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10 mM glucosamine prevents activation of proADAMTS5 (aggrecanase-2) in transfected cells by interference with post-translational modification of furin

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Summary

Objective: Glucosamine has been previously shown to suppress cartilage aggrecan catabolism in explant cultures. We determined the effect of glucosamine on ADAMTS5 (a disintegrin-like and metalloprotease domain (reprolysin type) with thrombospondin type-1 motifs 5), a major aggrecanase in osteoarthritis, and investigated a potential mechanism underlying the observed effects.

Design: HEK293F and CHO-K1 cells transiently transfected with ADAMTS5 cDNA were treated with glucosamine or the related hexosamine mannosamine. Glucosamine effects on *FURIN* transcription were determined by quantitative RT-PCR. Effects on furin-mediated processing of ADAMTS5 zymogen, and aggrecan processing by glucosamine-treated cells, were determined by western blotting. Post-translational modification of furin and N-glycan deficient furin mutants generated by site-directed mutagenesis was analyzed by western blotting, and the mutants were evaluated for their ADAMTS5 processing ability in furin-deficient CHO-RPE.40 cells.

Results: Ten mM glucosamine and 5–10 mM mannosamine reduced excision of the ADAMTS5 propeptide, indicating interference with the propeptide excision mechanism, although mannosamine compromised cell viability at these doses. Although glucosamine had no effect on furin mRNA levels, western blot of furin from glucosamine-treated cells suggested altered post-translational modification. Glucosamine treatment led to decreased glycosylation of cellular furin, with reduced furin autoactivation as the consequence. Recombinant furin treated with peptide N-glycanase F had reduced activity against a synthetic peptide substrate. Indeed, site-directed mutagenesis of two furin N-glycosylation sites, Asn^{387} and Asn^{440}, abrogated furin activation and this mutant was unable to rescue ADAMTS5 processing in furin-deficient cells.

Conclusions: Ten mM glucosamine reduces excision of the ADAMTS5 propeptide *via* interference with post-translational modification of furin and leads to reduced aggrecanase activity of ADAMTS5.

Key words: Aggrecanase, Glucosamine, ADAMTS, Furin, Catabolism.

Introduction

Osteoarthritis (OA) is a common disorder characterized by loss of articular cartilage as a result of degenerative changes in the joint. An early phenomenon in cartilage degradation is proteolytic loss of aggrecan, which forms giant aggregates with hyaluronan in the extracellular matrix (ECM)[1]. These aggregates bind water, and are constrained by a collagen network, which gives cartilage its compressibility. Aggrecan proteolysis diminishes cartilage compressibility and exposes collagen and other cartilage ECM proteins to degradation, thereby setting in motion a vicious cycle of cartilage breakdown[2]. Two secreted metalloproteases, ADAMTS4 and ADAMTS5 (a disintegrin-like and metalloprotease domain (reprolysin type) with thrombospondin type-1 motifs), referred to as aggrecanases, cleave aggrecan efficiently and are implicated as key mediators of OA[3,4]. Therefore, inhibition of these proteases, such as by endogenous inhibitors, medicinal compounds, or biosynthetic interference, is of significant therapeutic interest[5]. ADAMTS5, also known as aggrecanase-2, has attracted considerable interest as a target in OA[5,6] since mice with a targeted deletion of *Adamts5* are resistant to both immune and mechanically induced arthritis[7,8].

ADAMTS5 is synthesized as a zymogen (proADAMTS5) which undergoes proteolytic excision of its propeptide by proprotein convertases (PCs) such as furin and PACE4[9,10]. ADAMTS4 and ADAMTS5 each require propeptide excision for proteolytic activity, but they are activated somewhat differently. ADAMTS4 is processed intracellularly[11], whereas ADAMTS5 is processed extracellularly by furin and/or other PCs. ADAMTS4 and ADAMTS5 zymogens may also be deposited in cartilage ECM, where they are activated by secreted PCs such as PACE4[10]. Both furin and PACE4, which activate ADAMTS5 efficiently, cleave the consensus cleavage site, $RRRR^{261}\downarrow$, which is present at the junction of the ADAMTS5 propeptide and catalytic domain[9,10,12].

The hexosamines glucosamine and mannosamine were previously shown to suppress aggrecan catabolism in cartilage explant cultures[13–19]. Both hexosamines can interfere with N-glycosylation[20,21], and mannosamine is a recognized inhibitor of glycosylphospatidyl-inositol (GPI) anchor formation[14–19]. The effects of hexosamines on ADAMTS4

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biosynthesis, but not on ADAMTS5, were previously investigated at the molecular level. C-terminal processing of furin-activated ADAMTS4 by a GPI-anchored metalloprotease located at the cell surface was inhibited by mannosamine[15,22]. Treatment of cells with hexosamines also led to a prevalence of unprocessed ADAMTS4 zymogen[15]. ADAMTS4 lacks N-linked glycans[4], so the observed effects could not be attributed to inhibition of its N-glycosylation by hexosamines. These published observations led us to examine the effects of glucosamine on ADAMTS5 activity, and to elucidate the molecular mechanisms of the observed effects. Unlike ADAMTS4, ADAMTS5 is N-glycosylated and does not bind to the cell surface, but has been shown to reside in the ECM[23,24]. In cultured cells, which make little ECM, such as HEK293F cells, ADAMTS5 is present in the conditioned medium (CM)[9]. Here, we show that hexosamine treatment of cells interferes with the activation of ADAMTS5 through an indirect mechanism involving the loss of furin activity. These findings provide one possible explanation for previously observed suppressive effects of glucosamine on cartilage catabolism and are thus relevant to OA biochemistry.

Materials and methods

CELL CULTURE AND TREATMENTS

HEK293F cells and CHO-K1 cells (ATCC, Manassus, VA) were cultured in Dulbecco's Modified Eagles Medium (DMEM, high glucose) supplemented with 10% FBS and antibiotics. Furin-deficient CHO-RPE.40 cells[25] were cultured in Ham's F12 medium (high glucose) supplemented with 10% FBS and antibiotics.

EXPRESSION PLASMIDS AND SITE-DIRECTED MUTAGENESIS

Constructs for expression of full-length human ADAMTS5 and the propeptide and catalytic domain of human ADAMTS5 (ADAMTS5 Pro-Cat) were previously described[9] (Fig. 1). Asn residues within two of three consensus N-linked glycosylation sites in furin (GenBank accession no. NP_002560) were mutated singly (Asn^{387}, Asn^{440}), or in combination ($Asn^{387+440}$), by replacement with Gln using site-directed mutagenesis (Quikchange site-directed mutagenesis kit, Stratagene, La Jolla, CA). The mutations were introduced into full-length untagged human furin and a furin construct with an N-terminal FLAG tag introduced immediately downstream of the furin autoactivation site[26].

Expression plasmids were transiently transfected into HEK293F, CHO-K1 and CHO-RPE.40 cells using FUGENE6 (Roche Diagnostics, Indianapolis, IN). HEK293F and CHO-K1 cells were treated with glucosamine−HCl (neutralized to pH 7.4 before use) or mannosamine (Sigma−Aldrich, St. Louis, MO). Before treatment, cells were transferred to serum-free DMEM containing 5 mM glucose for 3−5 h[13]. Fresh medium containing each compound in a range of concentrations was added to cells. CM was collected, and cells were harvested in phosphate buffered saline after 24 h or 48 h followed by lysis in 1% Triton X-100, 10 mM Tris.HCl, pH 7.6 containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). In some experiments, HEK293F and CHO-RPE.40 cells were transfected (or co-transfected) with furin (either wildtype or Asn → Gln mutants) and/or ADAMTS5 Pro-Cat plasmid. As a control, empty vector used for furin cloning (pCI-Neo, Promega, Madison, WI) was co-transfected with ADAMTS5 Pro-Cat in CHO-RPE.40 cells. Serum-free media and cell lysates were collected after 48 h and processed as described above.

QUANTITATIVE RT-PCR

HEK293F cells were treated with glucosamine and harvested after 48 h as described above. Total RNA was extracted using QIAGEN complete RNA isolation kit (QIAGEN, Valencia, CA). RNA concentration and purity were determined with a NanoDrop (ND-100) spectrophotometer (Nanodrop Technologies, Wilmington, DE). All RNA samples used had an A260/280 ratio of >1.8. Two μg of total RNA per condition was reverse transcribed to cDNA in a total volume of 20 μl using the Superscript III kit (Invitrogen, Carlsbad, CA). For quantitative PCR, the *FURIN* primers 5′-CAGCGGTGGCCAACAGTGTG-3′ (forward) 5′-GCGGGCGGTGAGGCGACA-3′ (reverse) and 18s ribosomal RNA primers, 5′-GGGAGGTAGTGACGAAAAATAACAAT-3′ (forward), 5′-TTGCCCTCCAATGGATCCT-3′ (reverse) were used. SYBR-Green (Applied Biosystems, Foster City, CA) incorporation was detected using an iCycler (Bio-Rad. Hercules, CA) and relative levels of *FURIN* mRNA were quantitated using the ΔΔct method as per manufacturer's protocol.

WESTERN BLOTTING, ENZYMATIC DEGLYCOSYLATION OF FURIN, AND DETERMINATION OF AGGRECANASE ACTIVITY

For western blots, CM and cell lysate were electrophoresed by reducing SDS-PAGE in 6% or 7.5% gels prior to electroblotting to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Peptide N-glycosidase F (PNGaseF, New England Biolabs, Ipswich, MA) treatment was carried out as per manufacturer's instructions following reduction of the sample. ADAMTS5 was detected using rabbit polyclonal antibody RP2 (recognizing the propeptide) (Triple Point Biologics, Forest Grove, OR)[9] or anti-myc mouse monoclonal antibody 9E10 (Sigma−Aldrich, St. Louis, MO). Anti-GAPDH mouse monoclonal antibody (Chemicon, Temecula, CA) was used to detect intracellular and extracellular GAPDH to ensure equivalent protein loading. Anti-furin mouse monoclonal

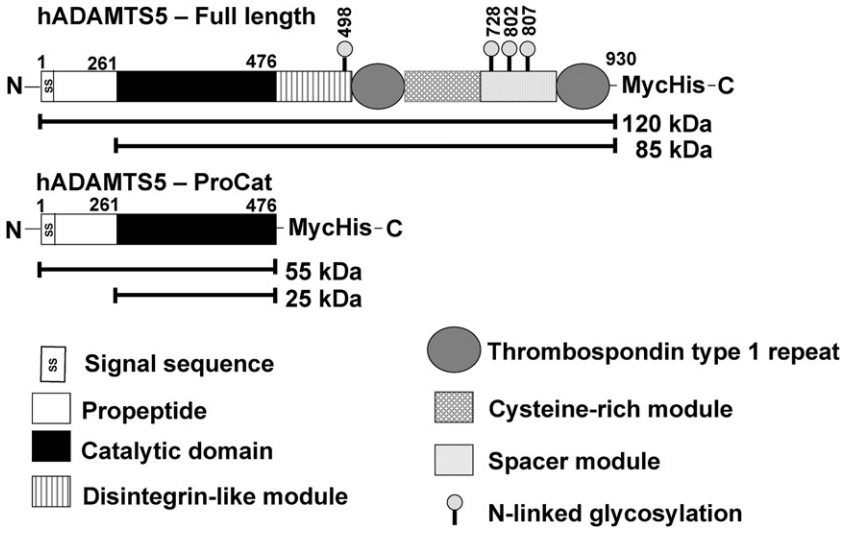

Fig. 1. ADAMTS5 constructs used in the analysis of glucosamine effects. Domain organization of ADAMTS5 and ADAMTS5 Pro-Cat, predicted molecular weights of the relevant protein species, N-linked glycosylation site and furin processing site are shown. The key to the various modules is at the bottom of the figure.