Hepatocyte Growth Factor/c-Met Has Proliferation-promoting And Anti-apoptosis Effects On Rabbit Nucleus Pulposus Cells In Vitro.

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Introduction: Currently, the massive medical expenditure spent on low back pain becomes a serious social problem. Intervertebral disc degeneration is a major cause of back pain and is related to the progress of apoptosis of the component cells due to non-physiological mechanical loading and exposure of inflammatory cytokines as well as reactive oxygen species (ROS). To restrain the intervertebral disc degeneration, the enhancement of the proliferative ability of remaining intervertebral disc cells and suppression of apoptosis are required, but there has been no established method proven to be effective.

Hepatocyte growth factor (HGF) is effective in enhancing cell proliferation, suppressing cell death, demonstrating anti-inflammatory property, etc., and has been reported to possess an anti-apoptotic function even on cells with poor proliferative capacity in models with spinal cord injury and cardiac infarction. However, no study has clarified the effect of HGF on intervertebral disc cells.

The purpose of this study is to clarify the effect of HGF on intervertebral disc component cells in terms of cell proliferation enhancement and cell death suppression under ROS and inflammatory cytokine stimulation.

Methods: Isolation of intervertebral disc component cells: Nucleus pulposus (NP) cells were isolated from intervertebral discs from Japanese white rabbits (14-18 weeks old male). The cells were cultured in monolayer for 10 to 14 days until 60-70% confluence. For use in the experiments, the cells were trypsinized and subcultured. All experiments were completed using the second passages of cells.

C-Met, the specific receptor of HGF, gene expression: HGF (1-100 ng/mL) and/or TNF-α (100 ng/mL) were administrated into culture for 24-48h. The cDNA was synthesized from m-RNA of the NP cells. C-Met gene expression was analyzed with the cDNA by real-time RT-PCR.

HGF treatment and apoptosis induction: Human recombinant HGF (1-100 ng/mL) was pretreated and apoptosis was applied. Apoptosis was induced by H2O2 (0.1mM) or TNF-α (100 ng/mL) as ROS generators. H2O2 and HGF was treated for 0.5-3 hours after HGF exposure. TNF-α was simultaneously administrated with HGF treatment for 24-48 hours.

Cell proliferation: The cell proliferation was analyzed using two cell counting kit 8 assays. One assay was incubation for 1, 3, 5 and 7 days under HGF treatment at varying concentration. The other assay was incubation for 1, 3, 5 and 7 days after HGF and H2O2 treatment.
Apoptosis detection and signal analysis: In situ detection of DNA fragmentation in apoptotic cells was performed with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining after HGF and H2O2 treatment. Apoptosis related gene, caspase-3 gene expression was analyzed by real time RT-PCR after HGF and TNF-α treatment. Caspase-3 activities were also assessed using Caspase-Glo 3/7 assay as an index of apoptosis.

Statistical analysis: Multi-way ANOVA with the Tukey-Kramer test was used. P-values less than 0.05 were regarded statistically significant.

**Results:**

C-Met expression in NP cells: The m-RNA expression of c-Met was detected in the cultured NP cells of Japanese white rabbits. The c-Met expression level was significantly increased by TNF-α treatment. Both HGF and TNF-α treatment significantly suppressed the c-Met expression compared with TNF-α only. (Fig. 1).

HGF induced NP cell proliferation and ameliorated cell damage caused by ROS: HGF treatment at 10 ng/ml caused significantly increased cell viability of NP cells. There was no significant increase of cell viability with 1 ng/ml of HGF on day 7, though significant on day 3 and 5 (Fig.2A). The cell viability of NP cells was remarkably decreased by ROS treatment for 5 and 7 days culture. Supplementation with 10 ng/ml of HGF successfully rescued these cell viability decrease that was induced by ROS (Fig.2B).

HGF suppressed ROS-induced apoptosis of NP cells: A number of apoptotic nuclei with double-strand DNA breaks were observed in the HGF-untreated group, while apoptotic cells were rarely demonstrated in the HGF-treated populations (Fig.3A). The caspase-3 gene expression increased in NP cells under the TNF-α treatment. Supplementation of HGF significantly down-regulated caspase-3 gene expression in NP cells (Fig.3B). The caspase-3 activity was significantly cleaved in NP cells under the H2O2 treatment. Supplementation of HGF significantly prevented this activation of caspase-3 in NP cells (Fig.3C). Taken together, HGF suppressed ROS-induced apoptosis of cultured intervertebral disc cells.

**Discussion:**

This study demonstrated the expression of c-Met on the cultured NP cells for the first time. The c-Met expression level significantly fluctuated under the influence of external stimulation, such as HGF and TNF-α. Also, in in-vivo intervertebral discs, the expression level of c-Met fluctuated in accordance with background conditions, such as age, level of degeneration, etc., which suggests the possibility that the effect of HGF fluctuates depending on the background conditions.

The administration of HGF resulted in enhanced cell proliferative ability of the NP cells not only under normal condition but also under ROS stimulation. These results propose the possibility that HGF improves the suppressed proliferative ability of NP cells exposed to stress-induced damage.

The HGF treatment led to decreased expression of apoptotic factors and suppressed progress of cell death under the ROS stimulation where apoptosis is induced. The result may suggest HGF has the potential to improve the decrease of NP cells due to apoptosis under non-physiological stress.

This study clearly demonstrated that HGF has the proliferative ability of intervertebral disc NP cells under non-physiological stress and protect NP cells from ROS induced apoptosis. These effects of HGF might be utilized to prevent the progression of intervertebral disc degeneration.

**Significance:** We first analyzed the effects of HGF on NP cells in vitro. Our study suggests that HGF may prevent the progression of intervertebral disc degeneration.
Fig. 1

** Fig. 2A and Fig. 2B **

- **Fig. 2A**: Cell viability over days with different treatments (HGF and ROS) at various concentrations.
- **Fig. 2B**: Cell viability over days with different treatments (ROS and HGF) at various concentrations.

*Note: Graphs illustrate the effect of various treatments on cell viability over time.*
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### Fig. 3A

(TUNEL staining)

- **ROS(-)HGF(-)**
  - 50 μm

- **ROS(+)HGF(-)**
  - 50 μm

### Fig. 3B

**caspase-3/GAPDH**

### Fig. 3C

**caspase-3 activity**

* (*p < 0.05, **p < 0.01, ***p < 0.001*)