng/ml; lane B), human chitinase-1 (100 ng/ml; lane C) or IGF-1 (50 ng/ml) in unsupplemented culture medium. Cells were lysed after 60 min of exposure and cell lysates were analysed by Western blotting with either phosphorylation-specific (P-MAPK) or phosphorylation-independent (Total MAPK) antiserum against the MAP kinases ERK1 and ERK2. Controls with BSA and human chitinase-1 were performed for all cell lines with identical results. A similar dose dependency of MAP kinase phosphorylation was observed for HFLs (results not shown).

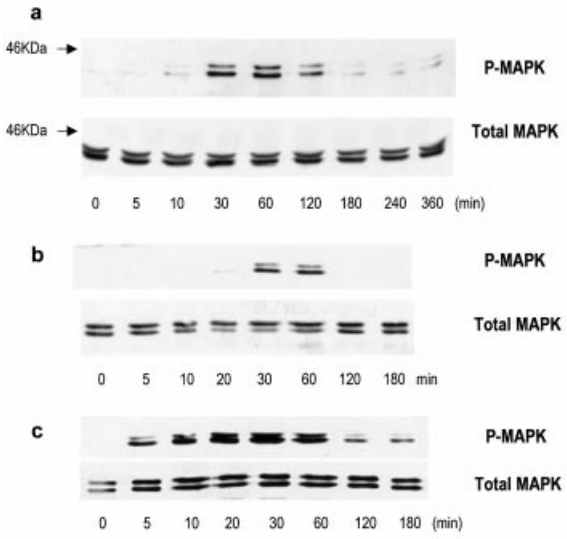

Figure 5 Time course of HC-gp39-induced ERK1/ERK2 phosphorylation

Human synovial cells (**a**), fibroblasts (**b**) or human articular chondrocytes (**c**) were grown to near-confluence and serum-starved for 48 h, followed by exposure to 50 ng/ml HC-gp39 for times ranging from 0 to 360 min as indicated. Cell lysates were analysed for ERK1/ERK2 phosphorylation as described for Figure 4.

illustrated in Figure 4(c), ERK1/ERK2 phosphorylation was also stimulated in these cells following addition of HC-gp39. The lowest effective concentration in all cases was similar to that eliciting a mitogenic response. IGF-1 was used as a positive

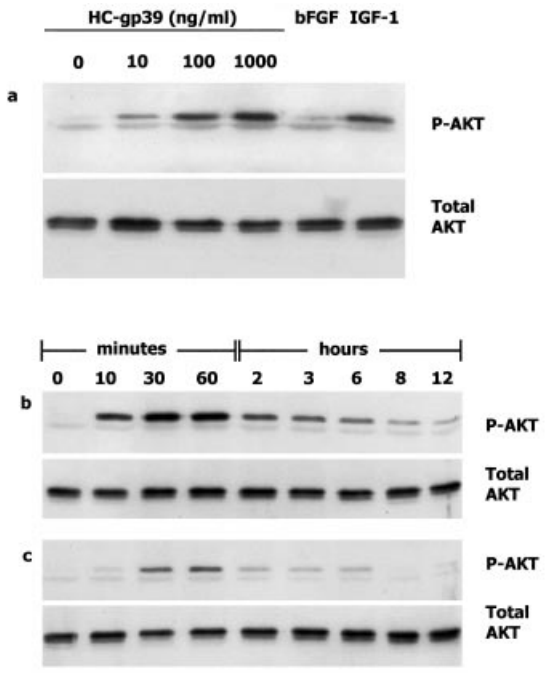

Figure 6 HC-gp39 induces phosphorylation of AKT in a concentration- and time-dependent manner

Human synovial cells were grown to near-confluence and serum-starved for 48 h, followed by exposure to HC-gp39 at the indicated concentrations for 60 min (**a**), or by exposure to 100 ng/ml of HC-gp39 for the indicated time periods up to 12 h (**b**). A time course for AKT phosphorylation in response to 100 ng/ml of IGF-1 is shown in (**c**). The levels of AKT phosphorylation relative to total AKT levels in cell lysates were determined by Western blotting using an antiserum specific for phosphorylated AKT (P-AKT) and a pan-specific antiserum (Total AKT). Similar results were obtained with human articular chondrocytes. bFGF, basic fibroblast growth factor.

control in these experiments. No ERK1/ERK2 phosphorylation was observed in cells exposed to medium supplemented with BSA or human chitinase, or when HC-gp39 was denatured by reduction and alkylation.

The time course of MAP kinase phosphorylation revealed a maximal effect between 30 and 60 min after addition of HC-gp39 to synovial or HFL-1 cells (Figures 5a and 5b). Articular chondrocytes responded much more quickly to HC-gp39, with a signal detectable as early as 5 min following addition of HC-gp39 (Figure 5c). Although the levels of ERK1/ERK2 phosphorylation peaked at 60 min, low levels were still evident up to 3 h after stimulation in human synovial cells and chondrocytes, whereas this event was much more transient in HFLs, where ERK1/ERK2 phosphorylation was undetectable at 2 and 3 h. Thus HC-gp39 initiates a signalling cascade in connective-tissue cells that is commonly associated with stimulation of mitogenesis.

Signalling through the PI-3K pathway has been associated with cell survival, and activation of AKT is pivotal in this aspect. The strong inhibition by the PI-3K inhibitor, LY294002, indicated that signalling through this kinase was essential for the mitogenic response to HC-gp39. Phosphorylation of AKT was therefore analysed in cells exposed to this protein. HC-gp39 induced a dose-dependent AKT phosphorylation in human synovial cells, with an effective range similar to that of IGF-1 (Figure 6a). Phosphorylation peaked between 30 and 60 min, but some activated AKT was still detectable 6 and even 12 h after stimulation (Figure 6b). Stimulation of human synovial cells

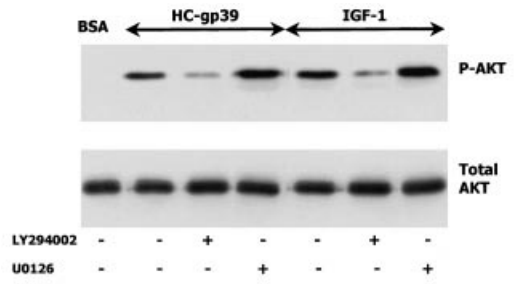

Figure 7 Stimulation of AKT phosphorylation by HC-gp39 is dependent on PI-3K activity

Synovial fibroblasts were serum-starved as above and exposed to the MEK1/MEK2 inhibitor U0126 or to the PI-3K inhibitor LY294002 for 1 h prior to addition of HC-gp39 (100 ng/ml). Cell lysates were prepared after 60 min of stimulation and levels of phosphorylated and total AKT were analysed as described for Figure 6.

with IGF-1 resulted in activation of AKT with a similar time course (Figure 6c). HC-gp39 also induced a dose-dependent activation of AKT in human skin fibroblasts and human chondrocyte cultures (results not shown).

Although AKT is usually activated through a PI-3K-dependent pathway [35], MAP kinase-dependent phosphorylation has been reported [36]. To determine whether both pathways were used for AKT phosphorylation in cells exposed to HC-gp39, the effects of the PI-3K-specific inhibitor, LY294002, or the MEK1/2 inhibitor, U0126, were investigated. AKT phosphorylation was inhibited by LY294002, but not by U0126, indicating that AKT activation in this system is entirely dependent on PI-3K activity (Figure 7). A consistently stronger phosphorylation signal for AKT was observed in the presence of U0126, i.e. when MEK1/2 activity was inhibited. IGF-1-induced phosphorylation of AKT was also enhanced by U0126. Similarly, phosphorylation of ERK1/ERK2 was inhibited by U0126, as expected, and a stronger signal was obtained in the presence of the PI-3K inhibitor LY294002 (results not shown). These data suggest that MAP kinase-dependent signalling events can modulate the level of AKT activation and vice versa.

DISCUSSION

Although the chitinase-like protein, HC-gp39, has been characterized extensively with respect to its expression patterns [1,7,11, 12,14] and possible association with degenerative diseases [8,10,16,37] insight into its physiological action has only been obtained recently, prompted by the identification of a family of growth factors, in the fruit fly *D. melanogaster*, as chitinase-like proteins [24]. The fact that one member of the mammalian family of these proteins also has very potent growth-promoting activity, as demonstrated by the work of de Ceuninck [25], indicates that this function may be conserved across a wide range of species. The results presented here demonstrate that HC-gp39 initiates MAP kinase and PI-3K signalling cascades in human connective-tissue cells, leading to the phosphorylation of ERK1/ERK2 and AKT, respectively. This finding is consistent with the well-established roles of these signalling pathways in the propagation of mitogenic signals [33–35]. The inhibition of the mitogenic response of the cells to HC-gp39 by either the MAP kinase (PD98059 and U0126) or PI-3K (LY294002) inhibitors suggests that both pathways are required for the cells to complete progression through the mitotic cycle. The analysis of ERK1/ERK2 and AKT phosphorylation in the presence of either inhibitor shows that each inhibitor works in a specific manner with respect to these two signalling pathways, supporting the suggestion that signalling through only one of these pathways is insufficient for the cells to complete mitosis. Although AKT has been thought to play a major role, mainly in cell survival, recent data suggest that PI-3K activation is required for the progression of mitosis, promoting the entry of quiescent cells into the S phase [38], and the downstream phosphorylation of AKT is in part responsible for the propagation of this signal. The strong inhibition of the mitogenic response to HC-gp39 by the PI-3K inhibitor, LY294002, is consistent with this role for PI-3K in mitogenesis. Jones and Kazlauskas [39] have shown that the mitogenic response to platelet-derived growth factor involves the sequential activation of the MAP kinase and PI-3K pathways, suggesting that these different phases of signalling are a general requirement in mitogenic signalling.

The activation of cytoplasmic signal-transduction pathways suggests that HC-gp39 interacts with one or several signalling components on the plasma membrane. Whether this occurs through specific receptors on the cell surface of connective-tissue cells remains to be determined, and the nature of potential HC-gp39 ligands is at the moment highly speculative. Some suggestions may be derived from the recently elucidated molecular structure of the murine protein YM1 (Chi3-L3) [40], which belongs to the vertebrate chitinase-like protein family and, like HC-gp39, possesses no enzymic activity. Perhaps not surprisingly, this protein is structurally very similar to the catalytic domain of the *Serratia marcescens* chitinase A [23]. Further analysis by Chang et al. [41] indicates that YM1 binds preferentially to oligomers of glucosamine as well as to heparin, suggesting that this protein may act by binding to a carbohydrate ligand, possibly cell-membrane-associated heparin sulphate proteoglycans. Interestingly, YM1 did not bind to *N*-acetylglucosamine oligomers, the native substrate for the chitinases. We have observed a similar lack of interaction between chitin oligomers and HC-gp39, suggesting that binding to chitin, possibly as a defence mechanism against fungal or parasitic infection, is probably not the major function of this protein.

The human chitinase secreted by macrophages is structurally very similar to HC-gp39, except that the sequence configuration of the active site favours interaction with *N*-acetylglucosamine oligomers. The lack of mitogenic activity and its inability to interfere with the responses to HC-gp39 suggest that the substrate specificity of this enzyme is unrelated to the binding affinity of HC-gp39. Whether other family members, such as YKL39 (Chi3-L2) or YM1 (Chi3-L3), promote the growth of connective tissue or other cell types, is currently not known.

It has recently been reported that YM1 possesses chemotactic activity towards eosinophils [42,43]; however, Webb et al. [44] report that this activity is rather weak. This discrepancy in activity may be due to differences in the purification of the protein. We have found that the biological activity of HC-gp39 depends on its native structure, as no mitogenic response was observed when the protein was reduced and alkylated, and the protein is prone to denaturation under a variety of conditions. A chemotactic activity has been reported for the porcine homologue of HC-gp39 [45], suggesting a role in angiogenesis. However, these effects also required relatively high concentrations of the protein and await further confirmation.

Increased synthesis of HC-gp39 has been reported in a number of pathological conditions where fibrosis is a prominent part of the disease pathology, such as rheumatoid arthritis [1,10], cirrhosis of the liver [18] and the formation of atherosclerotic plaques [37], and HC-gp39 may play a role in initiating or maintaining this response. For example, increased expression of

HC-gp39 is detectable at early stages in the development of degenerative lesions in articular cartilage [8], and the synovial hyperplasia, which is very often associated with the progression of osteoarthritis, could be initiated or promoted by increased levels of this protein. In addition, HC-gp39 could potentiate the action of other growth factors on connective-tissue cells. We have shown that HC-gp39 acts synergistically with IGF-1 and this may also be true for other growth factors such as basic fibroblast growth factor, which have been implicated in synovial hyperplasia. de Ceuninck et al. [25] did not observe any synergistic effects with IGF-1; however, the concentrations used were quite high. In our work synergistic effects were only obvious at subnanomolar concentrations of HC-gp39, whereas at higher concentrations the response was additive. In addition, the cells used in the above study were shown to contain high levels of endogenous HC-gp39, and it is not clear how this would affect the cellular responses to exogenously added protein. It is currently not known whether cells will become refractory to HC-gp39 following chronic exposure, or if there are other factors that attenuate its activity.

Chakravarthy et al. [46] have suggested that IGF-1 extends the replicative life span of satellite cells through activation of PI-3K and phosphorylation of AKT. The similarity between the responses to IGF-1 and HC-gp39 that we have observed suggest that the latter may play a role in supporting the replicative capacity of connective-tissue cells in a similar manner. In this respect it is interesting to note that expression of the murine homologue of HC-gp39 in the involuting mammary gland [19] occurs at the same time as the removal of the terminally differentiated mammary epithelial cells by apoptosis [47]. Thus in this situation HC-gp39 may function to maintain or promote the integrity of the connective-tissue elements of the mammary gland in a highly pro-apoptotic environment.

Whether HC-gp39 stimulates mitogenesis in human articular chondrocytes is not clear at the moment. These cells produce HC-gp39 in the absence of FCS; however, these experiments have usually been performed with confluent cells, and thus any mitogenic response is difficult to evaluate. Our results demonstrate that HC-gp39 activates the same MAP kinase and PI-3K signalling pathways in articular chondrocytes as it does in the fibroblast lines, indicating that these cells also respond to this protein. It has been shown that phosphorylation of MAP kinases is required for IGF-1-dependent cell survival of human articular chondrocytes *in vitro* [48]. Thus the expression of HC-gp39 in degenerate cartilage could influence the capacity of chondrocytes to divide and survive in a biomechanically stressed environment.

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REFERENCES

1 Hakala, B. E., White, C. and Recklies, A. D. (1993) Human cartilage GP-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J. Biol. Chem. **268**, 25803–25810

2 Arias, E. B., Verhage, H. G. and Jaffe, R. C. (1994) Complementary deoxyribonucleic acid cloning and molecular characterization of an estrogen-dependent human oviductal glycoprotein. Biol. Reprod. **51**, 685–694

3 Boot, R. G., Renkema, G. H., Strijland, A., Vanzonneveld, A. J. and Aerts, J. M. F. G. (1995) Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. J. Biol. Chem. **270**, 26252–26256

4 Hu, B., Trinh, K., Figueira, W. F. and Price, P. A. (1996) Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J. Biol. Chem. **271**, 19415–19420

5 Boot, R. G., Blommaart, E. F. C., Swart, E., Ghauharali-van der Vlugt, K., Bijl, N., Moe, C., Place, A. and Aerts, J. M. F. G. (2001) Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J. Biol. Chem. **276**, 6770–6778

6 Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E. and Aerts, J. M. F. G. (1995) Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. J. Biol. Chem. **270**, 2198–2202

7 Connor, J. R., Dodds, R. A., Emery, J. G., Kirkpatrick, R. B., Rosenberg, M. and Gowen, M. (2000) Human cartilage glycoprotein 39 (HC gp-39) mRNA expression in adult and fetal chondrocytes, osteoblasts and osteocytes by in-situ hybridization. Osteoarthritis Cartilage **8**, 87–95

8 Volck, B., Ostergaard, K., Johansen, J. S., Garbarsch, C. and Price, P. A. (1999) The distribution of YKL-40 in osteoarthritic and normal human articular cartilage. Scand. J. Rheumatol. **28**, 171–179

9 Johansen, J. S., Hvolris, J., Hansen, M., Backer, V., Lorenzen, I. and Price, P. A. (1996) Serum YKL-40 levels in healthy children and adults. Comparison with serum and synovial fluid levels of YKL-40 in patients with osteoarthritis or trauma of the knee joint. Br. J. Rheumatol. **35**, 553–559

10 Johansen, J. S., Jensen, H. S. and Price, P. A. (1993) A new biochemical marker for joint injury. Analysis of YKL-40 in serum and synovial fluid. Br. J. Rheumatol. **32**, 949–955

11 Shackelton, L. M., Mann, D. M. and Millis, A. J. (1995) Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling. J. Biol. Chem. **270**, 13076–13083

12 Nyirkos, P. and Golds, E. E. (1990) Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. Biochem. J. **269**, 265–268

13 Rehli, M., Krause, S. W. and Andreesen, R. (1997) Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. Genomics **43**, 221–225

14 Krause, S. W., Rehli, M., Kreutz, M., Schwarzfischer, L., Paulauskis, J. D. and Andreesen, R. (1996) Differential screening identifies genetic markers of monocyte to macrophage maturation. J. Leukoc. Biol. **60**, 540–545

15 Kirkpatrick, R. B., Emery, J. G., Connor, J. R., Dodds, R., Lysko, P. G. and Rosenberg, M. (1997) Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. Exp. Cell Res. **237**, 46–54

16 Johansen, J. S., Cintin, C., Jorgensen, M., Kamby, C. and Price, P. A. (1995) Serum YKL-40: a new potential marker of prognosis and location of metastases of patients with recurrent breast cancer. Eur. J. Cancer **31A**, 1437–1442

17 Cintin, C., Johansen, J. S., Christensen, I. J., Price, P. A., Sorensen, S. and Nielsen, H. J. (1999) Serum YKL-40 and colorectal cancer. Br. J. Cancer **79**, 1494–1499

18 Johansen, J. S., Moller, S., Price, P. A., Bendtsen, F., Junge, J., Garbarsch, C. and Henriksen, J. H. (1997) Plasma YKL-40: a new potential marker of fibrosis in patients with alcoholic cirrhosis? Scand. J. Gastroenterol. **32**, 582–590

19 Morrison, B. W. and Leder, P. (1994) neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. Oncogene **9**, 3417–3426

20 Rejman, J. J. and Hurley, W. L. (1988) Isolation and characterization of a novel 39 kilodalton whey protein from bovine mammary secretions collected during the non-lactating period. Biochem. Biophys. Res. Commun. **150**, 329–334

21 Renkema, G. H., Boot, R. G., Strijland, A., Donker-Koopman, W. E., van den Berg, M., Muijsers, A. O. and Aerts, J. M. (1997) Synthesis, sorting, and processing into distinct isoforms of human macrophage chitotriosidase. Eur. J. Biochem. **244**, 279–285

22 Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J. Biol. Chem. **268**, 18567–18572

23 Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. and Vorgias, C. E. (1994) Crystal structure of bacterial chitinase at 2.3 A resolution. Structure **2**, 1169–1180

24 Kawamura, K., Shibata, T., Saget, O., Peel, D. and Bryant, P. J. (1999) A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells. Development **126**, 211–219

25 de Ceuninck, F., Gaufillier, S., Bonnaud, A., Sabatini, M., Lesur, C. and Pastoureau, P. (2001) YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. Biochem. Biophys. Res. Commun. **285**, 926–931

26 Recklies, A. D., Baillargeon, L. and Ling, H. (2000) HC-gp39 is a growth factor for connective tissue cells. Arthritis Rheum. **43** (9, suppl.), S295

27 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

28 Golds, E. E., Mason, P. and Nyirkos, P. (1989) Inflammatory cytokines induce synthesis and secretion of *gro* protein and a neutrophil chemotactic factor but not β2-microglobulin in human synovial cells and fibroblasts. Biochem. J. **259**, 585–588

29 Recklies, A. D. and Golds, E. E. (1992) Induction of synthesis and release of interleukin-8 from human articular chondrocytes and cartilage explants. Arthritis Rheum. **35**, 1510–1519
30 Bracey, L. T. and Paigen, K. (1987) Changes in translational yield regulate tissue-specific expression of beta-glucuronidase. Proc. Natl. Acad. Sci. U.S.A. **84**, 9020–9024
31 Olmsted, J. B. (1986) Analysis of cytoskeletal structures using blot-purified monospecific antibodies. Methods Enzymol. **134**, 467–472
32 Johansen, J. S., Olee, T., Price, P. A., Hashimoto, S., Ochs, R. L. and Lotz, M. (2001) Regulation of YKL-40 production by human articular chondrocytes. Arthritis Rheum. **44**, 826–837
33 Moriguchi, T., Gotoh, Y. and Nishida, E. (1996) Roles of the MAP kinase cascade in vertebrates. Adv. Pharmacol. **36**, 121–137
34 Waskiewicz, A. J. and Cooper, J. A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Curr. Opin. Cell Biol. **7**, 798–805
35 Datta, S. R., Brunet, A. and Greenberg, M. E. (1999) Cellular survival: a play in three Akts. Genes Dev. **13**, 2905–2927
36 Nomura, M., Kaji, A., Ma, W. Y., Zhong, S., Liu, G., Bowden, G. T., Miyamoto, K.i. and Dong, Z. (2001) Mitogen- and stress-activated protein kinase 1 mediates activation of Akt by ultraviolet B irradiation. J. Biol. Chem. **276**, 25558–25567
37 Johansen, J. S., Stoltenberg, M., Hansen, M., Florescu, A., Horslev-Petersen, K., Lorenzen, I. and Price, P. A. (1999) Serum YKL-40 concentrations in patients with rheumatoid arthritis: relation to disease activity. Rheumatology (Oxford) **38**, 618–626
38 Gille, H. and Downward, J. (1999) Multiple Ras effector pathways contribute to G1 cell cycle progression. J. Biol. Chem. **274**, 22033–22040
39 Jones, S. M. and Kazlauskas, A. (2001) Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat. Cell Biol. **3**, 165–172
40 Sun, Y. J., Chang, N. C., Hung, S. I., Chang, A. C., Chou, C. C. and Hsiao, C. D. (2001) The crystal structure of a novel mammalian lectin, Ym1, suggests a saccharide binding site. J. Biol. Chem. **276**, 17507–17514
41 Chang, N. C., Hung, S. I., Hwa, K. Y., Kato, I., Chen, J. E., Liu, C. H. and Chang, A. C. (2001) A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. J. Biol. Chem. **276**, 17497–17506
42 Owhashi, M. and Nawa, Y. (1987) Eosinophil chemotactic lymphokine produced by spleen cells of *Schistosoma japonicum*-infected mice. Int. Arch. Appl. Immunol. **82**, 20–25
43 Owhashi, M., Arita, H. and Hayai, N. (2000) Identification of a novel eosinophil chemotactic cytokine (ECF-L) as a chitinase family protein. J. Biol. Chem. **275**, 1279–1286
44 Webb, D. C., McKenzie, A. N. J. and Foster, P. S. (2001) Expression of the Ym2 lectin binding protein is dependent on IL-4 and IL-13 signal transduction: identification of a novel allergy-associated protein. J. Biol. Chem. **276**, 41969–41976
45 Malinda, K. M., Ponce, L., Kleinman, H. K., Shackelton, L. M. and Millis, A. J. (1999) Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. Exp. Cell Res. **250**, 168–173
46 Chakravarthy, M. V., Abraha, T. W., Schwartz, R. J., Fiorotto, M. L. and Booth, F. W. (2000) Insulin-like growth factor-I extends *in vitro* replicative life span of skeletal muscle satellite by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3′-kinase/Akt signaling pathway. J. Biol. Chem. **275**, 35942–35952
47 Wiesen, J. and Werb, Z. (2000) Proteinases, cell cycle regulation, and apoptosis during mammary gland involution (mini review). Mol. Reprod. Dev. **56**, 534–540
48 Shakibaei, M., Schulze-Tanzil, G., de Souza, P., John, T., Rahmanzadeh, M., Rahmanzadeh, R. and Merker, H. J. (2001) Inhibition of mitogen-activated protein kinase kinase induces apoptosis of human chondrocytes. J. Biol. Chem. **276**, 13289–13294

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