Glucosamine and its N-acetyl-phenylalanine derivative prevent TNF-α-induced transcriptional activation in human chondrocytes

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Abstract

Objectives

Glucosamine (GlcN) is used in the treatment of osteoarthritis as symptomatic slow-acting drug, but its mode of action is not completely known. We analyzed the influence of GlcN and its N-acetyl-phenylalanine derivative (NAPA) on mRNA transcription level of TNF-α-stimulated genes in cell culture.

Methods

Human immortalized chondrocyte cell line lbpva55 was stimulated with TNF-α and treated with GlcN and NAPA. mRNA transcription level of several genes, identified by complementary DNA microarray (cDNA microarray), was validated by Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR).

Results

Several genes, whose mRNA level was increased by TNF-α treatment and significantly reduced by GlcN and NAPA in lbpva55 cells, were identified. These include cytokine receptors TNF-R1 and TNF-R2, their associated factor TRAF-6, signaling intermediates IGFB-6 and Rnd1, as well as cell cycle regulating proteins CUL-2 and G1S protein 1. Down-regulation of mRNA expression level of some of these genes is in accordance with inactivation of NF-κB transcription factor. Moreover, we found down-regulation of c-jun mRNA level, a component of AP-1 transcription factor.

Conclusions

Our study suggests that GlcN and NAPA interfere with activation of NF-κB and AP-1 transcription factors, which are responsible for the expression of genes involved in diverse biological processes, such as cell growth and death, inflammatory and stress responses, accounting for the beneficial effects of GlcN in osteoarthritis.

Key words

Glucosamine, chondrocytes, osteoarthritis, chondrocyte gene expression, TNF-α stimulation.

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This work was supported by the grant "Progetto di Facoltà" from the "Sapienza" University of Rome.

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Received on January 16, 2007; accepted in revised form on May 16, 2007.

Competing interests: none declared.

Introduction

Osteoarthritis (OA) is a joint disorder, characterized by a progressive degradation of cartilage. An imbalance between synthesis and degradation of the extracellular matrix component leads to extensive articular damage, causing pain and disability in patients. The production of pro-inflammatory cytokines and chemokines has been demonstrated in OA cartilage (1-3). Cytokines stimulate chondrocytes to secrete an excess of proteases, which degrade both collagens and proteoglycans, causing abnormal matrix remodeling and loss, thereby inducing cartilage injury (4-7). The pharmacological therapy of OA is based on drugs aimed at controlling the symptoms of the disease, *i.e.* pain and limitation of function, using analgesic agents and non-steroidal antinflammatory drugs (NSAIDs) (8). During the last few years, there has been growing interest in symptomatic slow-acting drugs, such as glucosamine (GlcN). In particular, GlcN has been proved to be effective in relieving OA symptoms in several clinical trials (9-11). Successful clinical use of GlcN has prompted investigations of the molecular basis of its effect on cartilage cells. Recent reports show that GlcN can inhibit NO production, down-regulate iNOS and COX-2, both at level of mRNA expression and protein production and inhibit IL-6 secretion by human, rat and horse chondrocytes (12-16). GlcN can also modulate galactose-β-1,3-glucoronosyltransferase I enzyme, at the level of its mRNA expression and of its enzyme activity in proteoglycans synthesis (17). These studies demonstrate that GlcN has an anti-inflammatory and chondroprotective role. However, the rational for these activities is still not completely clear.

In a previous study, we reported an inhibitory effect of GlcN and its N-acetyl phenylalanine derivative (NAPA) on cathepsin B (18), which is involved in the degradation of cartilage components (19, 20), and found to be up-regulated in some pathological processes such as OA (21, 22).

The aim of the present work is to investigate the influence of GlcN and NAPA on the mRNA expression profile of a large panel of genes, in order to identify transcriptionally modulated genes, which could be involved in the anti-inflammatory and chondroprotective signal transduction cascades. The panel of genes was preliminarily identified by cDNA microarray experiments. A subset of these modulated genes was then validated by Q-RT-PCR.

We conducted our study on the human immortalized chondrocyte cell line lbpva55, derived from adult articular healthy cartilage (23). These cells appear to be a good system for studying the action of drugs on chondrocytes avoiding the problems arising by the use of primary cells due to their limited life span, rapid decrease of phenotypic differentiation molecular markers and inherent genetic variation.

Materials and methods

Cell culture and treatment

Cell culture was carried out as previously described (23). Briefly, human immortalized chondrocytes, lbpva55, were grown, in 100 mm plates, to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (HyClone, Cramligton, NE) and gentamycine, (Roche Diagnostic, Mannheim, Germany), then were transferred in DMEM supplemented with 10% FBS. After overnight incubation, the monolayer was rinsed with PBS and incubated with culture medium containing 1% Nutridoma-SP (Roche). Medium was changed twice a week and the cells were split once. After 14 days, cells were left untreated or treated with 10ng/ml recombinant TNF-α, (Pepro-Tech House, London, UK), 10ng/ml TNF-α, plus 10 mM 2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-β-D-glucose (NAPA), synthesized as previously reported (18), 10ng/ml TNF-α, plus 10 mM Glucosamine (GlcN) (Sigma) for 24 hours. To analyze early responsive gene, c-jun, the lbpva55 cells were incubated for 2 hours in 10mM GlcN or NAPA containing medium and then stimulated with 10ng/ml TNF-α, for 1 hour. Cells were subsequently harvested and processed for cDNA microarray and Q-RT-PCR.

RNA extraction and reverse-transcription

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturers' instruction. Briefly, a confluent 100 mm plate of lbpva55 cell line was washed with phosphate-buffered saline (PBS) (Sigma) and homogenized in 1ml TRIZOL reagent. RNA was stored at -80°C until used.

Complementary DNA (cDNA) was synthesized from 1µg of total RNA, using reverse transcriptase Improm II enzyme, (Promega Corporation, Madison, WI, USA), according to the manufacturers' instruction, and analyzed by Q-RT-PCR.

Microarray analysis

Alpha-^{32}P-dATP-labeled cDNA was prepared from 5µg of total RNA obtained as above described, and used to probe Human Cancer 1.2 cDNA array membranes (Clontech, BD Biosciences, Erembodegem, Belgium). Membranes were hybridized according to manufacturers' instruction and then exposed to a storage phosphor screen for 72 hours. Images were captured on a Typhoon 9410 phosphor imager (Amersham Biosciences AB, Uppsala, Sweden), using Image Quant software (Amersham). For spot intensity quantification, normalization and comparison, the software from Clontech, Atlas Image 2.7, was used.

Real Time-PCR

Q-RT-PCR analysis was performed using an ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). Amplification was carried out with 50ng of cDNA, in 96-well plates, using SYBR Green PCR Master mix (Applied Biosystems) in 25µl volume. Each sample was analyzed in triplicate. PCR conditions were: 94°C for 10 minutes and followed by 40 cycles of 94°C for 15 seconds, 60°C for 1 minute. Primers were designed using Primer Express software (Applied Biosystems) and were synthesized by Primm (Milan, Italy). The primer sequences are reported in Table I. The results were analyzed by using the Sequence Detection Systems software (Applied Biosystems), which automatically recorded the threshold cycle (C_t). Untreated cells sample (CTL) was used as a calibrator, the fold change for CTL was 1.0. Target gene C_t values were normalized against GAPDH, a house-keeping gene, whose expression is not expected to fluctuate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and expressed as fold change compared to CTL.

Table I. Primers used to quantify gene expression by Real Time-PCR.

Gene	Primer	GenBank
GAPDH	Fw: GGAGTCAACGGATTTGGTCGTA Rv: GGCAACAATATCCACTTTACCAGAGT	NM_002046
TNF-R1	Fw: GAAATGGGTCAGGTGGAGATTC Rv: GTTCTTCCTGCAGCCACACA	M33294
TNF-R2	Fw: GAGTGGTGAACTGTGTCATCATGA Rv: GTGAGGCACCTTGGCTTCTC	M55994
TRAF6	Fw: AGAACACCCAGTCACACATGAGA Rv: AGAGTCGGGTATAACGCTCAAACT	U78798
IGFBP-6	Fw: GCAGAGGAGAATCCTAAGGAGAGTAA Rv: TGGTCTCTGCGGTTCACATC	M62402
Rnd1	Fw: TGGAGCTGTCCCACCAGAA Rv: AGCTGCTTTGCTATTGCACAAC	Y07923
CUL-2	Fw: AATGCTGCTCCGAGAAATCAA Rv: CAAAGGAGTTAATAACCCCATGGA	U83410
G1S prot. 1	Fw: ATGTAGTATTCATTGGGCACGTAGAT Rv: TGTCAACCATTCCAGTCAAATACATT	X17644
c-jun	Fw: TCACGTGAAGTGACGGACTGTT Rv: GGAGGAACGAGGCGTTGAG	J04111

Table II. Genes up-regulated by TNF-α (T) and down-regulated by simultaneous treatment with NAPA (N) or GlcN (G), analyzed by cDNA microarray.

GenBank	GENE	T	N	G
	CELL CYCLE			
U83410	cullin homolog 2	↑	↓	↓
X17644	G 1 to S phase transition protein 1	↑	↓	↓
D13365	growt inhibitory factor metallothion III	↑	↓	↓
U72649	btg protein	↑	↓	↓
U09579	cyclin-dependent kinase inhibitor 1	↑	↓	↓
	KINASES			
M68520	cyclin-dependent protein kinase 2	↑	↓	↓
M34353	c-ros-1 tyrosine protein kinase	↑	↓	↓
L29220	CDC-like kinase 3	↑	↓	↓
X59727	extracellular signal- regulated kinase 4	↑	↓	↓
U43195	rho-associated kin	↑	↓	↓
M34182	PKAC-gamma	↑	↓	↓
U50648	PKR	↑	↓	↓
	MEMBRANE PROTEINS			
M63618	bullous pemphigoid antigen 1	↑	↓	↓
X53586	Integrina α-6	↑	↓	↓
M33294	TNF-R1	↑	↓	↓
M55994	TNF-R2	↑	↓	↓
U10485	lymphoid-restricted membrane protein	↑	↓	↓
	SIGNALING INTERMEDIATES			
U78798	TRAF6	↑	↓	↓
Y07923	rho6 (Rnd1)	↑	↓	↓
M62402	IGF-protein binding 6	↑	↓	↓
X02811	PDGFB	↑	↓	↓
J04164	interf-induc prot	↑	↓	↓
M25639	macroph inhib	↑	↓	↓
AF007871	torsina	↑	↓	↓

Statistics

Each experiment was repeated at least three times. The statistical significance of the differences between mean values was determined by a two-tailed *t*-test; $p \leq 0.05$ was considered significant. Where appropriate, results are expressed as the mean ± Standard Error of the Mean (SEM).

Results

Gene expression profile in lbpva55 cell line was analyzed by cDNA microarray analysis. We found several genes, whose mRNA level was increased at least two fold 24 hours after stimulation with TNF-α and decreased to control value upon treatment with NAPA and/or GlcN (Table II). These include membrane proteins, signaling intermediates as well as cell cycle regulating proteins.

We selected some genes listed in Table I for further analysis by Q-RT-PCR.

TNF-α stimulation of lbpva55 induces an increase of Tumor Necrosis Factor-α-receptor 1 (TNF-R1) and TNF-α-receptor 2 (TNF-R2) mRNA level, which is brought back to its background values by GlcN ($p < 0.02$) and NAPA ($p < 0.01$) (Fig. 1A).

TNF receptor associated factor 6 (TRAF6) mRNA transcription level was enhanced by TNF-α and significantly decreased by NAPA ($p < 0.005$) and GlcN ($p < 0.01$) (Fig. 1B).

We found a transcriptional up-regulation of Insulin Growth Factor-Binding Proteins (IGFBP-6) mRNA by TNF-α, which was down-regulated by TNF-α plus NAPA ($p < 0.01$), while no difference was observed between the sample stimulated with TNF-α as compared with that stimulated with TNF-α plus GlcN (Fig.1C).

TNF-α enhanced mRNA transcription level of Rnd1, which was statistically significant down-regulated by NAPA treatment ($p < 0.02$), while any significant effect could be detected in the sample treated with GlcN (Fig. 1D).

Cullin2 (CUL-2) and G1S phase transition protein 1 (G1S prot.1) were up-regulated by TNF-α. The decrease in mRNA expression level of CUL-2 in TNF-α plus GlcN or NAPA-treated cells with respect to TNF-α-treated cells

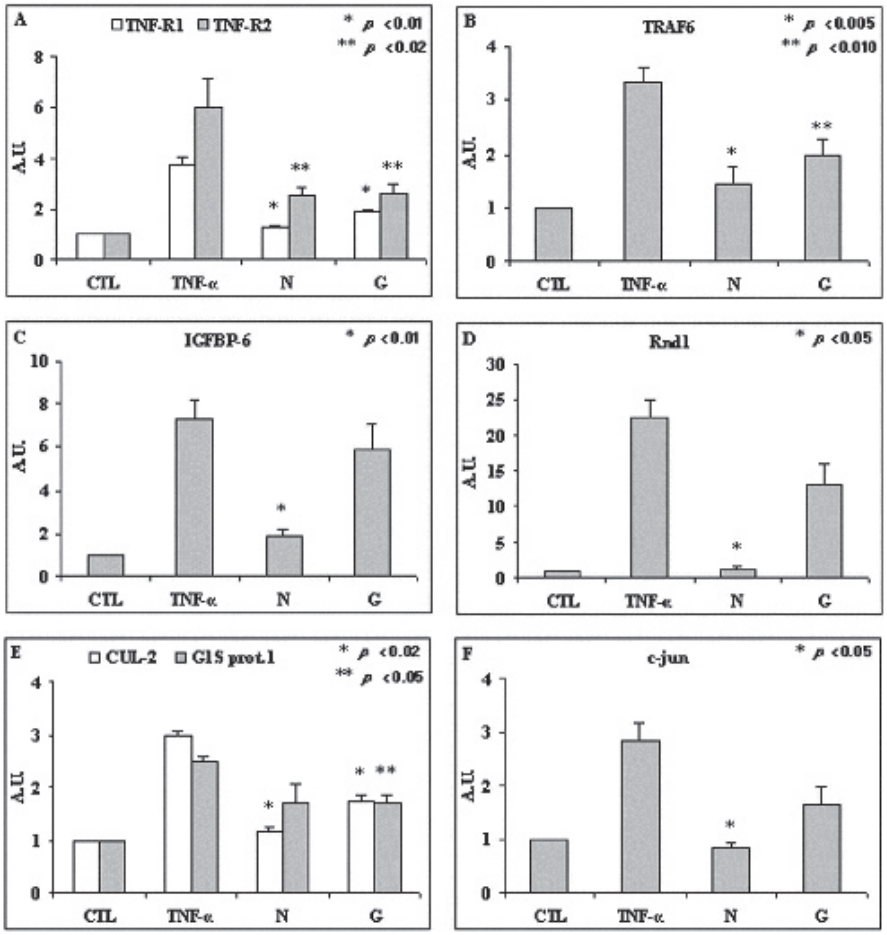

Fig.1. Effect of glucosamine (G) and 2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-β-D-glucose (N) on the mRNA expression of TNF-R1 and TNF-R2, TRAF6, IGFBP-6, Rnd1, CUL-2, G1S prot. 1, c-jun genes. lbpva55 cell line was treated with 10ng/ml TNF-α (TNF-α) and simultaneously with 10mM G or N for 24 hours (A, B, C, D, E) or pre-treated with G or N for 2 hours and then stimulated with TNF-α for 1 hour (F). mRNA was analyzed by Q-RT-PCR. Results are expressed in arbitrary units, A.U., and represent the mean ± SEM of data obtained by three different experiments. Statistical analysis was performed using two-tailed *t*-test, $p \leq 0.05$ was considered statistically significant.

was statistically significant ($p < 0.02$) as was the decrease of G1S prot.1 mRNA decrease in the presence of GlcN ($p < 0.05$). Conversely no significant effect of NAPA treatment could be observed in this case (Fig. 1E).

In order to analyze early responsive gene expression, we studied transcription factor elements. TNF-α, after 1 hour stimulation, up-regulated the c-jun mRNA level, which was significantly brought back by 2 hours pre-treatment with NAPA ($p < 0.05$), while GlcN down-regulation was not statistically significant (Fig. 1F).

Discussion

In this study, we have analyzed the effect of glucosamine and its N-acetyl-phenylalanine derivative on the mRNA expression pattern of human immortalized chondrocytes lbpva55 (23), stimulated with TNF-α, to mimic the inflammatory stimuli present in OA cartilage. It should be pointed out that the concentration used in this study of GlcN and TNF-α represent supraphysiological levels, but we selected doses of both molecules that provide robust responses.

Based on the results of a preliminary screening by microarray analysis we focused our attention on mRNA expression of genes coding for TNF-R1, TNF-R2, TRAF6, IGFBP-6, CUL-2, G1S prot.1 and Rnd1.

TNF-R1 (24) and TNF-R2 (25) are the two TNF cell surface receptors. TNF-α binding to TNF-R1 triggers a number of intracellular events that ultimately

result in the activation of transcription factors, such as NF-κB and AP-1. These transcription factors can induce the expression of genes involved in several biological processes such as cell growth and death, development, inflammatory response (24). TNF-R2, has been described to improve TNF-α-induced cell death through TNF-R1 (25). GlcN and NAPA affect the TNF-R1 and TNF-R2 mRNA level, restoring their background values and suggesting that the two molecules interfere with the TNF-α pathway by reducing the synthesis of its cell surface receptors.

TRAF6 is a member of a group of six closely related TRAF proteins (26), it regulates diverse physiological and pathological processes (26-28), playing critical roles in signal transduction of TNF receptor and IL1 receptor/Toll-like receptor (IL1/TLR) linking the activation of these receptors to downstream events, which culminate in regulation of gene transcription. Biological effects of TRAF6 are mediated by activation of NF-κB and AP-1 transcription factors. TNF-α enhances TRAF6 mRNA expression in lbvpa55 cell line, GlcN lowers TRAF6 mRNA expression to CTL levels. The same effect is observed also for NAPA, although to a lesser extent. These results suggest that GlcN and NAPA inhibit NF-κB activation by down-regulating the TNF-R1, TNF-R2 and TRAF6 mRNA expression.

IGFBPs are members of a protein family involved in regulation of insulin growth factor (IGF) activity by regulating IGF turnover, transport and tissue distribution (29). NAPA and, to a lesser extent, GlcN affect IGFBP-6 mRNA expression, which is upregulated by TNF-α treatment. IGFBP-6 expression is under the control of NF-κB, down-regulation of its transcription level is in accordance with the hypothesis that GlcN and NAPA can interfere with NF-κB activation.

Moreover, NAPA interfere also with AP-1 transcription factor. In fact, we found that NAPA was able to down-regulate the c-jun mRNA expression level.

Rnd1 is a small GTPase, which has been described to be a negative regulator of actin assembly and cell adhesion (30). Rnd1 overexpression in growing fibroblast, induce loss of polymerized actin structure and of cell-matrix adhesion. In osteoarthritis, chondrocytes lose cell-matrix adhesion and consequently cartilage homeostasis via CD44 (31). It could be hypothesized that Rnd1 overexpression is involved in this process. GlcN and, to a higher extent, NAPA, counteracting the Rnd1 mRNA overexpression, could prevent the loss of cartilage homeostasis.

CUL-2 and G1S prot.1 are cell cycle regulating proteins, which have been found to positively control cell cycle (32, 33). During early osteoarthritis phases, chondrocyte response is manifested by enhanced cell proliferation and only later by cell death. In our experimental model, GlcN and NAPA only slightly affects the TNF-α-induced CUL-2 and G1S prot.1 up-regulation, suggesting that both drugs counteract the TNF-α stimulation without affecting cell viability.

Recently, during the writing of this manuscript, a paper has been published describing the action of GlcN on primary chondrocytes of rat, challenged by Il-1β (34). mRNA expression profiling was analyzed by microarray and the author found that GlcN counteract the up-regulation of other genes compared to our panel. This discrepancy could be explained by the different cytokine used to stimulate, IL-1β instead of TNF-α, the different concentration of GlcN used, 20mM instead of 10mM and the different cells used, rat primary chondrocytes instead of human chondrocytes.

Taken together, these results suggest that both GlcN and NAPA exert similar effects on human lbpva55 chondrocyte cell line, although we found that NAPA is more efficient in decreasing mRNA expression of mostly TNF-α-modulated genes. In oder to find a mechanism by which these molecules act, we should consider their molecular structure.

Previously, we described the inhibitory effect of GlcN and NAPA on cysteine proteases such as cathepsin B. The two molecules, in solution, are stable pyranose hemiacetals in equilibrium with the open form bearing a free aldehyde group which reacts with the catalytic nucleophile SH group of cysteine proteases to form stable thioemiacetals and induce their inhibition (18). It could be hypothesized that both GlcN and NAPA can react with SH groups of other proteins present in the cells, inducing conformational changes and affecting their function. Several proteins, involved in the TNF-α pathway, present cysteine residues, such as IKKβ, a subunit of the kinase responsible of NF-κB inhibitor phosphorylation (35). More efficient mRNA modulation exerted by NAPA may be related to the higher hydrophobicity of this molecule with respect to GlcN.

In conclusion, our results demonstrate that GlcN and NAPA can affect different cellular pathways, probably interfering with the activation of molecules, which are involved at a very early stage in the TNF-α-signal transduction cascade.

Acknowledgements

The authors thank Dr. L. Roseti, who contributed to immortalize the chondrocyte cell line LBPVA55, and the Center of Excellence in Molecular Biology and Medicine (BEMM) for the use of their instruments.

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