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Effects of hypoxia/ischemia on catabolic mediators of cartilage in a human chondrocyte, SW1353

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ABSTRACT

Cells from a human chondrocyte cell line were studied in 1% oxygen and/or a lower glucose concentration (5.5 mM), compared to the routine culture conditions of normoxia and high glucose. HIF-1α, IL-1β, IL-6, IL-8, COX-2, TNFα, LIF, MMP-3, MMP-13, and reactive oxygen species (ROS) were evaluated, respectively. Effects of hypoxia inducing expression of HIF-1α were statistically significant at 72 h ($p < 0.05$). Increased production of ROS by hypoxia was also observed with passage of time ($p < 0.05$). The effects of hypoxia on HIF-1α and IL-1β were potentiated by 5.5 mM glucose, especially after 48 h ($p < 0.05$). IL-8 production was significantly induced in 1% O_2, with 5.5 mM glucose ($p < 0.01$). IL-8 mRNA expression and production in response to IL-1β were potentiated by hypoxia/ischemia ($p < 0.05$, $p < 0.01$, respectively). Up-regulation of IL-1β, ROS, and IL-8 by hypoxia/ischemia in human chondrocytes may occur in correlation with HIF-1α. IL-8 response to IL-1β may be potentiated synergically by hypoxia/ischemia, as an effector of hypoxia/ischemia. The results may suggest aggressive biology of the ordinary cartilage hypoxia/ischemia in the context of arthro-degeneration.

1. Introduction

Articular cartilage is distinct from other tissues as an avascular tissue, and chondrocytes survive hypoxia and poor nutrients. Silver et al. reported that the oxygen levels of healthy cartilage range from 7% to 10% of the superficial zone to values as low as 1% in the deep zone [1]. Accordingly, chondrocytes consume much less oxygen, compared with most other cell types [2], and can survive anoxia for days [3,4]. Most cell types have adaptive mechanisms to low oxygen tension, however, only a limited number of cell types can live on anaerobic glycolysis through prolonged deprivation of oxygen. Glucose is an important metabolic fuel for differentiated chondrocytes and a common structural precursor for synthesis of matrix glycosaminoglycans (GAGs). However, it is supplied from peri-articular vasculatures to the cartilage only by diffusion. Chondrocytes, as glycolytic cells, are able to sense the concentrations of glucose as well as oxygen, and respond appropriately by adjusting their metabolism [5]. In addition to glycolysis, glucose level in synovia and the glucose availability of chondrocytes may have roles in regulation of oxygen consumption of articular cartilage [6].

In most mammalian cells, responses to low oxygen and nutrients are mediated and controlled critically by hypoxia-inducible factor-1 (HIF-1) [7]. HIF-1 is a transcription factor consisting of constitutively produced α- and β-subunits [8–11]. However, with oxygen, oxygen-sensitive prolylhydroxylase catalyzes proteasome-mediated degradation of the α-subunits that are stabilized in deprivation of oxygen, therefore, the α-subunit is supposed to be the active component of HIF-1 responses to hypoxia [7,12].

The ordinary hypoxic and glycolytic conditions of cartilage are supposed to make cartilage prone to profound hypoxia/ischemia and vulnerable to damage, furthermore, the oxygen tensions showed a further decrease in osteoarthritis (OA) joints [13,14]. Some biochemical mediators have been proven as catabolic or protective for joint degeneration. The catabolic mediators include proinflammatory cytokines, such as IL-1, IL-6, IL-17, TNF-α, and PGE2; growth factors; glycoproteins, and oxidative stress mediators, such as ROS. IL-1β is known to play a major role in development of OA through induction of MMP-1, MMP-13, ADAMTS-4, IL-6, IL-8, and leukemia inducing factor (LIF) [15,16]. ROS mediates the destructive actions of IL-1β and TNF-α on cartilage through suppression of the synthesis of matrix proteins and induction of MMPs [17–19].

This study evaluated the effects of ordinary hypoxia/ischemia on chondrocytes through changes of catabolic mediators of cartilage in the context of arthro-degeneration.

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2. Materials and methods

2.1. Preparation of human chondrocytes

A human chondrocyte cell-line, SW1353 cells (ATCC, USA) was used in investigation of the effects of ischemia/hypoxia on chondrocytes. The cells were cultured in a monolayer with 25 mM-glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Korea) containing 10% FBS, penicillin 100 U/mL, and streptomycin 100 g/mL, and passaged weekly using trypsin–EDTA for detachment of cells from their monolayer. SW1353 cells of passage number between 27 and 36 were used for the experiments.

2.2. Culture conditions and treatment of chondrocytes

After over-night incubation, culture mediums were replaced with 3 mL of 25 mM-glucose DMEM or 5.5 mM-glucose DMEM. Chondrocytes in each glucose concentration were then cultured under 20% O_2 and 5% CO_2, or 1% O_2 and 5% CO_2 at 37 °C for 6 h, 24 h, 48 h, and 72 h, respectively. The hypoxic environment (1% O_2, 5% CO_2, 94% N_2) was created using a personal O_2/CO_2 multi-gas incubator, APM-300 (Astec, Korea), by replacing air with nitrogen gas [20,21]. Measurements of Oxygen partial pressures (PO_2) of the spent culture mediums were performed using an ABL800 basic analyzer (Radiometer, Denmark).

Chondrocytes under each condition were treated with human IL-1β (1 ng/mL) for 12 h in order to estimate the influences of hypoxia/ischemia on the responses of arthro-catabolic mediators to pro-inflammatory stimuli.

2.3. Detection and count of ROS producing chondrocytes

After conditioning, the chondrocytes were treated with 100 M carboxyl-2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probes, USA). Following incubation with H2DCF-DA for 1 h at 37 °C, the cells were washed twice with PBS and kept in the respective culture conditions for another 2 h. As a result of oxidation by intracellular ROS, the non-fluorescent dye was converted into its fluorescent form, dichlorofluorescein (DCF). SW1353 cells were then harvested, followed by analysis using a 488 nm argon laser EPICS XL-MCL flow cytometer controlled by EXPO 32-ADC software (Beckman Coulter, USA). The ROS value was determined by the fluorescence intensity of DCF.

2.4. Microarray analysis for ischemia/hypoxia-responsive gene profile in human chondrocytes

A microarray assay was performed for evaluation of gene expression in response to hypoxia/ischemia using the Human GE 4X44K V2 microarray kit (Genomax Technologies, Singapore). The qualified RNA of each condition was amplified and labeled using Agilent's low RNA input linear amplification kit PLUS. After hybridization, the microarrays were scanned using Agilent's DNA microarray scanner. As analysis of the images was performed using feature extraction software, the data were normalized and clustered using Agilent's gene spring software (Agilent Technologies, USA).

2.5. Western blotting for hypoxic makers in human chondrocytes

After 72 h incubation in each condition, total lysates were prepared in a PRO-PREP buffer (iNtRON, Seoul, Korea). Protein concentrations were determined by a Bradford assay using bovine serum albumin as a standard. Thirty-micrograms of the protein samples were separated on 10% SDS–polyacrylamide gels, and then transferred to nitrocellulose membranes. The membranes were soaked in 5% non-fat dried milk in TBST (10 mmol/L Tris–HCl pH 7.5, 150 mmol/L NaCl and 0.05% Tween-20) for 1 h and then incubated for 16–18 h with primary antibodies against HIF-1α Abcam, USA), GLUT-1 (Abcam, USA) and β-Actin at 4 °C. Membranes were washed three times with TBST for 10 min and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 4 °C. After incubation with the secondary antibody, the membranes were rinsed three times with TBST for 10 min and antigen–antibody complex was detected using an enhanced chemiluminescence detection system (LAS-3000, Fujifilm, Tokyo, Japan).

2.6. Real time RT-PCR analysis for hypoxia/ischemia-responsive genes of chondrocytes

Total RNA was harvested from SW1353 cells under each condition using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Korea), according to the manufacturer's instructions. Optical density (OD) was measured at 260 and 280 nm for determination of RNA concentrations. Among the arthro-catabolic mediators induced by hypoxia/ischemia, IL-1β, IL-6, IL-8, TNF-α, LIF, MMP-3, MMP-13, COX-2, and HIF-1α gene expressions were determined by real time RT-PCR under each condition. Complementary DNA (cDNA) was synthesized using 1 μg total RNA with oligo-(dT) primers and a Maxime RT premix kit (iNtRON Biotechnology, Korea) at 45 °C for 60 min, followed by RT inactivation at 95 °C for 5 min. Real-time RT-PCR for each gene was performed in triplicate using a 20 μL mixture containing LightCycler FastStart DNA SYBR Green I mix, primers, and 2 μL of cDNA. The relative expression levels of the genes were calculated by the formula $2^{-(\Delta\Delta Ct)}$ in comparison with control samples and β-actin as an internal reference gene. The primers used for PCR were as follows: human HIF-1α (sense, 5′-GTA GTT GTG GAA GTT TAT GCT AAT ATT GTG T-3′, antisense, 5′-CTT GTT TAC AGT CTG CTC AAA ATA TCT T-3′, 102 bp); IL-1β (sense, 5′-CGT CAG TTG TTG TGG CCA T-3′, antisense, 5′-GCG TGC AGT TCA GTG ATC GTA-3′, 183 bp); IL-8 (sense, 5′-ACA CTG CGC CAA CAC AGA AAT TA-3′, antisense, 5′-TTT GCT TGA AGT TTC ACT GGC ATC-3′, 185 bp), and β-actin (sense, 5′-CGG GAA ATC GTG CGT GAC ATT-3′, antisense, 5′-GAT GGA GTT GAA GGT AGT TTC GTG-3′, 327 bp).

2.7. Human IL-8 ELISA assay of chondrocytes

ELISA for human IL-8 was performed according to the manufacturer's instructions using kits from R&D systems (Minneapolis, USA). Briefly, 100 μL per well of the standard and spent culture mediums were incubated in plates coated with anti-human IL-8 antibody at room temperature for 2 h. After washing, the plates were further incubated with 200 μL of prepared biotinylated antibody at room temperature for 1 h, followed by addition of 200 μL of tetramethylbenzidine substrate solution in darkness. Following incubation at room temperature for 20 min, the reaction was stopped by addition of 50 μL of stop solution. Absorbance was measured at 450 nm for IL-8.

2.8. Statistical analysis

All values are expressed as means ± standard deviations (SD). Student's *t*-test was used for statistical analysis, and differences between groups were considered significant at p values < 0.05.

3. Results

PO_2 of the culture mediums after 72 h was 140.6 ± 7.5 mmHg in 1% O_2 with 25 mM glucose and 147.6 ± 2.1 mmHg in 1% O_2 with

5.5 mM glucose, which was significantly lower than 165.3 ± 5.1 mmHg in 20% O_2 with 25 mM glucose ($p = 0.01$, $p = 0.04$, respectively) or 172.3 ± 9.4 mmHg in 20% O_2 with 5.5 mM glucose ($p = 0.006$, $p = 0.02$, respectively).

To verify influences and effects of hypoxia/ischemia on chondrocytes, HIF-1α and GLUT-1 were evaluated as markers for hypoxia/ischemia; IL-1β and ROS as catabolic markers of cartilage.

HIF-1α and GLUT-1 were up-regulated by SW1353 cells in the hypoxic conditions and lower glucose synergically (Fig. 1A). Suppressed expression of HIF-1α mRNA was observed in SW1353 cells in 1% O_2 with 25 mM glucose at 6 h, 24 h, and 48 h, compared with that of 20% O_2 with 25 mM glucose as controls; however, an increase was observed with passage of time, with over-expression of HIF-1α at 72 h in 1% O_2 with 25 mM glucose, compared with controls ($p < 0.05$) (Fig. 1B). The lower glucose concentration enhanced the effect of hypoxia on HIF-1α expression with passage of time, which was statistically significant at 72 h, compared with that of 1% O_2 with higher glucose ($p < 0.05$) (Fig. 1B). Hypoxia-induced HIF-1α expression was analyzed in combination with lower glucose, compared with that of normoxia with a high glucose concentration. The combined conditions as an ischemia potentiated the effects of hypoxia after 48 h in correlation with time ($p < 0.05$) (Fig. 1B).

Suppressed expression of IL-1β mRNA in SW1353 cells was observed at 6 h, 24 h, and 48 h in 1% O_2 or 5.5 mM glucose, and over-expression was observed after 48 h, compared with that of 20% O_2 or 25 mM glucose (Fig. 1B), similar to the HIF-1α expression; 5.5 mM glucose was found to enhance IL-1β expression by hypoxia, compared to 25 mM glucose with 1% O_2, which was statistically significant at 72 h ($p < 0.05$) (Fig 1B). The combined hypoxia with lower glucose enhanced IL-1β expression, and potentiated synergically hypoxia-induced expression of IL-1β after 48 h in correlation with time ($p < 0.05$) (Fig. 1B).

ROS-positive chondrocytes were counted and sorted using a fluorescence activated cell sorting (FACS) system. Increased ROS production was induced by hypoxia of 1% O_2 with either 25 mM glucose or 5.5 mM glucose with passage of time (Fig. 2A and B). Similar to the IL-1β expression, ROS production by hypoxia in chondrocytes was consistent with HIF-1α expression, indicating that ROS increased as much as the influences of hypoxia/ischemia in human chondrocytes.

Global gene expression in response to hypoxia/ischemia was evaluated in human chondrocytes using a microarray assay. The results of microarray analysis showed up-regulation of HIF-1α and IL-1β expression by 1.3 and 1.6-fold, respectively. Among the reliable 27,113 genes expressed in hypoxia with lower glucose, the normalized ratios for cytokines/chemokines were 28.4 for IL-8, 1.25 for IL-6, 7.42 for LIF, 0.47 for IL-17A, and 0.4 for TNF-α as catabolic mediators; 5.78 for MMP-1, 1.78 for MMP-3, 0.24 for MMP-13, and 1.42 for ADAMTS-4 as collagenases. In particular, IL-8 expression was ranked as the 11th most responsive gene to hypoxia/ischemia as a pro-inflammatory cytokine and catabolic mediator of cartilage in SW1353 cells.

Among the cytokines/chemokines, collagenases, and other enzymes that were found to be up-regulated on microarray analysis, expression of IL-6, IL-8, COX-2, TNF-α, LIF, MMP-3, and MMP-13 was verified using real time RT-PCR. Semi-quantitative mRNA expression, even IL-8 expression, showed considerable variation

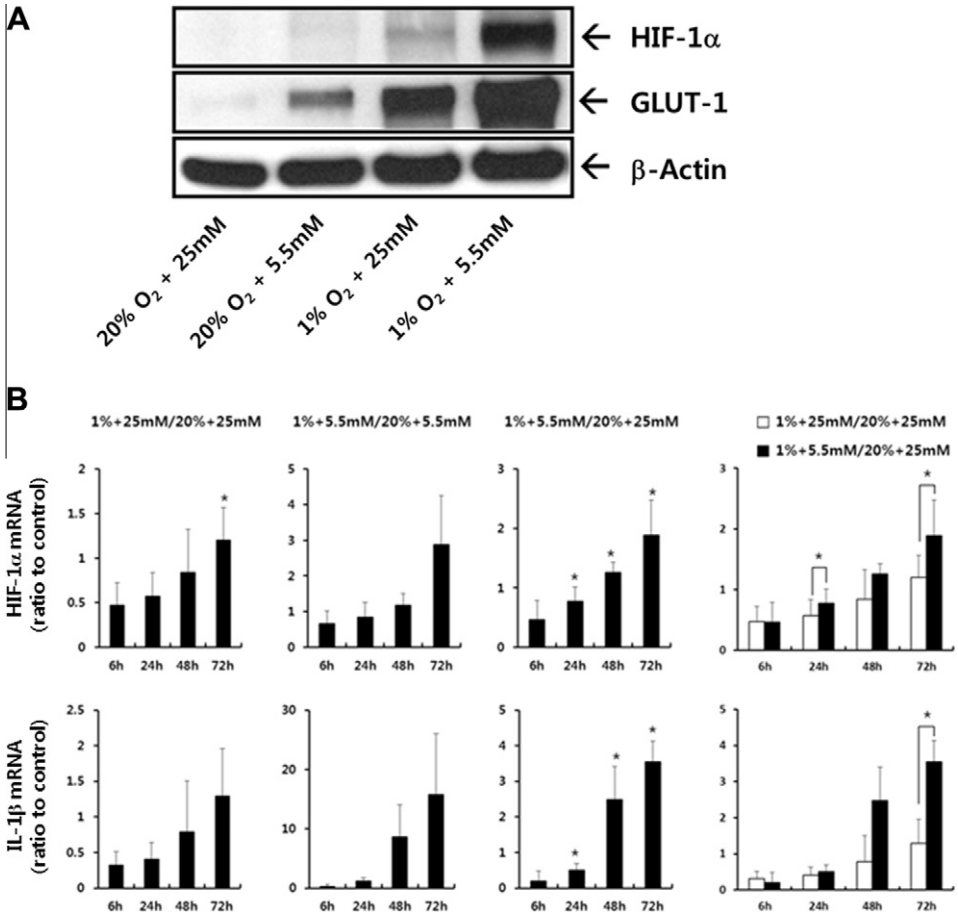

Fig. 1. HIF-1α, GLUT-1 and IL-1β in SW1353 cells were up-regulated under the hypoxic condition, the effects of hypoxia were potentiated by the lower glucose (5.5 mM) ((A) Western Blot, (B) real time RT-PCR). Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

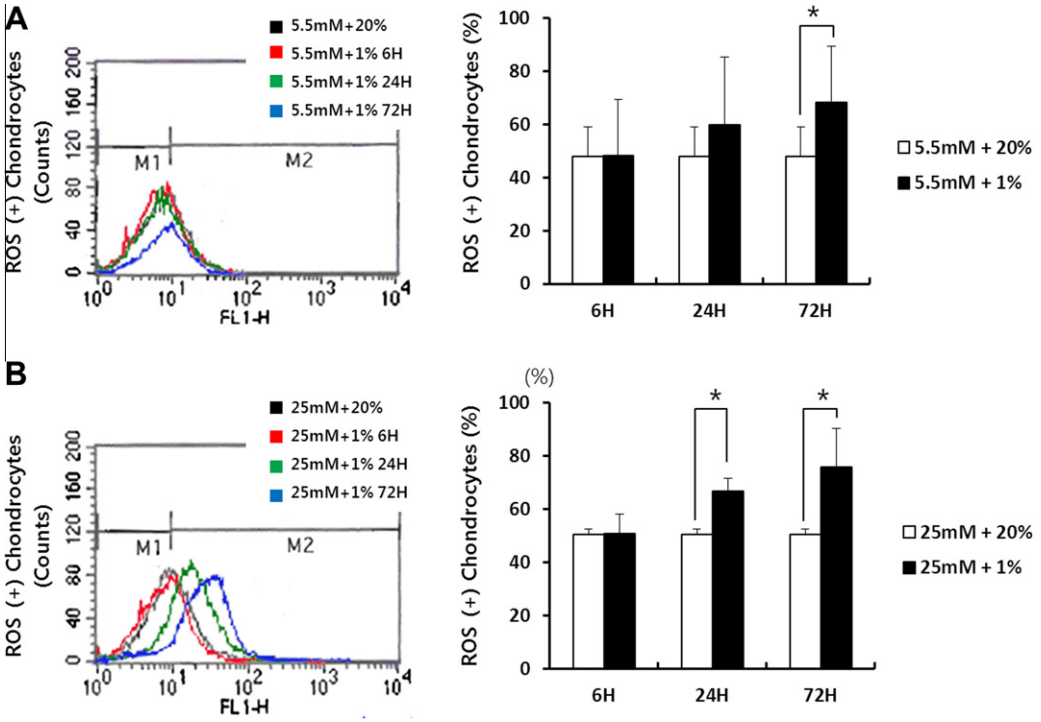

Fig. 2. Hypoxia induced production of ROS in SW1353 cells under each condition of higher glucose (A) and lower glucose concentration (B). Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

from experiment to experiment (Fig. 3A). However, production of IL-8 in chondrocytes was significantly enhanced by hypoxia in 1% O_2 with 5.5 mM glucose, compared with that of 20% O_2 with 5.5 mM glucose ($p < 0.01$) (Fig. 4A). The increases of IL-8 by hypoxia did not differ between glucose concentrations under the same oxygen concentration (Fig. 4B).

Analysis of IL-8 response to pro-inflammatory stimuli in hypoxia/ischemia was performed. After conditioning under the conditions of hypoxia/ischemia, the same as those used for real time RT-PCR, SW1353 cells were treated with human IL-1β. IL−1β-induced expression of IL-8 mRNA was potentiated by hypoxia in either 25 mM or 5.5 mM glucose ($p < 0.05$, $p < 0.01$, respectively) (Fig. 3B). IL-1β-induced IL-8 production was also potentiated by several folds by hypoxia ($p < 0.01$) (Fig. 4A and B).

4. Discussion

This study evaluated the effects of hypoxia/ischemia on human chondrocytes. Remarkable up-regulation of HIF-1α, IL-1β, ROS, and IL-8 by hypoxia was demonstrated in combination with lower glucose, compared to normoxia with higher glucose.

Considerable up-regulation of HIF-1α expression in hypoxia was significantly potentiated by lower glucose concentration. HIF-1α, the oxygen-sensitive subunit of HIF-1, acts as a survival

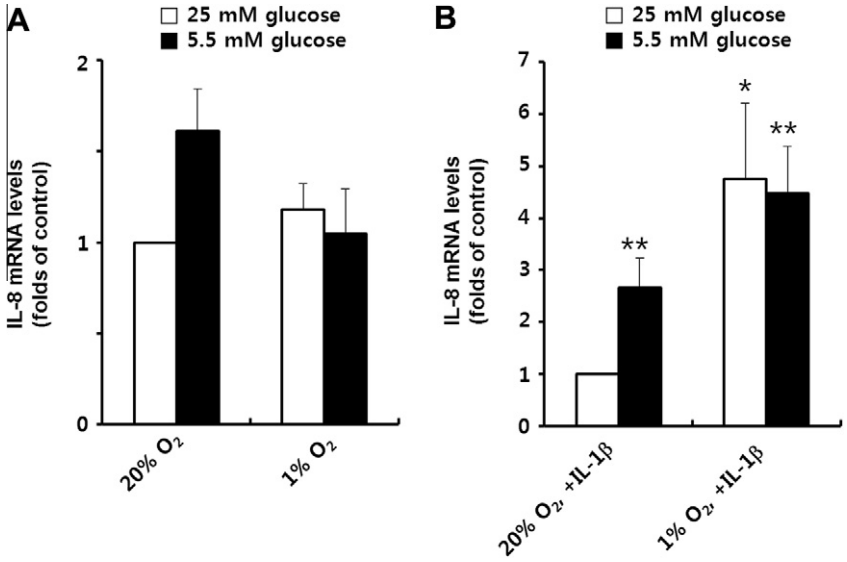

Fig. 3. IL-8 showed variable expressions by hypoxia/ischemia on real time RT-PCR (A). The expression of IL-8 responsive to IL-1β demonstrated a potentiation by hypoxia and lower glucose concentration (B). Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

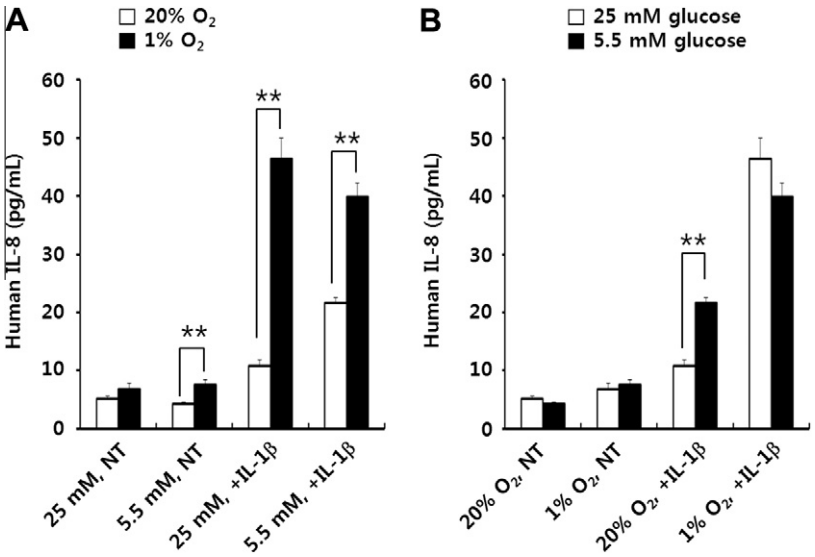

Fig. 4. Production of IL-8 was increased by hypoxia, which was prominent in lower glucose concentration ((A), (B) NT). IL-1β-induced IL-8 production was potentiated by hypoxia ((A), (B)+IL-1β). Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. NT, not treated.

factor and can help to restore oxygen homeostasis. Coimbra et al. reported on expression of HIF-1α in cultured human chondrocytes in normal and osteoarthritic cartilage, which was enhanced and stabilized by hypoxia [22]. Chondrocytes living with hypoxia/ischemia may have a critical requirement for HIF-1 for their differentiation, growth arrest, and survival by maintenance of metabolism [23]. Consistently, in this study, HIF-1α was influenced by glucose concentration as well as oxygen tension, thus, it was up-regulated by hypoxia/ischemia in human chondrocytes.

IL-1β expression and ROS production were analyzed as effectors of hypoxia/ischemia. As for the relevance of hypoxia to IL-1, lipopolysaccharide (LPS) stimulates release of IL-1β from alveolar macrophages under hypoxia without changes in mRNA expression [24]. Hypoxia enhances IL-1 activity in human endothelial cells with increase of IL-1α mRNA expression [25]. In contrast, IL-1β mRNA expression is up-regulated by hypoxia in rat monocytes through activation of the PKC/Raf-1/MAPK/NF-B pathway [26]. In this study, like the rat monocytes, human chondrocytes showed an up-regulated level of IL-1β mRNA by hypoxia/ischemia that was significantly enhanced by lower glucose concentration. The results of HIF-1α and IL-1β mRNA expression suggest that up-regulation of IL-1β may be consistently correlated with the influence of hypoxia/ischemia in human chondrocytes, and hypoxia/ischemia may exert pro-inflammatory and catabolic effects on cartilage.

In the current study, up-regulated ROS production by hypoxia was observed in human chondrocytes in a time-dependent manner. NADPH oxidases produce ROS in proportion to the rate of oxygen consumption; however, ironically, hypoxia has been reported to induce production of ROS from mitochondria, like in tissues consuming high oxygen. The increased ROS from mitochondria are required for activated transcriptions by hypoxia [27] and for hypoxic stabilization of HIF-1α via links with complex III of the mitochondrial electron transport chain [28]. Increased ROS production in human chondrocytes may indicate a role of hypoxia in arthro-degeneration.

Conditions simulating ordinary cartilage hypoxia/ischemia induced up-regulation of IL-1β expression and ROS production in human chondrocytes; these results were consistent and showed correlation with HIF-1α expression. The results may imply that hypoxia/ischemia on chondrocytes may evoke the catabolic metabolism of cartilage, and the hypoxia/ischemia of cartilage could be an independent predisposing factor for arthro-degeneration.

As for the growth factors and pro-inflammatory mediators, hypoxia induces expression of platelet-derived growth factor (PDGF), platelet-activating factor (PAF), IL-6, and IL-8, and enhances COX-2 induction [29,30]. Low SaO2 and altitude hypoxia increase the serum levels of IL-6 in human with no change of IL-1β, IL-1ra, TNF-α, or CRP levels [31]. In this study, increased expression of IL-1β, IL-8, IL-6, LIF, MMP-3, and ADMATS-4 by hypoxia/ischemia was demonstrated on microarray analysis; however, semi-quantitative mRNA expressions of the genes were not consistent or significant.

Even though the mRNA expressions of the catabolic mediators were not as remarkable as those observed on microarray analysis, significantly increased production of IL-8 by hypoxia/ischemia was observed in human chondrocytes. And pro-inflammatory response of IL-8 to IL-1β was also enhanced by hypoxia/ischemia. The results imply that hypoxia/ischemia may induce production or release of IL-8, and may potentiate the pro-inflammatory catabolism of cartilage.

As a member of the superfamily of CXC chemokines, IL-8 has diverse roles in pro-inflammatory responses and angiogenesis. A variety of cells, including lymphocytes, monocytes, endothelial cells, fibroblasts, hepatocytes, and keratinocytes are known to produce IL-8 [32,33]. Regarding hypoxia on IL-8 and pro-inflammatory responses, acute hypoxia up-regulates IL-8 in human alveolar macrophages as a potent pro-inflammatory stimulant in patients at risk of acute respiratory distress syndrome (ARDS) [34]. And the potential for release of IL-8 in human endothelial cells, and induction of IL-8 expression and secretion in human cancer cells via AP-1 and NF-kB like factor binding elements by hypoxia has been demonstrated [35,36]. Phorbol 12-myristate 13-acetate, LPS, TNF, and IL-1 are known as potent IL-8 inducers [37].

Results of the current study demonstrated significantly up-regulated levels of IL-1β, ROS, and IL-8 in correlation with HIF-1α by hypoxia/ischemia in human chondrocytes, similar to human cartilage. Furthermore, hypoxia/ischemia, showed a synergism in the pro-inflammatory catabolism of chondrocytes. This is the first study reported in human chondrocytes suggesting aggressive biology of ordinary cartilage hypoxia/ischemia. Hypoxia/ischemia may predispose chondrocytes to initiate catabolic processes, provoke inflammation, sensitize them to inflammatory stimuli, and potentiate the pro-inflammatory responses of chondrocytes. According to the results, we can suggest that cartilage hypoxia/ischemia can be a vicious natural predisposing condition for cartilage

degeneration, and may contribute to age-related joint diseases, when considering progressive hypoxia/ischemia and anaerobic metabolism with aging, and further decrease of oxygen levels in OA cartilage.

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