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Glucosamine Inhibits IL-1β-Mediated IL-8 Production in Prostate Cancer Cells by MAPK Attenuation

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ABSTRACT

Inflammation is a complex process involving cytokine production to regulate host defense cascades. In contrast to the therapeutic significance of acute inflammation, a pathogenic impact of chronic inflammation on cancer development has been proposed. Upregulation of inflammatory cytokines, such as IL-1β and IL-8, has been noted in prostate cancer patients and IL-8 has been shown to promote prostate cancer cell proliferation and migration; however, it is not clear whether IL-1β regulates IL-8 expression in prostate cancer cells. Glucosamine is widely regarded as an anti-inflammatory agent and thus we hypothesized that if IL-1β activated IL-8 production in prostate cancer cells, then glucosamine ought to blunt such an effect. Three prostate cancer cell lines, DU-145, PC-3, and LNCaP, were used to evaluate the effects of IL-1β and glucosamine on IL-8 expression using ELISA and RT-PCR analyses. IL-1β elevated IL-8 mRNA expression and subsequent IL-8 secretion. Glucosamine significantly inhibited IL-1β-induced IL-8 secretion. IL-8 appeared to induce LNCaP cell proliferation by MTT assay; involvement of IL-8 in IL-1β-dependent PC-3 cell migration was demonstrated by wound-healing and transwell migration assays. Inhibitors of MAPKs and NFκB were used to pinpoint MAPKs but not NFκB being involved in IL-1β-mediated IL-8 production. IL-1β-provoked phosphorylation of all MAPKs was notably suppressed by glucosamine. We suggest that IL-1β can activate the MAPK pathways resulting in an induction of IL-8 production, which promotes prostate cancer cell proliferation and migration. In this context, glucosamine appears to inhibit IL-1β-mediated activation of MAPKs and therefore reduces IL-8 production; this, in turn, attenuates cell proliferation/migration. J. Cell. Biochem. 108: 489–498, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: IL-1β; IL-8; GLUCOSAMINE; PROSTATE CANCER

Prostate cancer is the most frequently diagnosed cancer in Western males [Amanatullah et al., 2000] and a growing body of evidence suggest that inflammation is highly related to the development of various cancers, such as lung cancer, gastric cancer, cervical cancer, bladder cancer, pancreatic cancer, colorectal cancer, and prostate cancer [Aggarwal et al., 2006; Mantovani, 2007]. Therefore, controlling inappropriate inflammation would appear to be one strategy that might help control cancer progression.

Inflammation is an essential part of the host defense system in response to internal or external stimuli [Hanada and Yoshimura, 2002]. As a fine-tuned acute inflammation consequence lasting for a short period of time, it is responsible for innate immunity or humoral immunity and thus provides help of therapeutic significance to host. However, when inflammation becomes chronic and lasts for a long period of time, the result may be pathogenic consequences to the body, including tumorigenesis [Lawrence and Gilroy, 2007]. Chronic inflammation has been linked to various steps in tumor formation, including cellular transformation, proliferation, invasion, angiogenesis, and metastasis [Coussens and Werb, 2002; Mantovani, 2005, 2007; Aggarwal et al., 2006]. By examining various studies on sexually transmitted infections, clinical prostatitis and inflammation markers, a close correlation between chronic prostatic inflammation and prostate cancer has been proposed [Platz and De Marzo, 2004; De Marzo et al., 2007]. Among the inflammatory cytokines, interleukin 1 (IL-1), IL-6, IL-8, and IL-10 have been reported as present in the prostate cancer cells [van der Poel, 2007], indicating the significance of these inflammatory factors in prostate cancer progression.

The main function of IL-1 is to affect various inflammatory processes; however it also possesses growth-promoting properties. For example, IL-1β appeared to increase tumor angiogenesis and invasiveness [Apte et al., 2000]. In addition, IL-1β null mice either fail to develop tumors or tumors develop slower, when compared with wild-type mice [Voronov et al., 2003, 2007]. This supports the idea that IL-1 system plays an important role not only in inflammation but also in the tumor progression. Meanwhile, IL-8,

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secreted by various normal or neoplastic human cell types [Xie, 2001], was first identified as a chemotactic factor that promotes cellular directional migration and was isolated from activated monocytes/macrophages. Recently, IL-8 has been demonstrated to be important in immune-related diseases as well as in tumor progression [Shi et al., 2001; Brat et al., 2005] with a specific role as a proangiogenic factor in prostate cancer formation [Veltri et al., 1999; Araki et al., 2007]. In fact, elevation of the level of IL-8 is associated with a higher Gleason score and metastatic disease [Uehara et al., 2005]. Interestingly, not only inflammatory cells but also prostate cancer cells can produce IL-8 [Inoue et al., 2000], suggesting that IL-8 may be actively secreted from cancer cells and acts in an autocrine manner [Kamohara et al., 2007]. Therefore, IL-8 is now regarded as a potential progression factor in the development of prostate cancer [Veltri et al., 1999; Lee et al., 2004]. As IL-1 and IL-8 have been shown to be important in cancer progression and development and IL-8 secretion is often enhanced by other inflammatory cytokines, for example IL-1 [Brat et al., 2005], it would be intriguing to know whether IL-8 expression in prostate cancer cells is regulated by IL-1β.

Glucosamine was first identified as a clinical supplement for the treatment in osteoarthritis where it possibly acts as an anti-inflammatory agent [McCarty, 1994] and indeed its significance to the inflammatory response has also been documented [Walsh et al., 2007]. As prostate cancer is highly correlated with the inflammatory response, it would also be of interest to know whether glucosamine is able to affect the putative IL-1β-mediated regulation of IL-8 and thus it might be a potentially effective option for the treatment of both harmful inflammation and prostate tumors.

In order to clarify the detailed regulation machinery of IL-8 by IL-1β and the role of glucosamine on such an effect in prostate cancer cells, a series of experiments were carried out using three prostate cancer lines with the aim of characterizing the signaling pathways mediating IL-1β-induced IL-8 expression. This involved measuring IL-8 mRNA expression and protein secretion, delineating the impact of glucosamine on IL-1β-regulated IL-8 production and identifying glucosamine's potential acting targets within the signaling pathways that are involved in the IL-1β response.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Reverse transcriptase and Taq polymerase were obtained from Promega (Madison, WI). Recombinant human IL-8, IL-8 antibody and IL-8 ELISA kit were purchased from R&D Systems (Minneapolis, MN). Recombinant IL-1β was obtained from PeproTech (Rocky Hill, NJ). To monitor cellular MAPK (p38, ERK, JNK) phosphorylation, rabbit polyclonal anti-phospho-p38 (Thr180/Tyr182) and rabbit polyclonal anti-p38 antibodies; rabbit polyclonal anti-phospho-p44/42 (Thr 202/Tyr204) and rabbit anti-p44/42 antibodies; rabbit anti-phospho-JNK (Thr183/Tyr185) and rabbit monoclonal anti-JNK antibodies were purchased from Cell Signaling (Danvers, MA). The transwell insert system was obtained from Corning (Lowell, MA). Unless otherwise specified, all other chemicals and reagents used in this project were purchased from Sigma Chemicals (St. Louis, MO).

CELL CULTURE

The prostate cancer cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere in various media containing 10% fetal bovine serum (Hyclone), penicillin and streptomycin: LNCaP in RPMI-1640; PC-3 in Minimum Essential Medium (MEM), and DU-145 in Dulbecco's Modified Eagle's Medium (DMEM).

GLUCOSAMINE'S EFFECT ON IL-1β-MEDIATED IL-8 SECRETION

Overnight plated DU-145, PC-3, and LNCaP cells in 6-well plates were washed three times with serum-free medium and treated with IL-1β (10 ng/ml) in the absence or presence of glucosamine (10, 3, or 1 mM) for 24 h. To determine the levels of IL-8 secreted into cultured media, the supernatants from cells were collected and the IL-8 concentration was analyzed using a commercial available ELISA kit according to the instructions from the manufacturer (R&D Systems).

IL-8 mRNA DETERMINATION BY SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Overnight plated prostate cancer cells were washed three times with serum-free medium and exposed to IL-1β (10 ng/ml) with or without the inclusion of glucosamine for 6 h. Total RNAs were prepared from treated cells using Tri-Reagent (Sigma) according to the manufacturer's instructions. The isolated RNA samples were resuspended in RNase-free diethypyrocarbonate (DEPC)-treated water and kept at −80°C. A two-step semi-quantitative RT-PCR method was used to measure mRNA expression of the IL-8, the IL-8 receptor CXCR1 and CXCR2. The final cDNA yields were measured against the signal obtained from the internal standard housekeeping genes GAPDH or β-actin of the same samples after amplification for 35 PCR cycles using appropriate parameters. The primer sequences are listed in Table I. The PCR products were subjected to electrophoresis on a 2% agarose gel with 1 μg/ml ethidium bromide. The DNA signal on the gel was captured and analyzed by ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

TABLE I. Primer Sequences Used for Semi-Quantitative RT-PCR

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Size (bp)
GAPDH	5′-ATC ACC ATC TTC CAG GAG CG-3′	5′-CCT GCT TCA CCA CCT TCT TG-3′	574
IL-8	5′-ACT TCC AAG CTG GCC GTG GCT-3′	5′-TCA CTG GCA TCT TCA CTG ATT-3′	345
β-Actin	5′-GGC ACC ACA CCT TCT ACA AT-3′	5′-CGT CAT ACT CCT GCT TGC TG-3′	834
CXCR1	5′-ATG TCA AAT ATT ACA GAT CC-3′	5′-AGA TTC ATA GAC AGT CCC CA-3′	500
CXCR2	5′-GAG GAC CCA GGT GAT CCA GG-3′	5′-GAG AGT AGT GGA AGT GTG CC-3′	250

DETERMINATION OF CELL VIABILITY BY MTT ASSAY

To study the functional significance of IL-8 and glucosamine in prostate cancer cells, The MTT assay [Mosmann, 1983] was adopted to monitor cellular proliferation. Prostate cancer cells were plated at 10,000 cells/well in a 96-well plate format, serum starved for 24 h and treated with IL-8 for additional 48, 72, or 96 h. On termination of treatment, the treated cells were incubated with 0.5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) at a dilution of 1:10 from a stock solution (5 mg MTT/ml) based on the volume of culture medium; this was carried out for 4 h at 37°C. At the end of incubation, the MTT solution was removed and 200 μl of isopropanol was added to each well and mixed thoroughly to dissolve the dark-blue formazon crystals formed. The proportion of viable cells was determined by reading the optical density using a test wavelength of 570 nm with a reference wavelength of 630 nm using an ELISA reader (Power Wave X340, Bio-Tek Instrument Incorporation, Winoski, VT).

WOUND-HEALING ASSAY

In each well of a 24-well plate, PC-3 cells were seeded overnight to be near 100% confluent and serum starved for 24 h. A yellow pipette tip was used to make a straight scratch, simulating a wound, keeping the pipette tip under an angle of around 30 degrees to keep the scratch width relatively consistent. On each well, two separate wounds were made through the cells as duplicates within the same well. Cells were gently rinsed once with 1× PBS, which was followed by the addition of serum-free medium containing IL-1β (10 ng/ml) or 5% serum in the presence or absence of 10 mM glucosamine or containing IL-8 (200 or 400 ng/ml). Before adding any treatment, images of the wound edges were recorded by microscopy using 10× objective and this was designed as 0 h. The treated cells were then incubated at 37°C and the migration profile of cells into wounded areas observed using an inverted microscope and photographed after 24 h. The extent of closure was determined by measuring the increase in the cell number within the wound area over the 24 h. The obtained values were means from independent experiments with duplicate wounds within triplicate wells.

TRANSWELL MIGRATION ASSAY

A co-culture system was used as an alternative method of examining the migration of prostate cancer cells in response to treatment or to factors secreted from stimulated cells. In brief, 3×10^5 cells/well were seeded overnight onto 24-well plate, followed by 24 h serum starvation. Transwell inserts with 8 μm pore size and 6.5 mm-diameter polyethylene terephthalate filters (Corning) were coated on top and bottom sides by immersion in polylysin for 12 h and left to air-dry overnight. The coated inserts were then placed in the wells of 24-well plates with serum-started cells (bottom wells). Subconfluent PC-3 cells (10,000) were then seeded onto each inner insert of each well using 100 μl of serum-free medium. A variety of treatments were included in the bottom wells and the plates with inserts were then incubated for 24 h. At the termination of the experiment, medium was removed from inserts and the remaining cells on the upper side of the chamber were removed using a cotton swab. The inserts were then fixed with 4% paraformaldehyde for 15 min and then stained with crystal violet solution (Sigma) for 30 s. The cells that had migrated to the underside of the filter membrane were counted using a microscope. Five fields per filter were counted with a 40× objective from triplicate wells of each treatment group.

IDENTIFICATION OF THE SIGNALING PATHWAYS MEDIATING IL-1β-INDUCED IL-8 PRODUCTION AND THE POTENTIAL TARGET OF GLUCOSAMINE

To pinpoint whether MAP kinases and/or NFκB are the sites where IL-1β exerts its induction of IL-8 expression in prostate cancer cells, a panel of chemical inhibitors (SB203580 for p38; SP600125 for JNK; PD98059 for ERK and pyrrolidine dithiocarbamate [PDTC] for NFκB) were utilized to evaluate the inhibitors' effects on IL-1β-mediated-IL-8 production. Cells were pretreated with the inhibitors 30 min before IL-1β (10 ng/ml) was added for an additional 24 h. The activation profiles of MAPKs by IL-1β in terms of phosphorylation were also examined biochemically using specific antibodies against phospho-p38, phospho-JNK and phospho-ERK in the presence or absence of glucosamine. The aim of this approach was the identification of the MAPK targets of glucosamine effect.

STATISTICAL ANALYSIS

Experimental data were expressed as standard errors of the means (mean ± SEM) and were analyzed using one-way ANOVA followed by the Dunett's test to compare the difference between the treatment groups and the control group. Any *P* values smaller than 0.05 were considered to be significant.

RESULTS

GLUCOSAMINE EFFECT ON IL-1β-MEDIATED IL-8 SECRETION

To first examine whether IL-1β would regulate IL-8 production in prostate cancer cells and if glucosamine could affect such regulation, two androgen-independent cell lines, DU-145 and PC-3 and one androgen-dependent cell line, LNCaP, were exposed to IL-1β (10 ng/ml) in the presence or absence of glucosamine (10, 3, or 1 mM) and the concentration of IL-8 in the cultured media was determined. Constitutive secretion of IL-8 could not be detected in LNCaP (Fig. 1C), was low in DU-145 (~300 pg/ml, Fig. 1A) and was high in PC-3 (~1.7 ng/ml, Fig. 1B). The overall IL-1β-mediated secretion of IL-8 was highest in DU-145 (~160 ng/ml; Fig. 1A) compared to PC-3 (~30 ng/ml; Fig. 1B) and LNCaP (~15 ng/ml; Fig. 1C). Glucosamine at all concentrations (10, 3, or 1 mM) tested significantly inhibited IL-1β-induced IL-8 secretion in DU-145 (Fig. 1A) and produced moderate reductions in IL-1β-mediated IL-8 secretion in PC-3 and LNCaP cells (Fig. 1B,C). In addition, the IL-8 secretion amounts were not different between the treatment with just glucosamine and the control group: 0.5 ± 0.1 versus 0.3 ± 0.2 ng/ml in DU-145 (Fig. 1A); 1.8 ± 0.5 versus 1.7 ± 0.4 ng/ml in PC-3 (Fig. 1B); undetectable in LNCaP (Fig. 1C), suggesting that glucosamine alone did not appear to affect basal IL-8 secretion in any cell line.

REGULATION OF IL-8 mRNA EXPRESSION BY IL-1β

To clarify whether IL-8 production regulated by IL-1β and glucosamine occurred at the transcriptional level, the IL-8 mRNA concentration was determined by RT-PCR. Basal expression of IL-8

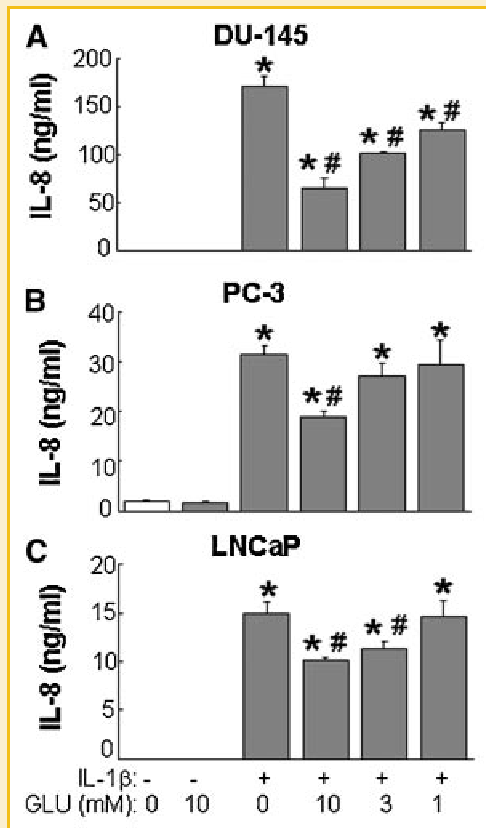

Fig. 1. Inhibition of IL-1β-induced IL-8 production in prostate cancer cells by glucosamine. Twenty-four-hour serum-starved prostate cancer cells DU-145 (A), PC-3, (B) or LNCaP (C) were either untreated, treated with glucosamine alone (10 mM) or treated with IL-1β (10 ng/ml) in the presence or absence of glucosamine (10, 3, or 1 mM) for 24 h. The IL-8 concentration in the cultured media was determined by ELISA and the results are expressed as mean ± SEM from four separate experiments. * $P < 0.05$ compared with control treatment; #$P < 0.05$ compared with IL-1β treatment.

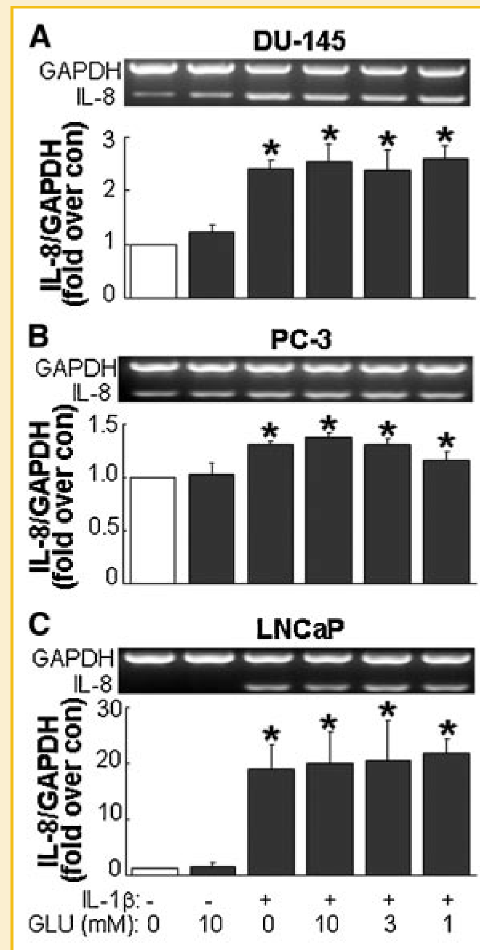

Fig. 2. Induction of IL-8 mRNA expression by IL-1β in prostate cancer cells. Twenty-four-hour serum-starved prostate cancer cells DU-145 (A), PC-3 (B), or LNCaP (C) were either untreated, treated with glucosamine alone (10 mM) or treated with IL-1β (10 ng/ml) in the presence or absence of glucosamine (10, 3, or 1 mM) for 6 h. The IL-8 mRNA levels were examined by semi-quantitative RT-PCR with GAPDH as an internal control and the results are expressed as mean ± SEM from four separate experiments. * $P < 0.05$ compared with control treatment.

mRNA was noted in DU-145 and PC-3, but not in LNCaP (Fig. 2). IL-1β treatment resulted in an elevation of IL-8 mRNA in all three cell types (Fig. 2A–C), with the greatest induction in LNCaP (Fig. 2C). The addition of glucosamine did not affect IL-1β-induced IL-8 mRNA expression (Fig. 2).

FUNCTIONAL SIGNIFICANCE OF IL-1β, IL-8, AND GLUCOSAMINE IN PROSTATE CANCER CELLS

To further examine the functional significance of IL-8 in prostate cancer cells, the cognate receptors for IL-8, CXCR1 and CRCR2, were determined in all three cell lines; it was found expression of these receptors seemed to remain constant across all treatments (Fig. 3A–C), suggesting a constitutive expression of CXCR1 and CXCR2 and that they were not regulated by either IL-1β or glucosamine. To address whether IL-1β, IL-8 or glucosamine exert any effects on the proliferation or migration of prostate cancer cells, 24 h serum-starved cells were subject to recombinant human IL-8 treatment and the cellular proliferation was evaluated by MTT assay. In both DU-145 and PC-3 cells, IL-8 did not seem to affect cellular proliferation (Fig. 4A,B); however IL-8 slightly increased cellular proliferation of LNCaP cells with the most dramatic effect being at 96 h (Fig. 4C). To characterize the impacts of IL-1β, IL-8 and glucosamine on cell migration, wound-healing and transwell assays were used. Low-level spontaneous migration was observed in control group (serum-free; Fig. 5) and this basal migration appeared to be inhibited by glucosamine (Fig. 5 and 6A). There was a dramatic induction of migration by 5% serum and a moderate induction of migration by IL-1β and these effects were attenuated by glucosamine (Fig. 5 and 6A). Meanwhile, addition of exogenous IL-8 also caused significant enhancement of PC-3 migration (Fig. 6B). When the transwell assay was used to determine PC-3's migration profile on treatment with IL-1β, IL-8 and glucosamine, it was found that the addition of IL-8 (200 or 400 ng/ml) resulted in significant PC-3 migration (Fig. 6C). In both wound-healing and transwell assays, addition of glucosamine along with IL-8 did not significantly affect the IL-8-mediated migration (Fig. 6B,C),

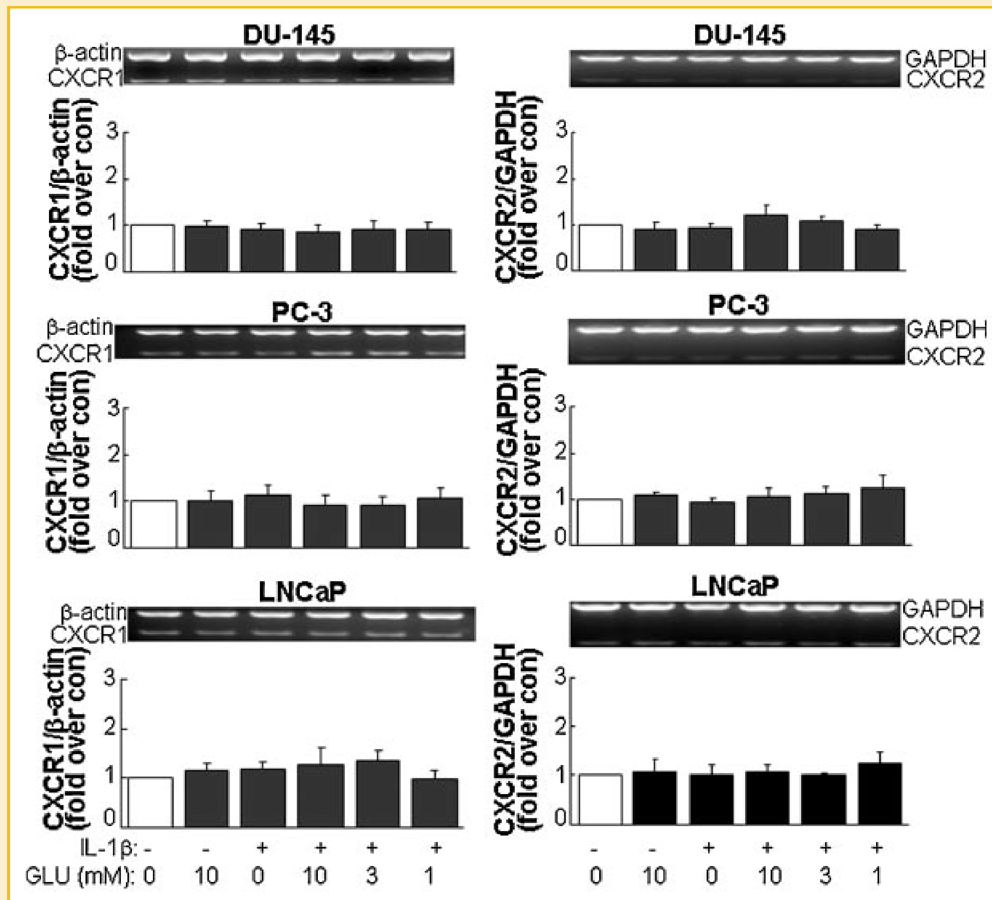

Fig. 3. Expression the mRNAs encoding IL-8 receptor CXCR1 and CXCR2 in prostate cancer cells. Twenty-four-hour serum-starved prostate cancer cells DU-145 (A), PC-3 (B), or LNCaP (C) were either untreated, treated with glucosamine alone (10 mM) or treated with IL-1β (10 ng/ml) in the presence or absence of glucosamine (10, 3, or 1 mM) for 6 h. The mRNA expression profiles of CXCR1 and CXCR2 were quantified by semi-quantitative RT-PCR using β-actin as an internal control for CXCR1 and GAPDH as an internal control for CXCR2. The results are expressed as mean ± SEM from four separate experiments.

suggesting no effect of glucosamine on IL-8-mediated migration. Inclusion of IL-1β induced a more than five-fold increase in cell migration and this effect was inhibited nearly 50% by glucosamine (Fig. 6D). In addition, cotreatment with anti-IL-8 antibody but not control antibody was found to suppress IL-1β-induced migration by more than 50% (Fig. 6D); this suggests that IL-8 is involved in IL-1β-mediated PC-3 migration.

IDENTIFICATION OF THE SIGNALING PATHWAYS MEDIATING IL-1β-INDUCED IL-8 PRODUCTION AND THE POTENTIAL TARGET OF GLUCOSAMINE

To clarify the signaling pathways mediating IL-1β-induced IL-8 production in DU-145, PC-3 and LNCaP cells, inhibitors of MAPKs as well as of NFκB were included with the IL-1β treatment of prostate cancer cells. In DU-145 cells, IL-1β-induced IL-8 production was inhibited by inhibitors of p38, JNK and ERK, with the most dramatic impact being that of the p38 inhibitor. However, the NFκB inhibitor had no effect (Fig. 7A). Similarly in PC-3 cells, IL-1β-mediated IL-8 production was suppressed by all the MAPK inhibitors but not by the NFκB inhibitor; however, the reduction was relatively modest compared to DU-145 (Fig. 7B). In LNCaP cells, the inhibitors of JNK and ERK significantly neutralized the induction effect of IL-1β on IL-8 production (Fig. 7C). Interestingly, inclusion of the inhibitors of p38 or NFκB seemed to enhance IL-1β-induced IL-8 production (Fig. 7C).

As IL-1β-mediated IL-8 production in all three cell types appeared to be dependent on MAPK signaling pathways (Fig. 7), we investigated whether glucosamine may affect IL-1β-mediated MAPK activation and therefore blunt IL-1β-induced IL-8 production. IL-1β-induced phosphorylation of p38, JNK and ERK was monitored at 10 and 15 min in DU-145 cells. Phosphorylation of p38 was activated by IL-1β at both 10 and 15 min and cotreatment with glucosamine abrogated this phosphorylation at 15 min (Fig. 8A). JNK phosphorylation was activated by IL-1β at 15 min and this activation was also significantly inhibited by glucosamine (Fig. 8B). Similarly, ERK phosphorylation was induced by IL-1β at 10 and 15 min and its induction was reduced by glucosamine at 15 min (Fig. 8C). Therefore, phosphorylation of p38, JNK, and ERK were all activated by IL-1β in DU-145 cells and the induction was considerably suppressed by glucosamine (Fig. 8).

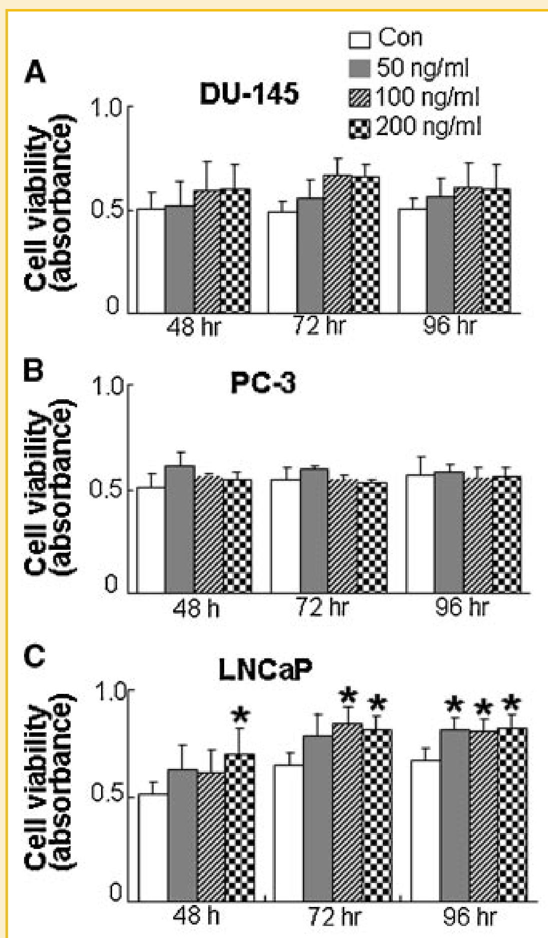

Fig. 4. Effects of IL-8 on prostate cancer proliferation. Prostate cancer cells DU-145 (A), PC-3 (B), and LNCaP (C) were either untreated or treated with IL-8 (50, 100, and 200 ng/ml) for 48, 72, or 96 h. Cellular proliferation was monitored by MTT assay. Data are expressed as the mean ± SEM from four individual experiments. Significant induction ($P < 0.05$) of proliferation by IL-8 within a time point was indicated by asterisk (*).

DISCUSSION

This study mimics the microenvironment of the prostate in order to investigate the potential impact of amplification of local cytokine IL-8 production induced by IL-1β, and the possible role that glucosamine may play in such regulation. Our results demonstrate that IL-8 mRNA expression and protein secretion is induced in prostate cancer cells by IL-1β and that this results in a stimulation of cell proliferation and migration. Induction of IL-8 secretion by IL-1β involves, at least in part, the MAPK signaling pathways (p38, ERK, and JNK) and glucosamine may target the MAPKs by down-regulating IL-1β-induced activation of MAPKs, which reduces IL-8 production as a result.

In supporting the significance of inflammation in cancer progression, our results demonstrated in prostate cancer cells that IL-1β-induced IL-8 production is an important mediator of prostate cancer proliferation/migration (Figs. 4–6). More importantly, IL-1β-induced cell migration was significantly attenuated by anti-IL-8 antibody treatment (Fig. 6), which strongly supports the hypothesis that IL-1β may induce IL-8 production by prostate cancer cells and, subsequently, the increased IL-8 promotes prostate cancer cell migration in an autocrine manner. In agreement with our findings, a previous study has pointed out that IL-8 is able to induce expression of matrix metalloproteinases (MMPs) and thus performs a proangiogenic function in cancer cells [Brat et al., 2005]. More importantly, in this study we are the first to demonstrate that when cancer cells are treated with glucosamine, IL-1β-induced IL-8 production (Fig. 1) and cell migration (Figs. 5 and 6) are significantly suppressed. This suggests that glucosamine may have potential therapeutic value in terms of targeting local (intra-prostate) inflammation and thus retarding cancer growth. Furthermore, in addition to maintaining energy homeostasis, it should be noted that an anti-inflammation role for glucosamine has been recognized previously and its use in the treatment of osteoarthritis is also established [McCarty, 1994]. Our findings further extend the value of glucosamine from that of anti-inflammation agent to that of an anti-cancer agent.

When considering the cellular target sites for a drug in order to regulate agonist-induced cytokine expression and subsequent secretion, there are a number of possible sites. The interaction may potentially occur either at the cell surface where the agonist binds its receptor or within an intracellular compartment with impact on a range of processes including gene transcription, post-transcription control, translation, post-translation control and secretion. In this study, we have addressed the significance of glucosamine in IL-1β-mediated IL-8 production, thus the possible target sites of glucosamine could be IL-1β binding to its receptor, or acting on the transcription, post-transcription control, translation, post-translation control and/or secretion of IL-8. To identify the molecular mechanisms of glucosamine, we first examined whether glucosamine alone would directly affect IL-8 production and we indeed confirmed that glucosamine only showed an inhibition effect in the IL-1β-stimulated condition (Fig. 1). This indicates that the glucosamine effect does not cause an extensive disturbance within the cell but that a specific targeted event on the IL1-β occurs. This property is very crucial when we consider that glucosamine is consumed by the body during oral supplement. Next, we went on to identify that the intracellular signaling pathways mediating IL-1β-elicited IL-8 production appeared to depend on the MAPKs, including p38, JNK and ERK in DU-145 and PC-3 cells, but only JNK and ERK in LNCaP cells (Fig. 7). Similar to our findings, previous studies have demonstrated in a variety of cell types that p38 [Neuder et al., 2009], JNK [Rasmussen et al., 2008], and ERK [Wurm et al., 2008] are involved in the regulation of IL-8 expression. One surprising discovery in our study is that in the all three prostate cancer cell types tested the IL-1β-induced IL-8 production did not appear to depend on the NFκB pathway (Fig. 7), which has been shown to act downstream of IL-1β stimulation to regulate transcription of target genes [O'Neill, 2008]. In addition, another study has also proposed NFκB to be a critical player in mediating bcl-xL-induced IL-8 transcription [Gabellini et al., 2008]. Thus further investigation is required to discover if our finding that NFκB-independent regulation of IL-1β-induced IL-8 production is unique to prostate cancer cells and whether this discrepancy has

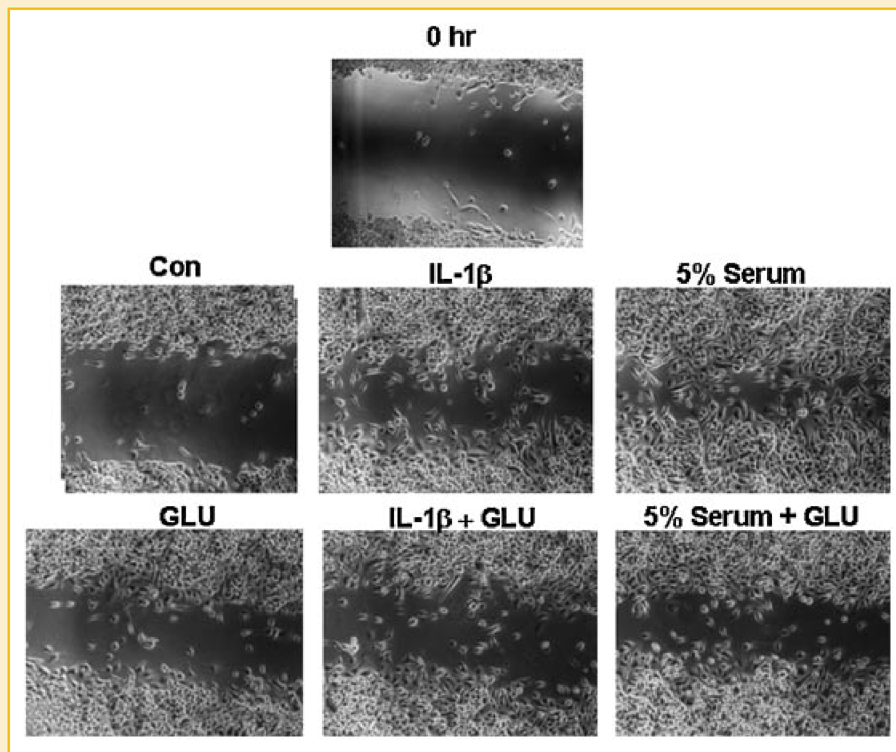

Fig. 5. Regulation of prostate cancer cell migration by IL-1β and glucosamine by wound-healing assay. Confluent PC-3 cells were serum-starved for 24 h and this was followed by a wound-healing assay after treatment with serum-free medium (Con), glucosamine (10 mM) IL-1β or 5% serum in the presence or absence of glucosamine (10 mM) for 24 h. The migration profiles were recorded by microscopy.

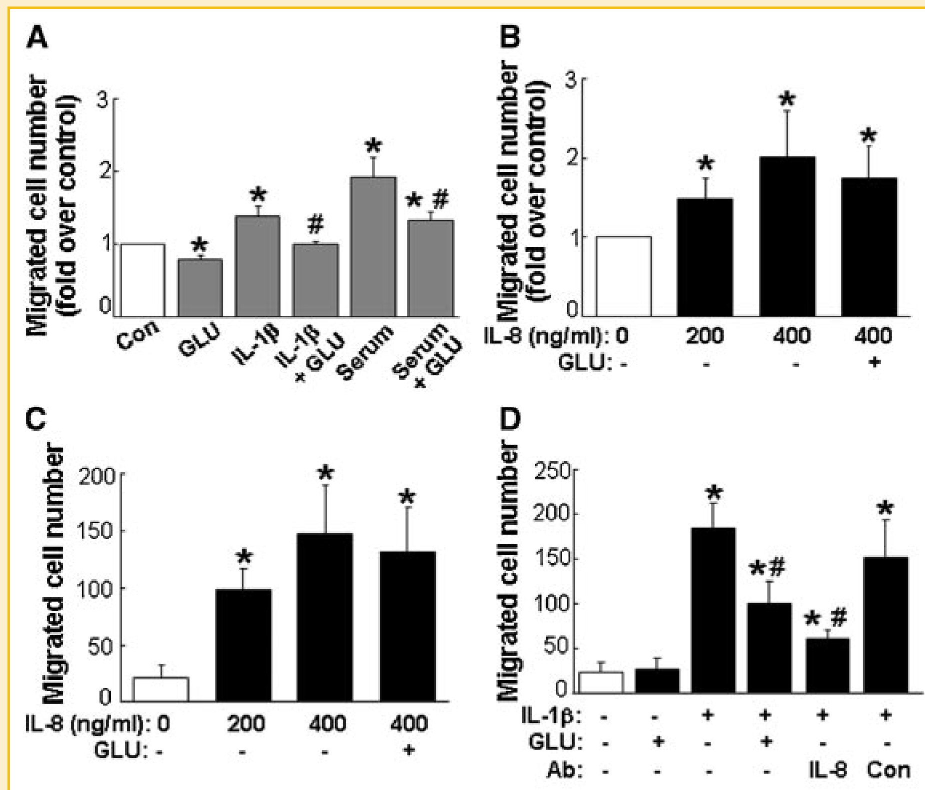

Fig. 6. Regulation of prostate cancer cell migration by IL-1β, IL-8 and glucosamine by wound-healing and transwell assays. Serum-starved confluent PC-3 cells were subject to the wound-healing assay with the treatment of serum-free medium (Con), glucosamine (10 mM) or IL-1β (10 ng/ml) or 5% serum in the presence or absence of glucosamine (10 mM) (A) or IL-8 (200 and 400 ng/ml) alone or with the inclusion of glucosamine (B) for 24 h. Alternatively, a co-culture transwell system was also used to analyze the migration or serum-starved PC-3 cells after the treatment of IL-8 alone or with the inclusion of glucosamine (10 mM) (C) or IL-1β (10 ng/ml) in the absence of presence of glucosamine (10 mM), anti-IL-8 antibody or control antibody (D) for 24 h. Data are expressed as the mean ± SEM from seven (A), three (B,C), or four (D) individual experiments. * $P < 0.05$ compared with control treatment; # $P < 0.05$ compared with IL-1β (A,D) or serum treatment (A) to denote the inhibition effect by glucosamine or IL-8 antibody.

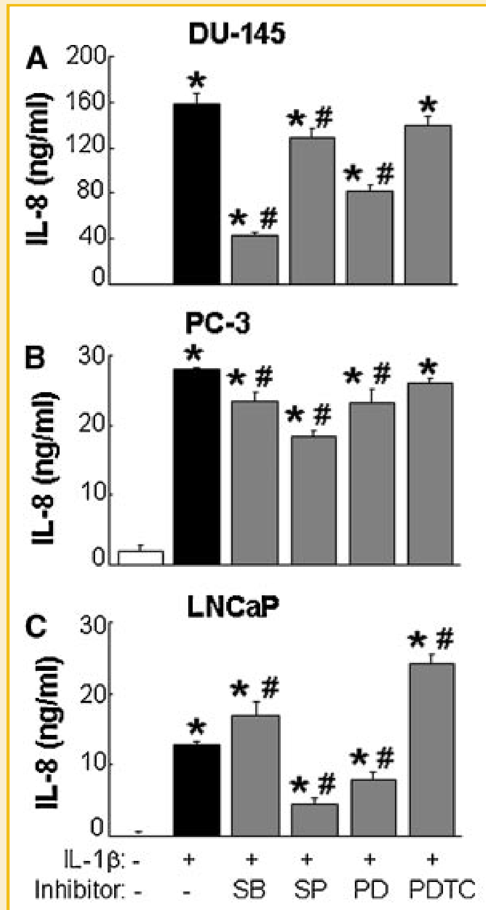

Fig. 7. Identification of the signaling pathway(s) mediating IL-1β-induced IL-8 production in prostate cancer cells. Prostate cancer cells DU-145 (A), PC-3, (B) or LNCaP (C) were pretreated with MAPK kinase inhibitors (SB: p38 inhibitor, 20 μM; SP: JNK inhibitor, 40 μM; PD: ERK inhibitor, 20 μM) or NFκB inhibitor (PDTC, 20 μM) for 30 min and then IL-1β (10 ng/ml) was included for additional 24 h. Data are expressed as the mean ± SEM from four individual experiments with each treatment analyzed in duplicate wells. * $P < 0.05$ compared with control treatment; # $P < 0.05$ compared with IL-1β treatment.

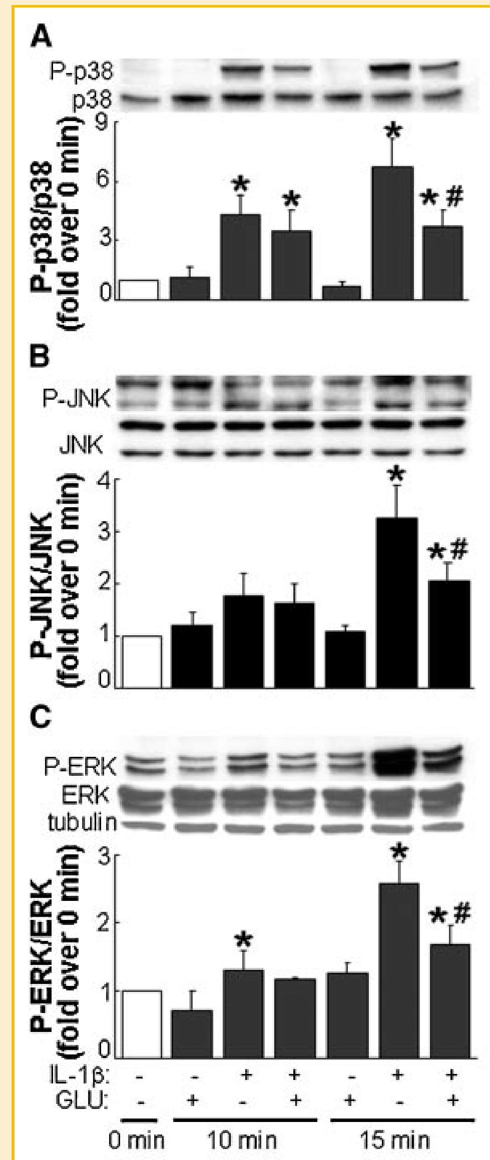

Fig. 8. Attenuation of the IL-1β-induced phosphorylation of MAPKs. Prostate cancer cells DU-145 were serum-starved for 24 h and exposed to the IL-1β (10 ng/ml) in the absence or presence of glucosamine (10 mM) for 10 or 15 min. Phosphorylation patterns of MAPK p38 (A), JNK (B), or ERK (C) was monitored by classical immunoblotting. Data are expressed as the mean ± SEM from four individual experiments. * $P < 0.05$ compared with 0 min point; # $P < 0.05$ compared with IL-1β treatment within the same time point.

pathological significance. Regardless of the answers to these questions, we clarified that IL-1β-induced phosphorylation (activation) of p38, JNK and ERK in DU-145 cells was significantly repressed by glucosamine (Fig. 8). This is in accordance with the finding that all three MAPK pathways are involved in IL-1β-induced IL-8 production (Fig. 7). Similar to our discovery, previous studies in macrophages and chondrocytes have also reported that glucosamine is able to inhibit phosphorylation of p38 and JNK [d'Abusco et al., 2007; Mendis et al., 2008]; however we are the first to describe glucosamine inhibition of ERK phosphorylation (Fig. 8). However, in this project, one unanswered question that remains is why and how does glucosamine affect IL-1β-induced IL-8 secretion (Fig. 1) but not IL-8 mRNA expression (transcription or post-transcription) in all three cell types (Fig. 2). Although the detailed mechanism of how glucosamine functions is still unknown and no glucosamine receptor or direct interacting partner has ever been identified, it has been shown that by changing extracellular glucose and glucosamine levels, a variety of cellular functions are modulated in a glycolysis-independent manner [Zachara and Hart, 2006]. In addition, a metabolic product of glucosamine, O-linked β-*N*-Acetylglucosamine (O-GlcNAc), has been proposed to regulate protein functions by altering protein phosphorylation, protein degradation, protein localization, protein–protein interactions, and gene transcription [Zachara and Hart, 2006]. Thus, whether glucosamine may interfere with IL-1β-mediated IL-8 production through the involvement of O-GlcNAc would seem to need further investigation.

In this study, we noted a constitutive and more profound IL-1β-induced secretion of IL-8 by androgen-independent DU-145 and PC-3 cells (Fig. 1A,B) in comparison with the androgen-dependent LNCaP cells (Fig. 1C). We also observed an effective induction of cell proliferation by IL-8 in LNCaP cells (androgen-dependent), but not in DU-145 or PC-3 cells (androgen-independent) (Fig. 4). Nonetheless, the expression of the IL-8 cognate receptors CXCR1 and CXCR2 in the three cell lines was determined to be consistent across all treatments (Fig. 3). Thus, presumably, all three cell types are able to respond to IL-8 stimulation. In this context, prostate cancer development often results in a shift from androgen-dependent to androgen-independent growth and this creates challenges during clinical treatment [Chen et al., 2004]. From these facts, an intriguing question arises, which is whether IL-8 is involved in the transition from androgen-dependence to androgen-independence and how such a scenario might occur; this remains to be determined in the future. In addition, we found that IL-8-promoted PC-3 cell migration was not inhibited by glucosamine (Fig. 6), suggesting that glucosamine is functioning specifically on IL-1β-mediated IL-8 production, rather than on IL-8's direct effect on cell migration. Another striking discovery in our study is that glucosamine seemed to inhibit IL-1β-mediated IL-8 production with various efficacies across the three cell types, with the most profound inhibition being on DU-145 cells (Fig. 1). The reason(s) for this difference among cell types are currently not clear; however this information strongly suggests that when considering glucosamine as a strategy to treat prostate cancer, dosage optimization for the different stages needs detailed consideration.

In summary, this study is the first to demonstrate in prostate cancer cells that a first acting pro-inflammatory cytokine, IL-1β, may induce IL-8 in a MAPK dependent manner and that consequently IL-8 acts in an autocrine manner to promote cell proliferation and/or migration. In addition, a common daily supplement, glucosamine, is able to attenuate the IL-1β-mediated IL-8 production and this is, at least in part, due to an inhibition of the IL-1β-mediated MAPK activation. Our findings highlight a new anti-cancer role for the anti-inflammatory molecule glucosamine and this is deserving of further investigation.

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