Original Paper

PEP-1-GRX-1 Modulates Matrix Metalloproteinase-13 and Nitric Oxide Expression of Human Articular Chondrocytes

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Glutaredoxin-1 • Protein transduction domain • Matrix metalloproteinase • Chondrocyte • Osteoarthritis.

Abstract

Background: The protein transduction domain (PTD) enables therapeutic proteins to directly penetrate the membranes of cells and tissues, and has been increasingly utilized. Glutaredoxin-1 (GRX-1) is an endogenous antioxidant enzyme involved in the cellular redox homeostasis system. In this study, we investigated whether PEP-1-GRX-1, a fusion protein of GRX-1 and PEP-1 peptide, a PTD, could suppress catabolic responses in primary human articular chondrocytes and a mouse carrageenan-induced paw edema model. **Methods:** Human articular chondrocytes were isolated enzymatically from articular cartilage and cultured in a monolayer. The transduction efficiency of PEP-1-GRX-1 into articular chondrocytes was measured by western blot and immunohistochemistry. The effects of PEP-1-GRX-1 on matrix metalloproteinases (MMPs) and catabolic factor expression in interleukin (IL)-1 β - and lipopolysaccharide (LPS)-treated chondrocytes were analyzed by real-time quantitative reverse transcription-polymerase chain reaction and western blot. The effect of PEP-1-GRX1 on the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light chain-enhancer of activated B cells (NF- κ B) signaling pathway were also analyzed by western blot. Finally, the inhibitory effect of PEP-1-GRX-1 on MMP-13 production was measured *in vivo* in a mouse carrageenan-induced paw edema model. **Results:** PEP-1-GRX-1 significantly penetrated into human chondrocytes and mouse cartilage, whereas GRX-1 did not. PEP-1-GRX-1 significantly suppressed MMP-13 expression and nitric oxide (NO) production in LPS-stimulated chondrocytes, and NO production in IL-1 β -stimulated chondrocytes, compared with GRX-1. In addition, PEP-1-GRX-1 decreased IL-1 β - and LPS-induced activation of MAPK and NF- κ B. In the mouse model of carrageenan-induced paw edema, PEP-1-GRX-1 significantly suppressed carrageenan-induced MMP-13 production as well as paw edema. **Conclusion:** These results demonstrate that PEP-1-GRX-1 can be transduced efficiently *in vitro* and *in vivo*.

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into human chondrocytes and mouse cartilage tissue and downregulate catabolic responses in chondrocytes by inhibiting the MAPK and NF- κ B pathway. PEP-1-GRX-1 thus has the potential to reduce catabolic responses in chondrocytes and cartilage.

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Introduction

Osteoarthritis (OA) is a common joint disorder characterized by reduced chondrocyte cellularity, cartilage degeneration, and joint dysfunction [1]. Multiple factors that disturb cartilage homeostasis and induce imbalance of anabolic and catabolic factors are involved in OA progression. However, since the pathogenic mechanisms during OA progression still remain largely unclear, the molecular events associated with OA development have been investigated to pursue novel therapeutic targets for the prevention and treatment of this disease [2].

Gene therapy using adenoviral and lentiviral vectors has been a useful strategy to deliver specific target molecules into articular cartilage [3-5]. However, the efficacy of gene delivery, duration of gene expression, and toxicity have remained as major limitations of this technique [6, 7]. Previously, it was reported that the basic domain of the human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) protein possesses the ability to traverse biological membranes efficiently in a process termed 'protein transduction' [8, 9]. The transduction domain is a region rich in positively charged amino acids and interacts with negatively charged phospholipids in the mammalian plasma membrane in a receptor- or transporter-independent fashion [10]. In addition, a variety of protein transduction domains (PTDs), including the third α -helix of Antennapedia homeodomain protein, VP22 protein from herpes simplex virus, the poly-arginine peptide, calcitonin-derived peptide, and Transportan, have attracted substantial interest in protein therapeutics [11]. In particular, PEP-1 peptide, an amphipathic peptide consisting of a hydrophobic tryptophan-rich sequence, a hydrophilic lysine-rich sequence, and a linker sequence, was developed for the application of therapeutic proteins without any chemical cross-linking or cloning [11]. It has been reported that PEP-1-mediated transduction was used for the delivery of biologically active molecules, including proteins, antibodies, small interfering RNA, and peptide nucleic acid, into various cell lines. We previously reported that a fusion protein, consisting of a PEP-1 PTD peptide and the 12-kDa FK506-binding protein (FK506BP12), penetrated effectively into human chondrocytes and cartilage explant cultures, and inhibited the expression of catabolic factors *in vitro* and *in vivo* [12]. In addition, a fusion protein of superoxide dismutase (SOD) with Tat peptide, Tat-SOD, inhibited IL-1 β -induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in chondrocytes [13]. Thus, the transduction of proteins using PTD can be regarded as a potential new therapy for OA.

Increasing reactive oxygen species (ROS) and oxidative stress are well correlated with the pathogenesis of arthritis. ROS play crucial roles in the inhibition of cartilage matrix synthesis, induction of chondrocyte apoptosis, and breakdown of cartilage matrix [14]. Elevated oxidative stress index and ROS level and decreased antioxidant parameters were found in the serum of patients with OA [15-17]. Dietary vitamins and selenium increased the expression of antioxidants such as glutathione (GSH) and Cu/Zn SOD and diminished the induction of OA in STR/1N mice [18], suggesting that a potent antioxidant might prevent OA. The glutaredoxin (GRX) system, one of the antioxidant systems in cells, consists of GRX, GSH, and NADPH-dependent glutathione reductase. GRX-1, a 12-kDa protein, is found mainly in the cytosol and also detected in the nucleus as well as the mitochondria; it functions as a hydrogen donor for ribonucleotide reductase in DNA synthesis [19].

In this study, we produced a fusion protein consisting of PEP-1 and GRX-1 and tested the transducing capacity of PEP-1-GRX-1 into a chondrocyte monolayer culture and mouse cartilage tissues. The effect of PEP-1-GRX-1 on LPS- and IL-1 β -stimulated catabolic factor expressions in human chondrocytes and a carrageenan-induced paw arthritis mouse model

was examined. The findings demonstrated the therapeutic potential of PEP-1-GRX-1 in terms of regulating the cartilage catabolic pathway of OA.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Welgene Inc. (Gyeongsan, Gyeongsangbukdo, South Korea). Fungizone (amphotericin B) and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer and HEPES buffer were purchased from Biosesang (Seongnam, Kyunggi, South Korea). Antibodies to iNOS and β -actin were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies to phosphorylated-c-Jun N-terminal kinase (p-JNK), phosphorylated p38 (p-p38), phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (p-I κ B α), I κ B α , p65, and phosphorylated extracellular signal-regulated kinase (p-ERK) were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and anti-His rabbit antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Chondrocyte isolation and culture

Human articular chondrocytes were obtained from the cartilage of patients with OA who underwent knee replacement surgery [20, 21]. Patient diagnoses were determined using the criteria set forth by the American College of Rheumatology. The collection and use of human samples were reviewed and approved by the institutional review board of Hallym University Sacred Heart Hospital (Anyang, South Korea; approval number 2013-I022). All patients provided written informed consent. The cartilage slices were carefully dissected and incubated sequentially with protease from *Streptomyces griseus*, collagenase from *Clostridium histolyticum*, and hyaluronidase from bovine testes (Sigma-Aldrich, St. Louis, MO, USA). Chondrocytes were cultured in DMEM supplemented with 20% FBS, 1% fungizone (150 units/mL), and penicillin/streptomycin (50 mg/mL) for 2-3 d and then maintained in DMEM containing 10% FBS.

Transduction of PEP-1-GRX-1 fusion protein

PEP-1-GRX-1 and GRX-1 were purified as previously described [12]. Briefly, *Escherichia coli* BL21 containing PEP-1-GRX-1 and GRX-1 expression vectors was cultured to an optical density of 0.6-1.0 at 600 nm and isopropyl- β -D-thiogalactoside (0.5 mM) was added to the culture to induce the overexpression of PEP-1-GRX-1 and GRX-1. The cells were harvested by centrifugation and lysed by sonication on ice. PEP-1-GRX-1 and GRX-1 in cell lysates were purified under conditions of binding (10 mM imidazole), washing (30 mM imidazole), and elution (200 mM imidazole) buffers using an Ni²⁺-nitrilotriacetic acid-Sepharose column. Purified proteins were desalted using PD-10 desalting column chromatography (GE Healthcare, Piscataway, NJ, USA).

To examine the capacity of PEP-1-GRX-1 and GRX-1 to penetrate chondrocytes, chondrocytes were seeded onto a 60-mm culture dish at a density of 6×10^5 and exposed to PEP-1-GRX-1 (0.2, 0.5, and 1 μ M) or GRX-1 (1 μ M) for 2 h. The cells were incubated with trypsin-ethylenediaminetetraacetic acid for 1 h to remove membrane-bound proteins, washed three times with phosphate-buffered saline (PBS), and then harvested by centrifugation. The harvested cells were subjected to immunoblot analysis.

Assay for PEP-1-GRX-1 and GRX-1

GRX-1 activity was assessed in a coupled assay with glutathione reductase. Briefly, enzyme solution or cell lysates from chondrocytes were preincubated with assay buffer, supplemented with 0.4 mM NADPH, 1 mM GSH, and 6 μ g/ml glutathione reductase from Bakers yeast at room temperature. 0.7 mM 2-hydroxyethyl disulfide as substrate was added to assay solution and the absorbance was measured at 340 nm for one minute. 1 unit of GRX-1 was defined as μ mole NADPH reduced per minute.

Immunoblot analysis

Chondrocytes were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.5, and 2 mM EDTA). Cell lysates were centrifuged at 13,000 rpm and the

protein concentration of each sample was measured by the bicinchoninic acid assay. Equal amounts of protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% nonfat milk and incubated with primary antibody and secondary antibody. Signals were detected using an enhanced chemiluminescence kit (Santa Cruz Biotechnology).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT PCR)

Total RNA was isolated from chondrocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA were generated using a reverse transcription reaction with Go Script (Promega, Madison, WI, USA) and oligo(dT) (Promega). Real-time PCR analysis was performed with a QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) using a StepOne Plus real-time PCR system (Applied Biosystems, Foster, CA, USA). The primer sequences of specific genes for real-time PCR were as follows: for iNOS, 5'-ACATTGATGAGAAGCTGTCC-3' (forward) and 5'-CAAAGGCTGTGAGTCCTGCA-3' (reverse); for MMP-13, 5'-AAGGACCCTGGAGCACTCATGTTT-3 (forward) and 5'-TGGCATCAAGGATAAGGAAGGGT-3' (reverse); and for GAPDH, 5'-TGATGACATCAAGAAGGTGGTGAAG-3' (forward) and 5'-TCCTTGGAGGCCATGTGGGCCAT-3' (reverse).

Enzyme-linked immunosorbent assay (ELISA)

Chondrocytes were incubated with PEP-1-GRX-1 and GRX-1 for 2 h, and were then simulated for 24 h with lipopolysaccharide (LPS) (Sigma-Aldrich). Twenty-four hours later, the culture medium was collected. For the experiment on joint tissues, mouse joint tissues frozen at -70°C were ground carefully into powder using a chilled mortar and pestle. Once the grinding was complete, the sample was transferred into a microcentrifuge tube and homogenization buffer (2 volumes) containing protease inhibitor (Sigma-Aldrich) was added to the tissue homogenate. After vortexing the tissue homogenates vigorously for 3–5 min three times, the supernatant was obtained by centrifugation at 13,000 rpm for 10 min. MMP-13 protein in the medium samples and cartilage homogenates was quantified by ELISA using the Quantikine Human Pro MMP-13 Immunoassay kit, in accordance with the manufacturer's instructions (R&D Systems).

Nitric oxide measurement

Nitric oxide (NO), a molecular mediator of inflammation, is oxidized to form nitrite. Nitrite concentration in the culture medium was measured using a Griess reagent kit (Promega), in accordance with the manufacturer's instructions. Briefly, culture medium was incubated with Griess reagent, consisting of *N*-(1-naphthyl)ethylenediamine and sulfanilic acid, for 30 min at room temperature and the absorbance of each sample was then measured at 548 nm.

Animal studies

All animal experiments were performed in accordance with protocols approved by the Care and Use of Laboratory Animals Committee of the National Veterinary Research & Quarantine Service of South Korea and the Hallym Medical Center Institutional Animal Care and Use Committee (HMC 2013-01-0530). To investigate the potential of PEP-1-GRX-1 to penetrate into mouse cartilage tissues, 16-week-old male C57BL/6N mice were purchased from Koatech (Pyeongtaek, Kyunggi, South Korea) and divided randomly into three groups (n=3 mice/group): PEP-1-GRX-1, GRX-1, and control. In these groups, PEP-1-GRX-1, GRX-1, and PBS, respectively, were injected intra-articularly into mice at a dose of 10 µg protein/10 µL per mouse. Six hours later, the knee joints of the mice were fixed in 4% paraformaldehyde, decalcified in the histological decalcifying agent Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA), dehydrated, embedded in paraffin, and sectioned.

To examine the anti-inflammatory and anti-catabolic effects of PEP-1-GRX-1 *in vivo*, a carrageenan-induced paw edema model was employed. Mice were assigned randomly to five groups (n = 4 mice/group) as follows: PBS, carrageenan, carrageenan+PEP-1-GRX-1 (0.2 mg/kg), carrageenan+PEP-1-GRX-1 (0.4 mg/kg), and carrageenan+GRX-1 (0.4 mg/kg). PEP-1-GRX-1, GRX-1, or PBS was injected subcutaneously into the plantar area of the right hindpaw. Two hours later, 40 µL of 2.5% carrageenan (Sigma-Aldrich) was injected subcutaneously into the plantar area of the right hindpaw to induce inflammation. The mice were sacrificed 4 d later.

Immunohistochemical analysis

Mouse knee joints injected intra-articularly with PEP-1-GRX-1, GRX-1, and PBS were fixed, de-calcified and embedded into paraffin to evaluate the penetration of PTD proteins into cartilage. Sections were deparaffinized with xylene, rehydrated, and incubated with 0.05% trypsin for 30 min at 37°C. Then, sections were blocked with 1.5% normal goat serum for 1 h and incubated with rabbit anti-His 804 polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology) overnight at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit antibody (1:200 dilution; Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. After incubation, the sections were incubated with Vectastain ABC reagent for 30 min, followed by exposure to 3,3'-diaminobenzidine (SK-4100; Vector Laboratories) for 8 min. Methyl Green (Sigma-Aldrich) was used as a counterstain.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). The significance of differences between two groups was tested using the Mann-Whitney U-test (GraphPad Prism 6). For all of the analyses, a P value less than 0.05 was considered to indicate statistical significance.

Results

Transduction of PEP-1-GRX-1 into human chondrocytes and mouse cartilage

The detailed structures of PEP-1-GRX-1 and GRX-1 are shown in Fig. 1A. To determine whether PEP-1-GRX-1 can be transduced into chondrocytes, human chondrocytes were incubated with PEP-1-GRX-1 (0.2, 0.5, and 1.0 μ M) and GRX-1 (1.0 μ M) for 2 h and transduced PEP-1-GRX-1 was detected by western blot analysis. Our data showed that

Fig. 1. Transduction of PEP-1-GRX-1 into a monolayer of cultured human chondrocytes and mouse cartilage tissue. (A) Schematic structures of PEP-1-GRX-1 and GRX-1. PEP-1 PTD, a 21-residue peptide carrier, consists of hydrophobic Trp R (tryptophan-rich), spacer, and hydrophilic Lys R (lysine-rich) domains. His, the six-histidine motif, was used in the detection and purification of the two proteins. (B) Transduction of PEP-1-GRX-1 into human primary chondrocytes. Chondrocytes were incubated with PEP-1-GRX-1 (0.2, 0.5, and 1 μ M) and GRX-1 (1 μ M) for 2 h and then with trypsin-ethylenediaminetetraacetic acid for 1 h. The levels of PEP-1-GRX-1 and GRX-1 in the cell lysates were measured by western blotting. (C) Activity assay of cell lysates from PEP-1-GRX-1- and GRX-1-transduced chondrocytes. Chondrocytes were incubated with PEP-1-GRX-1 (1 μ M) and GRX-1 (1 μ M) for 2 h and harvested. GRX-1 activity of each sample was measured using a coupled reaction with glutathione reductase. 1 unit = μ mole NADPH reduced per minute. (D) Transduction of PEP-1-GRX-1 into cartilage tissue of mouse joint. PEP-1-GRX-1, GRX-1, or phosphate-buffered saline was injected intra-articularly at a dose of 10 μ g protein/10 μ L per mouse into the knee joints of the mice. Six hours later, the knee joint tissues of the mice were harvested and immunostained with anti-His antibody. Immunohistochemistry data are representatives of two independent experiments using three different animals (n=3/group) (magnification; 200 $\times$). The arrows indicate the immune positive cells with His antibody. Scale bars = 100 μ m.

Fig. 2. PEP-1-GRX-1 suppresses lipopolysaccharide (LPS)-induced matrix metalloproteinase (MMP)-13 mRNA (A) and protein (B) levels. Chondrocytes were pretreated with PEP-1-GRX-1 (0.5 and 1 μ M) or GRX-1 (1 μ M) for 2 h and exposed to LPS (1 μ g/mL) for (A) 6 or (B) 24 h. (A) mRNA level was measured using real-time quantitative PCR. (B) The level of MMP-13 released into culture media was inhibited by PEP-1-GRX-1 in a dose-dependent manner. MMP-13 protein levels in culture media was measured by MMP-13 enzyme-linked

immunosorbent assay. Data are expressed as the mean $\pm$ standard deviation of duplicate data from three different donors. #### P < 0.001 vs. unstimulated control; *, P < 0.05 and ****, P < 0.001 vs. LPS-stimulated control; §§§, P < 0.005 and §§§§, P < 0.001 vs. LPS+GRX-1-treated group.

PEP-1-GRX-1 was transduced significantly into primary chondrocytes in a dose-dependent manner, while the control GRX-1 was not (Fig. 1B). The functional activity of GRX-1 was measured using a coupled assay with glutathione reductase. Cell lysates from PEP-1-GRX-1 transduced chondrocytes had more than 60% higher activity of GRX-1 compared to GRX-1 transduced chondrocytes (1.45 unit/mg vs 0.9 unit/mg) (Fig. 1C), revealing that PEP-1-GRX-1 penetrated chondrocytes effectively and conferred higher GRX activity than GRX-1. Next, the transduction of PEP-1-GRX-1 into *in vivo* mouse cartilage tissues was evaluated. PEP-1-GRX-1, GRX-1, or PBS was injected intra-articularly into the knee joints of mice. Six hours later, the knee joint tissues of the mice were harvested and immunostained with an anti-His antibody. Our results revealed that PEP-1-GRX-1 penetrated significantly into the superficial zone of cartilage, while control GRX-1 did not (Fig. 1D). These results demonstrate that PEP-1-GRX-1, GRX-1 protein fused with PEP-1 peptide, has the capacity to penetrate chondrocytes and cartilage tissues significantly without any additional stimulus.

PEP-1-GRX-1 inhibited MMP-13 or nitric oxide production induced by LPS- or IL-1 β treatment in chondrocytes

Chondrocytes produce NO and proinflammatory cytokines and activate catabolic processes in response to IL-1 β and LPS, which are implicated in the pathogenesis of OA [22-25]. We examined whether transduced PEP-1-GRX-1 has a regulatory function in chondrocyte catabolic signaling. Chondrocytes were incubated with PEP-1-GRX-1 for 2 h, followed by incubation with LPS. PEP-1-GRX-1 (1 μ M) suppressed LPS-induced MMP-13 expression significantly at both the mRNA and protein levels (Fig. 2A and B). However, PEP-1-GRX-1 failed to suppress IL-1 β -induced MMP-13 expression (data not shown).

We also determined whether PEP-1-GRX-1 can mediate NO production, which is involved in cartilage degradation. Chondrocytes were pretreated with PEP-1-GRX-1 and GRX-1 for 2 h and stimulated with LPS and IL-1 β for 6 and 24 h. LPS and IL-1 β induced the mRNA and protein expression of iNOS, which was suppressed significantly by PEP-1-GRX-1 (1 μ M) (Fig. 3A and B). However, GRX-1 failed to inhibit iNOS expression. In addition, PEP-1-GRX-1 significantly decreased the nitrite accumulation stimulated by LPS and IL-1 β in a concentration-dependent manner (Fig. 3C). On the other hand, PEP-1 peptide alone did not suppress iNOS or MMP-13 expression induced by IL-1 β and LPS (Fig. 4A-D). Our findings show that PEP-1-GRX-1 significantly suppresses NO production in LPS- and IL-1 β -stimulated chondrocytes as well as MMP-13 expression in LPS-stimulated chondrocytes.

PEP-1-GRX-1 suppressed MAPK and NF- κ B signaling activated by LPS and IL-1 β

Catabolic responses in articular cartilage are modulated via the MAPK and NF- κ B signaling pathways [26]. We investigated the effect of PEP-1-GRX-1 on these signaling

Fig. 3. PEP-1-GRX-1 suppresses lipopolysaccharide (LPS)- and interleukin (IL)-1 β -induced nitric oxide (NO) production in primary human chondrocytes. Chondrocytes were exposed to PEP-1-GRX-1 (0.5 and 1 μ M) for 2 h and then to LPS (1 μ g/mL) and IL-1 β (1 ng/mL) for (A) 6 or (B) 24 h. (B) The blot shown are representative of three or more independent experiments. (C) PEP-1-GRX-1 suppressed LPS- and IL-1 β -induced nitrite production, an indicator of nitric oxide synthase activity. Chondrocytes were exposed to PEP-1-GRX-1 (0.5 and 1 μ M) for 2 h and then to LPS (1 μ g/mL) or IL-1 β (1 ng/mL) for 24 h. Nitrite concentration in culture medium was measured using a Griess reagent kit. Data are expressed as the mean $\pm$ standard deviation of duplicate data from three different donors. ####, $P < 0.001$ vs. unstimulated control; *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.005$ vs. LPS- or IL-1 β -stimulated control; §§, $P < 0.01$ vs. LPS- or IL-1 β - + GRX-1-treated group. ns, non-significant.

Fig. 4. PEP-1 alone did not inhibit lipopolysaccharide (LPS)- and interleukin (IL)-1 β -induced iNOS and MMP-13 expression in primary human chondrocytes. Chondrocytes were pretreated with PEP-1 (1 μ M) for 2 h and exposed to LPS (1 μ g/mL) or IL-1 β (1 ng/mL) for (A) and (B) 6 or (C) and (D) 24 h. The levels of iNOS and MMP-13 were measured using (A) and (B) real-time quantitative PCR, (C) Western blot assay, and (D) ELISA. Data are expressed as the mean $\pm$ standard deviation of duplicate data from three different donors. ns, the difference between two groups is not significant.

pathways. Chondrocytes were incubated with PEP-1-GRX-1 (1 μ M) for 2 h prior to incubation with LPS and IL-1 β for 0, 15, 30, and 60 min. p38, JNK, and ERK1/2 were activated markedly

Fig. 5. PEP-1-GRX-1 suppresses lipopolysaccharide (LPS)- and interleukin (IL)-1 β -induced mitogen-activated protein kinase and nuclear factor- κ B activation in primary chondrocytes. Chondrocytes were incubated with PEP-1-GRX-1 (1 μ M) for 2 h and then with (A) LPS (1 μ g/mL) or (B) IL-1 β (1 ng/mL) for 15, 30, and 60 min. p38, JNK and ERK1/2 phosphorylation and p65 nuclear translocation were measured by western blotting. β -actin and laminin served as loading controls. The blots shown are representative of three or more independent experiments using cartilage from three different donors. Cyto, cytosolic; Nuc, nuclear.

Fig. 6. PEP-1-GRX-1 inhibits paw edema and matrix metalloproteinase (MMP)-13 expression in a mouse carrageenan-induced paw edema model. (A) Experimental design to examine the anti-inflammatory activity of PEP-1-GRX-1 in a mouse paw edema model. (B) Carrageenan-induced edema of mouse hindpaw was suppressed by PEP-1-GRX-1 (0.2 and 0.4 mg/kg), but not by GRX-1 (0.4 mg/kg). (C) Carrageenan-induced MMP-13 expression was inhibited by PEP-1-GRX-1. Joint tissue homogenates were prepared and MMP-13 level was measured using MMP-13 enzyme-linked immunosorbent assay kit. Data are expressed as the mean $\pm$ standard deviation of duplicate data from four independent experiments using four different animals ($n=4$ /group). ####, $P < 0.001$ vs. unstimulated control; ***, $P < 0.005$ vs. carrageenan-injected group; §, $P < 0.05$ vs. carrageenan + GRX-1-injected group. w/o carrageenan, without carrageenan; w carrageenan, with carrageenan.

13 expression was inhibited by PEP-1-GRX-1. Joint tissue homogenates were prepared and MMP-13 level was measured using MMP-13 enzyme-linked immunosorbent assay kit. Data are expressed as the mean $\pm$ standard deviation of duplicate data from four independent experiments using four different animals ($n=4$ /group). ####, $P < 0.001$ vs. unstimulated control; ***, $P < 0.005$ vs. carrageenan-injected group; §, $P < 0.05$ vs. carrageenan + GRX-1-injected group. w/o carrageenan, without carrageenan; w carrageenan, with carrageenan.

in the presence of LPS and IL-1 β . PEP-1-GRX-1 significantly inhibited the LPS- and IL-1 β -induced phosphorylation of p38, JNK, and ERK at 15 and 30 min (Fig. 5A and B). In addition, LPS- and IL-1 β -induced NF- κ B nuclear translocation was suppressed by PEP-1-GRX-1 (Fig. 5A and B). These findings suggest that PEP-1-GRX-1 significantly inhibited LPS- and IL-1 β -induced activation of catabolic signaling in chondrocytes.

PEP-1-GRX-1 inhibited MMP-13 production and edema in a mouse carrageenan-induced paw edema model

To determine the inhibitory effect of PEP-1-GRX-1 fusion protein *in vivo*, PEP-1-GRX-1 (0.2 or 0.4 mg/kg) or GRX-1 (0.4 mg/kg) was injected 2 h prior to 2.5% carrageenan injection into the right hindpaw of mice to induce inflammation. The mice were sacrificed 4 d later (Fig. 6A). A representative photograph of the hindpaw reveals that it was significantly thicker in carrageenan-injected mice, whereas the hindpaw thickness of PEP-1-GRX-1-treated mice was decreased significantly compared with that of GRX-1-injected mice (Fig. 6B). In addition, the MMP-13 level in PEP-1-GRX-1-injected mice was reduced in a dose-dependent manner, whereas the control GRX-1 did not affect it (Fig. 6C). These results demonstrate that PEP-1-GRX-1 significantly suppressed the catabolic response represented by MMP-13 in the mouse carrageenan-induced paw edema model.

Discussion

In this study, we demonstrated that PEP-1-GRX-1, a fusion protein consisting of PEP-1 peptide and GRX-1, penetrated efficiently into human chondrocytes and mouse articular cartilage tissue compared with GRX-1. PEP-1-GRX-1 significantly downregulated LPS-induced MMP-13 expression as well as LPS- and IL-1 β -stimulated NO production in chondrocytes through the inhibition of MAPK and NF- κ B activation. In addition, our mouse carrageenan-induced arthritis model data show that PEP-1-GRX-1 suppressed carrageenan-induced MMP-13 production and paw edema.

Previous studies demonstrated that ROS play an important role in a variety of inflammatory responses, including cytokine induction, recruitment of white blood cells to sites of injury, and glutathionylation of proteins, including actin, glyceraldehyde 3-phosphate dehydrogenase, c-Jun, and NF- κ B [27]. The glutaredoxin (GRX) system, one of the defense systems in cells, consists of GRX, glutathione (GSH), and NADPH-dependent glutathione reductase. The expression of GRX, which is responsible for the deglutathionylation process together with GSH, is induced by ROS and ROS-induced cytokines, such as IL-4, -6, -13, tumor necrosis factor- α (TNF- α), and interferon- γ [28-30]. Human cells contain the three members of the glutaredoxins (GRXs): the cytosolic GRX-1 and two mitochondrial members, GRX-2 and -5 [31]. GRX-1 regulates transcription factors such as nuclear factor 1 (NF1) [32], nuclear factor- κ B (NF- κ B) [33], and activator protein-1 (AP-1) [34]. These findings indicate that GRX could be one of the defense systems maintaining homeostasis and considered as a therapeutic target for various diseases via the control of oxidative signaling pathways [35].

ROS and NO are produced in LPS- or IL-1 β -stimulated articular chondrocytes, which possess NADPH oxidase [22, 36, 37]. In particular, strongly reactive NO can react easily with superoxide and subsequently form potent derivatives, including peroxynitrite, hydroxyl radical, and nitrogen dioxide, leading to catabolic responses in chondrocytes [36, 37]. Recently, GRX-1 was reported to be able to control the glutathionylation of molecules implicated in LPS- or IL-1 β -stimulated signaling pathways in a variety of cell types. GRX-1 suppresses oxidative stress-induced AKT glutathionylation and apoptosis in human retinal pigment epithelial cells [38]. It also regulates IL-1-mediated glutathionylation of TNF receptor-associated factor 6, an intermediate molecule involved in the IL-1R and TLR4 signaling pathways [39]. In addition, microvascular endothelial cells and macrophages from GRX-1-deficient mice showed enhanced LPS-stimulated glutathionylation of eNOS and, in a model of necrotizing enterocolitis (NEC), GRX-1-deficient mice were more susceptible to NEC development through the activation of TLR4 than the wild type [40]. In contrast, adenoviral overexpression of GRX-1 increased microglia activation and subsequently led to death of neuron cells [41]. GRX-1-deficient mice did not display increased vulnerability to acute injury of heart and lungs in ischemia/reperfusion and hyperoxia mouse model, whereas mouse embryonic fibroblasts from GRX-1-deficient mice were more tolerant to apoptosis induced by TNF- α plus actinomycin D. These findings suggest that variations in GRX-1 function may exist depending on cell types and animal models of disease [42].

An increased level of NO was found in human OA cartilage and human synovial fluid from rheumatoid arthritis and OA patients, suggesting that iNOS expression and the subsequent upregulation of NO are involved in the pathogenesis of arthritis [43-45]. Several lines of evidence demonstrated that NO suppressed the production of proteoglycans and collagen in rabbit cartilage and human chondrocytes [46, 47]. In addition, inflammatory cytokines led to the expression of cartilage-degrading enzymes such as MMPs by inducing NO production [48, 49]. In another study, iNOS inhibitors were shown to reduce cartilage degradation in an OA dog model and led to the suppression of IL-1 β -stimulated MMP production as well as the recovery of IL-1 β -inhibited glycosaminoglycan synthesis [36]. This accumulating evidence suggests that NO could be a target for OA therapy. We conclude that PEP-1-GRX-1 prevented LPS- and IL-1 β -associated inflammation in chondrocytes by modulating NO production and the MAPK and NF- κ B signaling pathways.

Several therapeutic strategies that suppress OA have been developed, but many obstacles were revealed prior to their clinical applications due to difficulties in the delivery of drugs to chondrocytes embedded deep within a dense cartilage matrix. Subsequently, various approaches for circumventing these obstacles were suggested. Oligonucleotide-based gene therapy has been employed for the treatment of various inflammatory diseases [50]. In practice, this approach required additional components, including cationic lipids, polymers, and nanoparticles, to increase cellular uptake across the cell membrane and prevent degradation by nucleases [51], but toxicity-related problems subsequently appeared as an obstacle to the usage of drugs. In recent years, growing interest has developed in the use of PTD for the treatment of a variety of diseases, including OA. For example, in terms of apoptosis regulation, anti-cell death protein FNK fused with PTD was reported to protect against chondrocyte death and to suppress graft degeneration [52-54]. Tat-Bcl-XL and Tat-BH4, homology region 4 domain from Bcl-2/Bcl-XL, suppressed apoptosis by inhibiting the activation of caspase in human islets [55]. In addition, intranasal treatment with Tat-human acidic fibroblast growth factor (Tat-haFGF) significantly suppressed amyloid protein deposition in the mouse brain and the apoptosis of neurons in a mouse model of Alzheimer's disease, showing that Tat-haFGF can attenuate AD progression [56]. Although many factors, including the type of PTD, cargo molecules, and cell types, were employed differently in the above-mentioned experiments, these results illustrate the potential of using PTD fusion proteins for therapeutic purposes. The potential advantages of protein transduction technology include the application of targeted intracellular functions of particular signaling pathways, direct regulation of protein levels inside cells, and reversibility with minimal toxicity [57].

In conclusion, we have demonstrated that PEP-1-GRX-1, a fusion protein of GRX-1 with PTD, had the potential to penetrate chondrocytes and cartilage tissue compared with GRX-1 and protected chondrocytes from LPS- and IL-1 β -induced catabolic responses by suppressing the MAPK and NF- κ B signaling pathways. In a mouse model of carrageenan-induced paw edema, PEP-1-GRX-1 suppressed the expression of MMP-13 significantly. Therefore, PEP-1-GRX-1 may have beneficial effects for managing the pathogenesis of OA.

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Disclosure Statement

The authors declare no conflict of interest.

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