



Research article

Matrix metalloproteinase-13 downregulation and potential cartilage protective action of the Korean Red Ginseng preparation



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ABSTRACT

Background: The present study was designed to prepare and find the optimum active preparation or fraction from Korea Red Ginseng inhibiting matrix metalloproteinase-13 (MMP-13) expression, because MMP-13 is a pivotal enzyme to degrade the collagen matrix of the joint cartilage.

Methods: From total red ginseng ethanol extract, *n*-BuOH fraction (total ginsenoside-enriched fraction), ginsenoside diol-type-enriched fraction (GDF), and ginsenoside triol-type-enriched fraction (GTF) were prepared, and ginsenoside diol type-/F4-enriched fraction (GDF/F4) was obtained from *Panax ginseng* leaf extract.

Results: The *n*-BuOH fraction, GDF, and GDF/F4 clearly inhibited MMP-13 expression compared to interleukin-1 β -treated SW1353 cells (human chondrosarcoma), whereas the total extract and ginsenoside diol-type-enriched fraction did not. In particular, GDF/F4, the most effective inhibitor, blocked the activation of p38 mitogen-activated protein kinase (p38 MAPK), c-Jun-activated protein kinase (JNK), and signal transducer and activator of transcription-1/2 (STAT-1/2) among the signal transcription pathways involved. Further, GDF/F4 also inhibited the glycosaminoglycan release from interleukin-1 α -treated rabbit cartilage culture (30.6% inhibition at 30 μ g/mL).

Conclusion: Some preparations from Korean Red Ginseng and ginseng leaves, particularly GDF/F4, may possess the protective activity against cartilage degradation in joint disorders, and may have potential as new therapeutic agents.

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1. Introduction

The extracellular matrix (ECM) provides tension and strength in human articular cartilage. ECM consists of mainly collagenous materials and aggrecans [1], which are maintained under the control of a normal turnover process between new ECM synthesis by residing chondrocytes and breakdown by matrix metalloproteinases (MMPs) and aggrecanases. In certain pathological conditions, such as osteoarthritis, however, some MMPs are highly induced and degrade ECM. Among the MMPs, MMP-13 is the most important collagenase to degrade and destabilize ECM in human articular cartilages [2–4]. In this regard, it is thought that MMP-13 inhibitor(s) and/or downregulator(s) may play a beneficial therapeutic role of chondroprotection.

Korean Red Ginseng (steamed white ginseng, *Panax ginseng* Meyer) is famous for possessing various biological effects, including enhancing vital energy, enhancing immune capacity, and inhibition of cancer cell growth. Its major constituents are various ginsenosides that have been reported to exhibit numerous pharmacological activities, including vitality enhancement, immune modulation, and anticancer activity [5–7]. However, few investigations or few clinical studies of ginsenosides on cartilage degradation disorders have been reported.

Among the ginsenosides from Korean Red Ginseng, some are not present in white ginseng products [8,9]. Examples are ginsenoside Rg3, Rg5, Rk1, and F4 that are only detected in red ginseng extract. Previously, one ginsenoside, Rg3, was found to inhibit MMP-13 expression in human osteoarthritic chondrocytes [10]. We have

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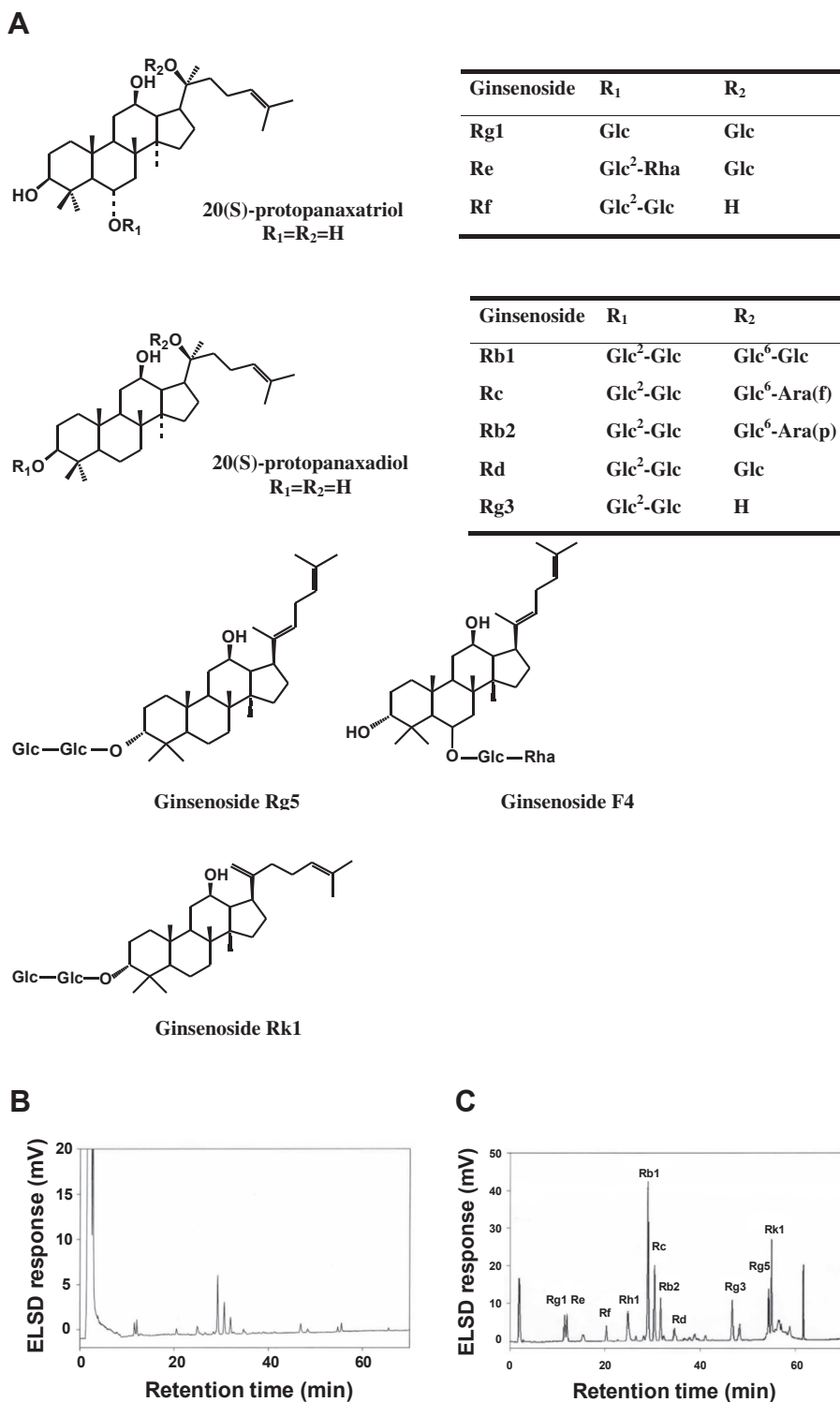


Fig. 1. The chemical structures of ginsenosides and representative high-performance liquid chromatograms (HPLC) of the preparations from Korean Red Ginseng and ginseng leaves used in this study. (A) Chemical structures of ginsenosides. (B) HPLC of Korean Red Ginseng total extract. (C) HPLC of *n*-butanol fraction. The retention time and % of the area of each ginsenoside were: 11.43 min and 4.19% for Rg1; 11.90 min and 6.47% for Re; 20.27 min and 2.33% for Rf; 24.67 min and 7.73% for Rh1; 28.93 min and 30.89% for Rb1; 30.30 min and 16.24% for Rb2; 31.63 min and 7.89% for Rc; 34.43 min and 3.37% for Rd; 46.40 min and 7.07% for Rg3(S); 47.93 min and 3.22% for Rg3(R); 54.30 min and 1.52% for Rk1; and 54.90 min and 2.47% for Rg5. (D) HPLC of ginsenoside diol-type-enriched fraction. (E) HPLC of ginsenoside diol-type-enriched fraction (GDF). (F) HPLC of GDF/F4. Note: Compared to GDF, GDF/F4 contains more hydrophobic ginsenosides such as F4, Rg3, and Rg5. ELSD stands for evaporative light scattering detection.

recently found that certain ginsenosides including Rc, Rd, Rf, F4, Rg1, and Rg3 inhibit MMP-13 induction from human chondrocytes, and some also block glycosaminoglycan (GAG) release from interleukin (IL)-1 α -treated cartilage culture to some degree [11]. These

previous findings strongly suggest that the Korean Red Ginseng products and/or some ginsenoside-enriched preparations may possess a significant inhibitory activity of MMP-13 expression and thereby block cartilage degradation. Thus, several ginseng

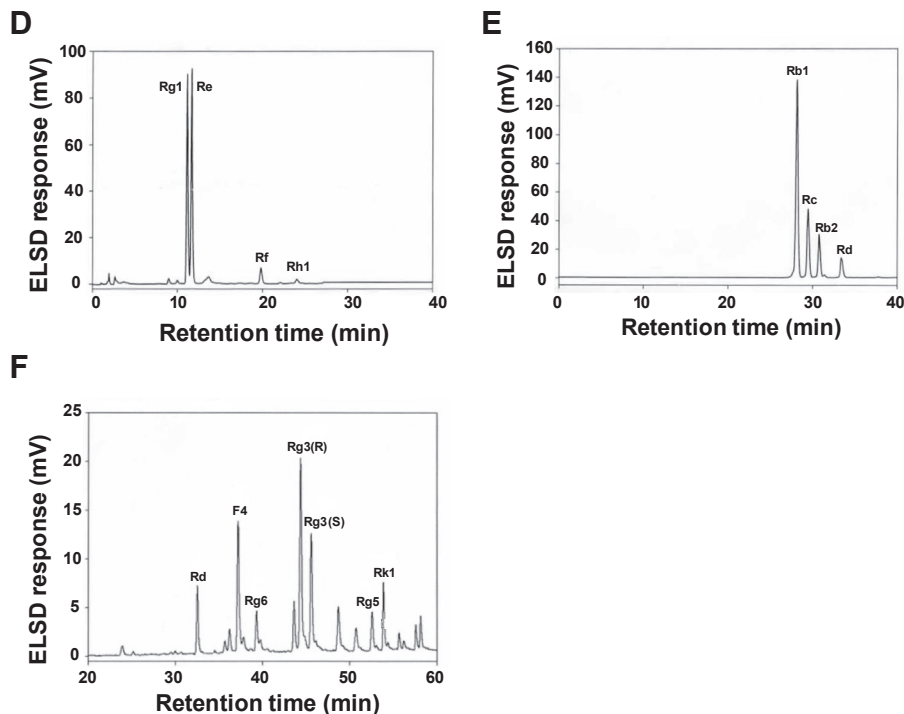


Fig. 1. (continued).

preparations have been designed and prepared in the present study. They were examined for MMP-13 downregulatory effect and cartilage protection to find a potential for a new chondroprotective agent. This is the first report of the preparations from Korean Red Ginseng and ginseng leaves to show MMP-13 downregulating properties.

2. Materials and methods

2.1. Chemicals

Human IL-1 α , IL-1 β , dexamethasone, diclofenac, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-MMP-13 antibody were purchased from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL (Grand Island, NY, USA). The protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). All antibodies relating to mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling were purchased from Cell Signaling Technologies (Dancers, MA, USA). Lamin B1 antibody was purchased from Bioworld technology (Minneapolis, MN, USA).

2.2. Preparation of ginseng products

Korean Red Ginseng was purchased from a local market (Seoul). Dried root powder was extracted three times with 70% ethanol by sonication for 3 h, followed by rotary evaporation at 4°C under reduced pressure (total ethanol extract, 28.1% of raw material). The extract was suspended in distilled water in a separatory funnel and partitioned with *n*-butanol three times. The combined fractions were evaporated to dryness (*n*-butanol fraction, total ginsenoside-enriched fraction, 6.5% of raw material), and the ethanol extract was loaded onto a Diaion HP-20 (Sigma–Aldrich) open column (100 cm \times 10 cm; the volume of the column was 7.8 L) and

sequentially eluted with a methanol gradient beginning with 100% water and 30%, 65%, and finally 80% methanol. The enriched ginsenoside fractions were obtained from 65% methanol (ginsenoside triol-type-enriched fraction, GTF, 0.7% of raw material) and 80% methanol eluate (ginsenoside diol-type-enriched fraction, GDF, 1.3% of raw material).

In a separate experiment to obtain ginsenoside diol-type-/F4-enriched fraction (GDF/F4), the dried ginseng leaves were extracted with 95% ethanol (total ethanol extract, 22.1% of raw material), and the extract was dried using a rotary evaporator. The dried extract was partitioned in distilled water and *n*-butanol three times (*n*-butanol fraction, total ginsenoside-enriched fraction, 5.7% of raw material). The *n*-butanol fraction was concentrated for column chromatography. The *n*-butanol fraction was adsorbed to Diaion HP-20 resin (Sigma–Aldrich), and was washed with water. Then, the column was eluted with 100% MeOH. The 100% MeOH fraction was concentrated to obtain a highly-enriched saponin fraction. To the fraction, two volumes of double concentrated vinegar (Ottugi, pH 2.3, acidity 13–14%) were added and then exposed for 30 min at an oscillation frequency of 2,450 MHz, with a microwave output power of 700 W (Samsung electronics, RE-C20DB, Seoul, Korea). The sample used for the experiment (GDF/F4) was finally obtained by passing the HP-20 resin eluted with 87% MeOH after washing with 73% MeOH (87% MeOH fraction, ginsenoside diol-type-/F4-enriched fraction, 0.4% of raw material). It is mainly composed of ginsenosides Rd, F4, Rg6, Rg3, Rg5, and Rk1. The composition of various ginsenosides in each product was examined by high performance liquid chromatography analysis, and the profiles are shown in Fig. 1.

2.3. Animals

Male New Zealand white rabbits (age 5 weeks) were purchased from Central Experimental Animal Co. (Seoul, Korea). The animals were maintained in the animal facility (KNU) at 20–22°C under 40–60% relative humidity and a 12-h/12-h (light/dark) cycle. The

experimental design using the animals was approved by the local committee for animal experimentation of Kangwon National University (KIACUC-12-0012). The animals were handled according to the guidelines described in the Food and Drug Administration (Korea) Guide for the Care and Use of Laboratory Animals throughout the experiments.

2.4. SW1353 cell culture and MMP-13 induction

SW1353 cells (human chondrosarcoma cell line) purchased from the American type culture collection (Manassas, VA, USA) were cultured and treated with IL-1 β according to previously described procedures [12]. In brief, the cells were maintained in DMEM with 10% FBS, glutamine, and penicillin/streptomycin. To induce MMP-13, IL-1 β (10 ng/mL) with/without test compounds was added to the cells in serum-free DMEM for 24 h. MMP-13 released in the media was examined by Western blotting analysis using anti-MMP-13 antibody. All test compounds were initially dissolved in dimethyl sulfoxide (DMSO) and diluted with serum-free DMEM to adjust the final DMSO concentration to 0.1% (v/v). Cell viability was checked using MTT bioassay [13]. No effect on cell viability or the MMP-13 expression level was observed by the treatment of 0.1% DMSO.

2.5. Cellular mechanisms of inhibition of MMP-13 induction

Using total cellular lysate, expression and phosphorylation of MAPKs and STAT-1/2 were examined. Total cellular protein was extracted with Pro-Prep solution (iNtRON Biotechnology, Kyungki-Do, Korea) containing 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate, and 1mM sodium fluoride. Expression of nuclear transcription factor- κ B (NF- κ B) p65, c-Jun, and c-Fos was identified in nuclear fractions. For an extraction of nuclear proteins, cells were resuspended in 400 μ L of buffer A (10mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF, 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin) and incubated on ice for 10 min. After 25 μ L of 10% NP-40 was added, cells were vortexed for 10 sec and centrifuged at 2,500 g for 2 min. The nuclear pellet was vigorously vortexed in buffer B (20mM HEPES, pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF, 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin) and centrifuged at 16,000 g for 10 min. BCA protein assay (Pierce, IL, USA) was used to determine protein concentration in the nuclear fraction. Proteins were separated, blotted, and visualized as described above.

2.6. Effects of GDF/F4 on GAG release from rabbit cartilage culture

According to the previously described procedures [12], articular cartilages were excised from the femoral condyles of rabbit knee and incubated in DMEM containing 5% FBS for 1–2 days. In addition, approximately 30 mg cartilage fragments per well were incubated in DMEM containing 1% FBS in 400 μ L/well. Cartilages were treated with 10 ng/mL of human IL-1 α (Sigma–Aldrich) in the presence or absence of test compounds for 3 days. The amounts of released GAG in the supernatant were measured with a Blyscan sulfated GAG assay kit (Biocolor, Carrickfergus, County Antrim, UK) based on dimethylmethylene blue assay, according to the manufacturer's protocol.

2.7. Statistical analysis

Experimental values are represented as arithmetic mean \pm standard deviation. Statistical analysis was evaluated using one-way analysis of variance followed by Dunnett's analysis (IBM

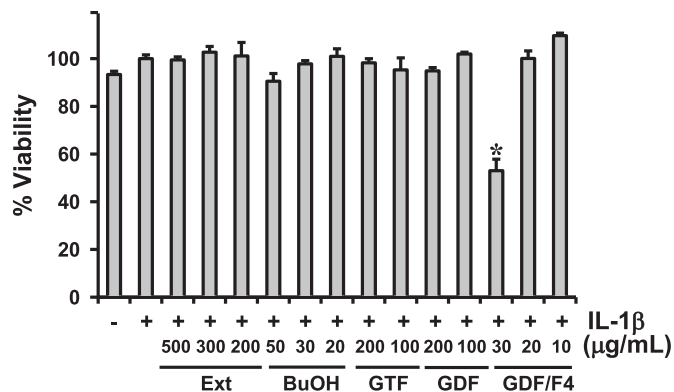


Fig. 2. Cytotoxicity of various preparations from Korean Red Ginseng on interleukin (IL)-1 β -treated SW1353 cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide bioassay was carried out to measure the cytotoxicity as described in the experimental section. All data are represented as arithmetic mean \pm standard deviation ($n = 3$). * $p < 0.05$, significantly different from the interleukin-1 β -treated control group. BuOH, *n*-butanol fraction; Ext, total ethanol extract; GDF, ginsenoside diol type-enriched fraction; GDF/F4, ginsenoside diol type-/F4-enriched fraction; GTF, ginsenoside triol-type-enriched fraction.

SPSS Statistics, Version 21, IBM Korea). A $p < 0.05$ was considered significantly different.

3. Results

3.1. Effects of ginseng preparations on MMP-13 expression

Initially, the cytotoxic effects of total red ginseng ethanol extract, *n*-butanol fraction (total ginsenoside-enriched fraction), GDF, GTF, and GDF/F4 were examined. As shown in Fig. 2, MTT assay demonstrated that the total extract and GTF did not show significant reduction of cell viability at the tested concentrations when incubated in SW1353 chondrocytes. However, 50 μ g/mL of *n*-butanol fraction reduced viability (7.0%), and 200 μ g/mL of GDF slightly reduced viability (5.1%), but the results are not statistically significant. GDF/F4 showed cytotoxicity at 30 μ g/mL (53.0%). Therefore, all other experiments of *n*-butanol fraction, GDF, and GDF/F4 fractions were carried out at lower concentrations than indicated.

When the MMP-13 downregulatory effects of these preparations were compared in SW1353 cells, the crude extract (up to 300 μ g/mL) and GTF (up to 200 μ g/mL) failed to downregulate MMP-13 expression (Fig. 3A and 3B). By contrast, the *n*-butanol fraction (30 μ g/mL) showed significant inhibition of MMP-13 expression (Fig. 3C). In particular, GDF and GDF/F4 showed clear inhibition at 10–100 μ g/mL and 5–20 μ g/mL, respectively, without cytotoxic effects (Fig. 3D and 3E). Dexamethasone (10 μ M) used as a reference agent strongly inhibited MMP-13 expression as expected. These results indicate that *n*-butanol fraction, GDF, and GDF/F4 possess MMP-13 downregulatory activity, with GDF/F4 having the strongest inhibition of MMP-13 induction among the preparations tested.

3.2. Cellular inhibitory action mechanisms of GDF/F4 on MMP-13 down-regulation

Next, the cellular mechanisms of MMP-13 downregulation by GDF/F4, the strongest downregulator, were examined. In SW1353 cells, IL-1 β treatment induced MMP-13 expression. Previously, this induction in IL-1 β -treated SW1353 cells was found to be mediated, at least in part, via activation of transcription factors, such as NF- κ B, activator protein-1 (AP-1), and STAT-1/2 [12,14]. Among upstream kinases, p38 MAPK and JAK activation were importantly involved [12]. When the effects on MAPK pathways were examined, GDF/F4

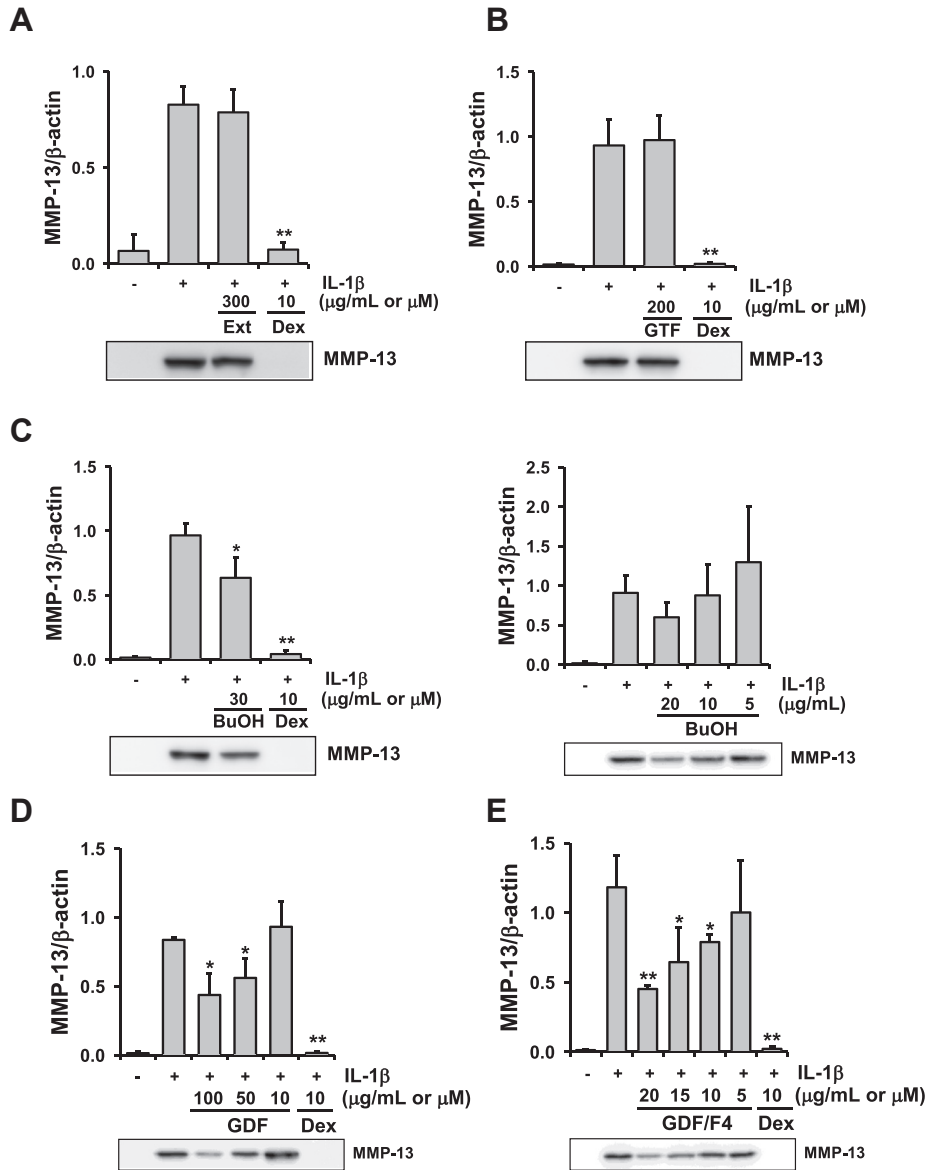


Fig. 3. Effects of various preparations from Korean Red Ginseng on matrix metalloproteinase-13 expression in interleukin (IL)-1 β -treated SW1353 cells. Western blotting analysis was used to examine the expression levels of matrix metalloproteinase (MMP)-13. Dexamethasone was treated at 10 μ M. (A) Effect of total ethanol extract (300 μ g/mL) on MMP-13 expression (B) Effect of GTF (200 μ g/mL) on MMP-13 expression (C) Effect of *n*-butanol fraction (5, 10, 20 and 30 μ g/mL) on MMP-13 expression (D) Effect of GDF (10, 50 and 100 μ g/mL) on MMP-13 expression (E) Effect of GDF/F4 (5, 10, 15 and 20 μ g/mL) on MMP-13 expression. All data are represented as arithmetic mean \pm standard deviation ($n = 3$). * $p < 0.05$, significantly different from the interleukin-1 β -treated control group. BuOH, *n*-butanol fraction; Dex, dexamethasone; Ext, total ethanol extract; GDF, ginsenoside diol type-enriched fraction; GDF/F4, ginsenoside diol type-/F4-enriched fraction; GTF, ginsenoside triol-type-enriched fraction.

inhibited the activation of p38 MAPK and JNK at 20 μ g/mL. Among the transcription factors, the activation of STAT-1/2 was blocked, but not that of NF- κ B and AP-1 (Fig. 4). Thus, it is suggested that GDF/F4 downregulates MMP-13 expression by blocking the activation of multiple points including MAPKs and the transcription factor, STAT-1/2.

3.3. Inhibition of GAG release from rabbit cartilage culture

To establish the cartilage protective effect of the new preparation, rabbit cartilage tissue culture was employed. IL-1 α treatment of rabbit cartilage induced MMPs, which degraded the matrix materials and released large amounts of GAG into the media for a 3-day culture (Fig. 5). Under this condition, GDF/F4 inhibited GAG release (30.6% and 19.3%) from rabbit cartilage at 30 μ M and 50 μ M,

respectively, whereas the reference compound, diclofenac (30 μ M), showed strong inhibition (64.1%) as expected. However, Korean Red ginseng total ethanol extract did not protect the GAG release at 200 μ g/mL under the same experimental conditions.

4. Discussion

The present study clearly demonstrates that the ginsenoside-enriched fraction (*n*-BuOH fraction) and the newly prepared GDF and GDF/F4 inhibited MMP-13 expression in human chondrocytes. However, the red ginseng total extract and GTF did not significantly inhibit MMP-13 induction. In addition, GDF/F4 was also found to give considerable protection of cartilage degradation in rabbit cartilage culture, although this was not statistically significant.

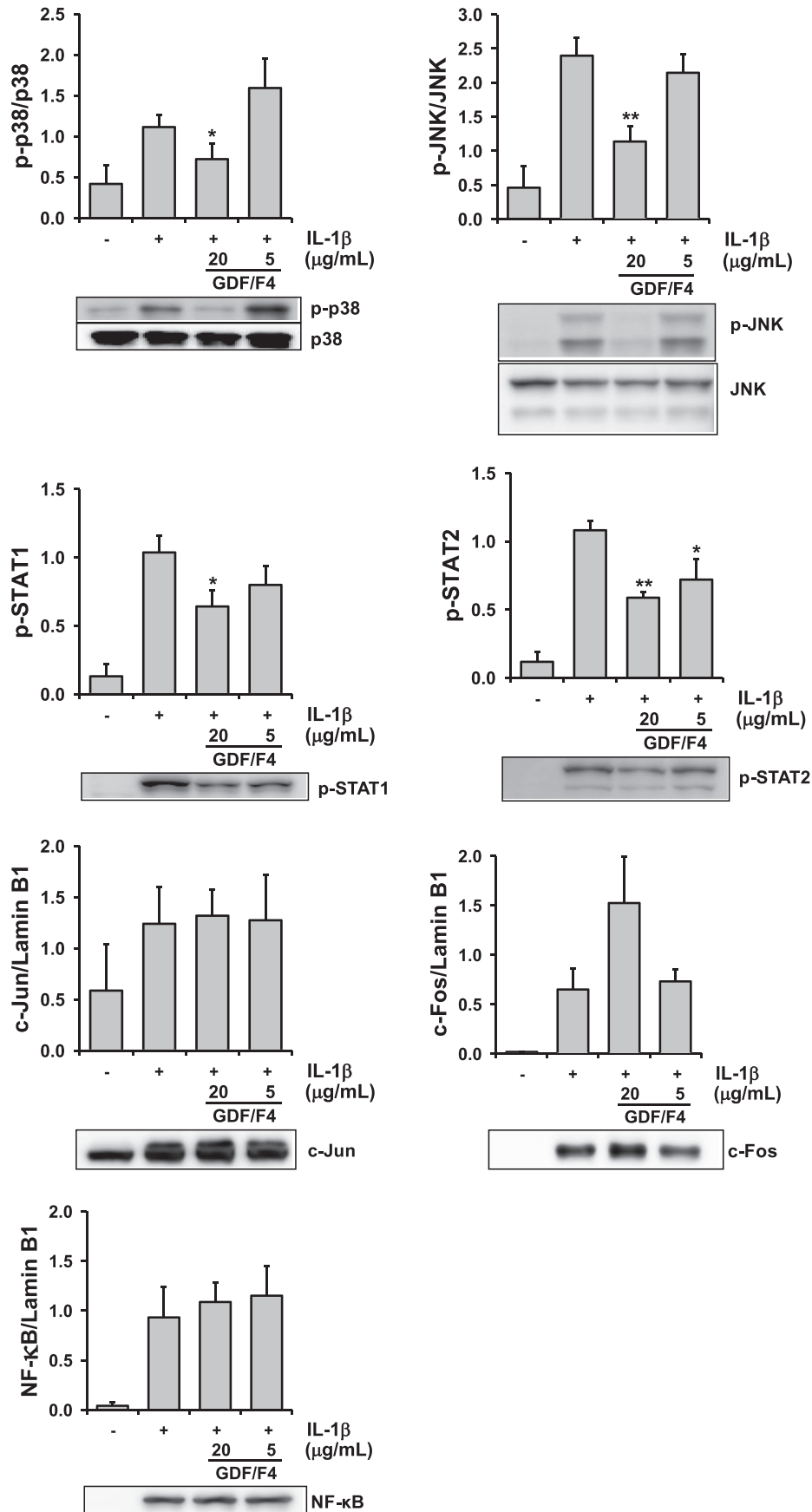


Fig. 4. Effects of ginsenoside diol type-/F4-enriched fraction (GDF/F4) on the various signal transduction pathways of matrix metalloproteinase-13 expression in interleukin-1 β -treated SW1353 cells. Western blotting analysis was used to examine the expression levels of various signaling molecules. All data are represented as arithmetic mean \pm standard deviation ($n = 3$). * $p < 0.05$, significantly different from the interleukin-1 β -treated control group. JNK, c-Jun-activated protein kinase; NF- κ B, nuclear factor- κ B; STAT, signal transducer and activator of transcription.

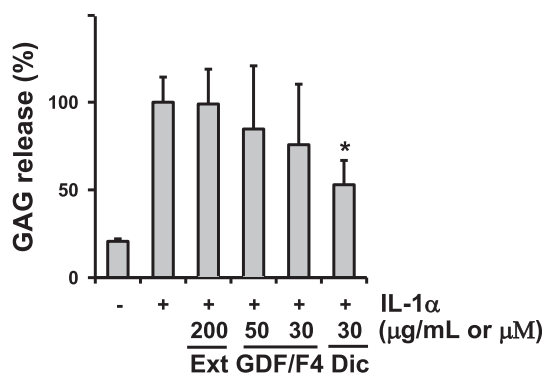


Fig. 5. Effects of ginsenoside diol type-/F4-enriched fraction (GDF/F4) on glycosaminoglycan (GAG) release from rabbit cartilage culture. Rabbit cartilage was incubated with interleukin (IL)-1 α in the presence or absence of the test samples. All data are represented as arithmetic mean \pm standard deviation ($n = 4$). * $p < 0.05$, significantly different from the interleukin-1 α -treated group. Dic, diclofenac; Ext, total ethanol extract.

Previously, it was found that ginsenosides Rc, Rd, Rf, F4, Rg1, and Rg3 possess MMP-13 downregulatory activity against IL-1 β -treated chondrocytes at concentrations of 1–50 μM [11]. The most prominent inhibitors are ginsenosides Rg3 and F4. In this study, GDF/F4 was newly prepared from *Panax ginseng* leaves because the leaves contain higher amounts of F4 and Rg3 than ginseng roots on a weight basis. However, the total ginseng extract (the ethanol extract) did not exert MMP-13 downregulation. The inactive result of the total extract is possibly explained by the fact that the contents of these active ginsenosides in the extract might be too low to exert MMP-13 downregulation, as shown in Fig. 2. Otherwise, it is reasonable to think that if these active ginsenosides are enriched in certain fractions, they may possess meaningful inhibitory action. Indeed, the *n*-BuOH fraction (total ginsenoside-enriched fraction, Fig. 2) having higher amounts of ginsenosides strongly inhibited MMP-13 induction. In this case, however, some cytotoxicity was observed on SW1353 cells at the concentrations of 50 $\mu\text{g/mL}$ or higher. The cytotoxic property of the *n*-butanol fraction could be, at least partly, explained by the previous findings that ginsenosides such as Rg3, Rg5, and Rk1 exert considerable cytotoxicity on SW1353 cells and several other cells at high concentrations [7,11,15].

Because the major active ginsenosides are diol-type and F4 [11], we designed a new preparation that contains high amounts of the diol-type ginsenosides and F4, i.e., GDF/F4. As expected, the most prominent active preparations for MMP-13 downregulation are GDF and GDF/F4, with GDF/F4 being the strongest. It is understood that the MMP-13 downregulatory action of these preparations might rely on the major ginsenosides of GDF (Rc and Rd) and GDF/F4 (Rc, Rd, Rg3, and F4). By contrast, the ginsenoside triol-type-enriched fraction (GTF) did not inhibit MMP-13 expression. Actually, among ginsenoside triol-type derivatives, Rf and Rg1 were found to inhibit MMP-13 expression weakly at high concentrations [11].

It was previously found that MAPKs, NF- κ B, AP-1, and STAT-1/2 are important to induce MMP-13 in IL-1 β -treated SW1353 cells [12,14]. GDF/F4 blocked the activation of MAPKs, including p38 MAPK and JNK and transcription factors STAT-1/2. However, one prominent MMP-13 downregulating ginsenoside, F4, was previously found to block only p38 MAPK activation under the same experimental conditions [11]. These differences may be because GDF/F4 contains several different ginsenosides in addition to F4.

It is important to indicate that most active MMP-13 downregulating ginsenosides are the components of Korean Red Ginseng, but not of white ginseng [8,9]. Rg3 and F4 are unique to Korean Red Ginseng. These results may suggest the importance of Korean Red Ginseng for treating cartilage degradation disorders.

In conclusion, some ginsenoside-enriched fractions (*n*-BuOH fraction, GDF, and GDF/F4) were, for the first time, found to inhibit MMP-13 expression from chondrocytes, at least in part, via blocking the activation of p38 MAPK, JNK, and STAT-1/2. GDF/F4 also showed some protective activity against cartilage degradation in rabbit cartilage tissue culture. Our study may open a new therapeutic area for red ginseng product(s). These products may be beneficial for chondroprotection in cartilage degradation-related disorders such as osteoarthritis.

Conflicts of interest

All authors have no conflict of interest to declare.

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