Original Paper

Nicotinamide Phosphoribosyltransferase Inhibitor APO866 Prevents IL-1 β -Induced Human Nucleus Pulposus Cell Degeneration via Autophagy

Changgui Shi^a Huiqiao Wu^a Di Du^b Hee-Jeong Im^{c,d} Ying Zhang^a Bo Hu^a
Huajiang Chen^a Xinwei Wang^a Yang Liu^a Peng Cao^a Ye Tian^a
Xiaolong Shen^a Rui Gao^a Andre J van Wijnen^e Xiaojian Ye^a Wen Yuan^a

^aDepartment of Spinal Surgery, Changzheng Hospital, Second Military Medical University of China, Shanghai, ^bDepartment of Orthopedics and Trauma Surgery, Changzheng Hospital, Second Military Medical University of China, Shanghai, China, ^cDepartments of Bioengineering, the University of Illinois at Chicago, Chicago, ^dJesse Brown Veterans Affairs Medical Center (JBVAMC) at Chicago, Chicago ^eDepartment of Orthopedic Surgery, Mayo Clinic, Rochester, USA

Key Words

Nucleus pulposus • IL-1 β • Nampt • APO866 • ECM • Autophagy

Abstract

Background/Aims: Intervertebral discs consist of an extracellular matrix (ECM) with a central gelatinous nucleus pulposus (NP) enclosed in an outer layer known as the annulus fibrosus. ECM metabolic disorders result in loss of boundary between the annulus fibrosus and NP, which can lead to intervertebral disc degeneration (IDD). Proinflammatory cytokines, such as interleukin (IL)-1 β , mediate the progression of IDD. Nicotinamide phosphoribosyltransferase (Nampt) catalyzes the first step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) and is known to be induced by IL-1 β . APO866 is an inhibitor of NAD biosynthesis and is involved in autophagy. LC3 (microtubule-associated protein 1 light chain 3) is a key regulator of autophagy and is used as an indicator of increased autophagy. Herein, we investigate the role of APO866 in regulating autophagy in NP cells and IL-1 β mediated NP cell degeneration and apoptosis. **Methods:** NP cells were extracted from IDD tissues and cultured in DMEM/F12 medium. Nampt was induced by different concentrations of IL-1 β (0, 0.5, 1, 5, 10 ng/mL) for 24 h or NP cells were treated with 10 ng/mL IL-1 β for 0, 6, 12, 48 h. QRT-PCR and western blots were used to detect Nampt and ECM-related protein expression in NP tissue of patients with IDD and in NP cells. Confocal analysis was used to detect membrane-bound LC3, Aggrecan, and Collagen II. **Results:** Nampt is expressed in NP tissue at higher levels in severe grades of IDD (Grade IV and V) compared with low grades (Grade II and III). In NP cells, 10 ng/mL IL-1 β induced Nampt expression for 48 h, increased expression of the degradative-associated

C. Shi, H. Wu and D. Du contributed equally to this work.

Xiaojian Ye
and Wen Yuan

Department of Spinal Surgery, Changzheng Hospital, the Second Military Medical University,
415 Fengyang Road, Shanghai 200003 (China)
Tel. +86-21-81885637, E-Mail yexj2002@163.com; smmuyuanwen@163.com

proteins, ADAMTS4/5 and MMP-3/13, and decreased expression of ECM-related proteins, Aggrecan and Collagen II. However, the Nampt inhibitor APO866 blocked IL-1 β induction, and the knockdown of Nampt expression increased the expression of ECM proteins that were inhibited by IL-1 β . Moreover, evidence provided by the autophagic markers LC3 and Beclin-1 indicated that APO866 induced NP cell autophagy. Furthermore, although APO866 inhibited the downregulated expression of ECM-related proteins by IL-1 β , this function was blocked by autophagy inhibitor, 3-methyladenine. **Conclusion:** APO866 protects NP cells and induces autophagy by inhibiting IL-1 β -induced NP cell degeneration and apoptosis, which may have therapeutic potential in IDD.

Introduction

Intervertebral disc degeneration (IDD) has been attributed to several etiological factors including aging, trauma, and genetic predisposition [1-4]. Intervertebral discs consist of an extracellular matrix (ECM) with a central gelatinous nucleus pulposus (NP) composed of proteoglycans and held together by a type II collagen network. The NP is enclosed in the annulus fibrosus, a fibrous outer layer composed of highly organized lamellae of type I collagen fibrils [5, 6]. The hydrated heterogeneous structure of the NP allows the intervertebral disc to withstand the application of biomechanical forces [7-9]. The primary proteoglycan in the NP is Aggrecan, which contains highly anionic glycosaminoglycan side chains, such as chondroitin sulfate and keratan sulfate, that enable the NP to absorb the water that provides the compressive strength required to counteract physical stress [10]. Collagen II is at the highest level in the NP and decreases toward the periphery of the intervertebral disc [11]. IDD is associated with the proteolytic degradation of ECM, leading to large structural changes [10]. The low cellularity of the discs limits repair processes, making them susceptible to injury and age-associated accumulation of tissue damage [12]. ECM metabolic disorders disrupt the boundary between the outer annulus fibrosus and inner NP, which impairs the mechanical function of the discs [13, 14].

The breakdown of ECM products during IDD is thought to elicit a proinflammatory response with mechanisms that are dependent on the context and inducing agent [15-17]. In response to infection, plasma and leucocytes are recruited to the affected site to activate other cell types whereas in response to tissue injury a vascular response is also instigated, a process that is more complex in avascular tissue [16]. Cytokines, such as interleukin (IL)-1 β , are thought to be mediators in the breakdown of ECM [4, 18-20]. Activation of cytokines is believed to proceed through toll-like receptor (TLR)-2 and TLR4 signaling and can lead to increased mRNA expression levels of several inflammatory and catabolic genes including IL-1 β , IL-6, IL-8, cyclooxygenase (COX)-2, matrix metalloproteinase (MMP)-1 and -13 [21-23]. The regulation of MMP-1 and -13 is thought to be through heme oxygenase-1 (HO-1) via its regulation of NADPH oxidases (NOX) 4 and reactive oxygen species (ROS) production in chondrocytes [24]. HO-1 can also reverse the effects of IL-1 β on the expression of MMP-1, 3, 9 and 13 to regulate ECM metabolism induced by pro-inflammatory cytokines in NP cells [25].

Nicotinamide phosphoribosyltransferase (Nampt), also known as a pre-B cell colony-enhancing factor (PBEF) or Visfatin, is a 52 kDa mammalian protein that is constitutively synthesized by several tissues and secreted by various cell types, however, its function is not clearly understood [26]. Nampt, encoded in humans by the *PBEF1* gene [27], catalyzes the first step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide and is thought to be involved in several physiological processes, such as cell differentiation, the stress response, and apoptosis [28, 29]. Nampt expression is induced by the presence of IL-1 β and lipopolysaccharide (LPS) indicating an association with inflammatory cytokines [25]. Furthermore, the IL-1 β -induced dedifferentiation of articular chondrocytes was found to be through the up-regulation of SIRT1 activity enhanced by both Nampt and ERK signaling [25]. Nampt activity is thought to be increased in cancer cells because genomic instability and persistent PARP1-dependent DNA repair lead to increased levels of NAD [30, 31].

APO866, also known as FK866, is an inhibitor of NAD biosynthesis that induces apoptosis and autophagy in many cancers but the role of autophagy in APO866-induced NP cell death is unclear [32-34]. LC3 and Beclin-1 are used as indicators of increased autophagy [35, 36]. During autophagy, the cytosolic form of LC3 (LC3-I) is recruited to autophagosomal

membranes when it is converted to LC3-phosphatidylethanolamine conjugate (LC3-II) [37]. Beclin-1 induces autophagy by interacting with several cofactors to regulate the lipid kinase Vps-34 protein and promote the formation of Beclin-1–Vps34–Vps15 complexes [38].

In this work, we used the autophagy markers LC3 and Beclin-1, to assess the involvement of APO866 in autophagy-mediated NP cell death and its impact on NAD biosynthesis through its influence on Nampt.

Materials and Methods

Human intervertebral disc degeneration tissue samples

Informed consent was given by the patients or relatives to obtain human intervertebral tissue at surgery. The experimental methods were carried out in accordance with approved guidelines and the study was authorized by the ethics committee of Second Military Medical University (Shanghai, China). Fifty NP samples with different degrees of IDD (n = 50; age 27 to 78 years, mean age 56.4 years) were obtained from patients between 2016 and 2017, who underwent disc resection surgery or spinal fusion to relieve lower back pain. Assessment of the disease state was by Pfirrmann grading according to a previous study [39]. No medications or anti-inflammatory drugs were used before surgery. In this study, grade-II (G-II) and grade-III (G-III) samples were set as the mild IDD group (n = 26), which was used as a relatively normal control; grade-IV (G-IV) and grade-V (G-V) samples were set as the severe IDD group (n = 24).

Isolation of nucleus pulposus cells

Cells from NP tissue were extracted according to a previous study [40, 41], specimens were minced and incubated with 2 U/mL protease (Pronase; Sigma-Aldrich, St Louis, MO, USA) in serum-free DMEM/F12 medium (Gibco, Grand Island, NY, USA) for 30 min at 37 °C after washing twice in PBS. The digested cells were then incubated with 0.25 mg/mL type II collagenase (Gibco) for 4 h at 37 °C. The cell suspension was passed through a 40 μ M cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) and then centrifuged at 800 $\times$ g for 5 min. Isolated NP cells were resuspended in DMEM/F12 containing 10% FBS (Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 1% L-glutamine. Cells were cultured and incubated at 37°C under an atmosphere of 5% CO₂.

Reagents

For the experiment, cell cultures were treated with either IL-1 β (Peprotech, Rocky Hill, NJ, USA), 3-methyladenine (3-MA) (Sigma-Aldrich), or APO866 (Selleck, Houston, TX, USA). Human recombinant Nampt (rNampt) was obtained from R&D Systems (Lille, France) and nicotinamide mononucleotide (NMN) was obtained from Sigma-Aldrich. rNampt was used at 100 ng/mL and NMN at 100 μ mol/L according to previous studies [42, 43].

qRT-PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We used Epoch spectrophotometry to measure RNA level. For qRT-PCR, we used SYBR-green PCR Master Mix in a Fast Real-time PCR 7500 System (Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR are listed in Table 1.

Western blots

NP cells and fresh-frozen IDD tissues (50 samples) were harvested and lysed in lysis buffer in Tris-HCl (20 mM; pH 7.4), KCl (140 mM), EDTA (3 mM) and 0.5% Triton X-100 supplemented with protease inhibitor cocktail (Sigma-Aldrich) according to a previous study [44]. Protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL) and aliquots containing 60 μ g of protein were separated by 10% sodium

Table 1. The sequences for the primers

gene	Primer direction	Sequence
Nampt	Forward	5' TTGCTGCCACCTTATC 3'
	Reverse	5' AACCTCCACCAGAACC 3'
ADAMTS4	Forward	5' GCCTTCAAGCACCCAA 3'
	Reverse	5' CCACATCAGCCATACCC 3'
ADAMTS5	Forward	5' CTCCCAGAAACAACGG 3'
	Reverse	5' GCCTACATTCACTGCC 3'
MMP-3	Forward	5' AAACCCACCTTACATAC 3'
	Reverse	5' TCCATAGAGGGACTGA 3'
MMP-13	Forward	5' GCAGTCTTTCTTCGGCTTAG 3'
	Reverse	5' AGGGTCCTTGGAGTGGTC 3'
Aggrecan	Forward	5' TCTACACGCTACACCC 3'
	Reverse	5' AAGACCTCACCCTCC 3'
Collagen II	Forward	5' TCCTCTGCGACGACATAA 3'
	Reverse	5' CAGTGGCGAGGTCAGTT 3'
GAPDH	Forward	5' TCCCATCACCATCTT 3'
	Reverse	5' ACGCCACAGTTTCC 3'

dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were subsequently blocked using skimmed milk in 5% Tris-buffered saline with Tween-20 (TBST) at 37°C for 1 h and incubated overnight with the following primary antibodies: anti-Nampt (ab58640, Abcam, 1:800 dilution), ADAMTS4 (ab185722, Abcam, 1:1000 dilution), ADAMTS5 (ab41037, Abcam, 1:500 dilution), MMP-3 (ab53015, Abcam, 1:500 dilution), MMP-13 (ab39012, Abcam, 1:1000 dilution), Aggrecan (ab36861, Abcam, 1:1000 dilution) and Collagen II (ab34712, Abcam, 1:2000 dilution), LC3 (ab128025, Abcam, 1:1000 dilution) and Beclin-1 (ab62557, Abcam, 1:1000 dilution). After washing in PBS, membranes were incubated with the corresponding horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 1 h at room temperature. Signals were detected with Immobilon Western chemiluminescence HRP substrate.

Plasmid constructs

To knockdown Nampt expression, three small interfering RNA constructs were generated as follows: Nampt shRNA 1# (5'-TGAGTAACCTTAGGAGTCG-3'), shRNA 2# (5'-TCTTGAAGACAATGGAGAA-3'), and shRNA 3# (5'-ATGTGCTGCTTAGTTCAAT-3'); a scramble negative shRNA was used as a control (5'-GACCTGTACGCCAACACAGTG-3'). The sequences were chemically synthesized (Genechem, Shanghai, China) and subcloned into a psilencer 4.1 vector (Invitrogen, NY, USA), followed by selection with puromycin (Gibco, Grand Island, NY, USA) to generate stable cell lines.

CCK-8 assay

Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to manufacturer's instructions. Briefly, 2000 cells/well were cultured in 96-well plates for 12 h. CCK-8 solution (10 μ L) was added to each well and the cells were counted after 1–5 days by measuring A₄₅₀ with an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Monodansylcadaverine (MDC) staining

The fluorescent dye, Monodansylcadaverine (MDC), is a special marker for autophagic vacuoles. MDC staining was performed according to a method previously described [45]. Cells in 6-well plates were treated with glucosamine and then incubated with 0.05 mM MDC for 30 min. The cells were then rinsed three times with PBS and fluorescence intensity was detected with a UV filter using fluoroscopy without cell fixation.

NAD detection

Intracellular NAD level was detected using a NAD⁺/NADH quantification kit (BioVision, Mountain View, CA, USA) as previously described [46]. Cells were added to NADH/NAD extraction buffer and then subjected to two freeze/thaw cycles for 20 min at –70°C, then 10 min at room temperature. They were then centrifuged at 12,000 $\times$ g for 5 min, and 50 μ L of the supernatant was mixed with 100 μ L NAD cycling mix and 2 μ L NADH cycling enzyme mix. NAD was converted to NADH by incubating at room temperature for 5 min. After adding 10 μ L NADH developer, the optical density was read at 450 nm every 30 min for 4 h. A standard curve was used to determine the total sample NAD level expressed as pmol/10⁴ cells.

Immunohistochemistry (IHC)

Tissue samples were fixed in formalin and embedded in paraffin before sectioning (4- μ m thickness) and deparaffinization in xylene. After dehydration in a graded series of ethanol, antigen retrieval was performed in citrate buffer for 30 min in a steamer, followed by washing in phosphate buffered saline (PBS). Tissue sections were then quenched in 3% hydrogen peroxide, blocked with PBS containing 10% goat serum (Sigma-Aldrich) for 1 h at 37 °C, and incubated with anti-Nampt antibody (ab58640, Abcam, 1:100 dilution) overnight at 4°C. The signal was amplified and visualized with diaminobenzidine-chromogen, followed by counterstaining with hematoxylin. Nampt immunostaining was analyzed using intensity and distribution measurements as previously described [47-49]. The staining intensity was scored as 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). The staining distribution was measured as the percentage of positive cells (0, none; 1, <25%; 2, 25-75%; 3, 75%-100%). Nampt expression was scored by multiplying the intensity and distribution. Cells were further divided according to Nampt expression into “low” (Nampt low) and “high” (Nampt high) groups, according to a cutoff point. The cutoff point for Nampt expression was calculated using the X-tile software program as previously described [50].

Immunofluorescence staining

Cells were grown on glass coverslips, fixed in formalin, rinsed in PBS, and permeabilized with 0.1% Triton-X. Cells were then incubated with primary antibodies Aggrecan (ab36861, Abcam, 1:100 dilution), Collagen II (ab34712, Abcam, 1:100 dilution) and LC3 (ab128025, Abcam, 1:100 dilution) at 4°C overnight

followed by goat anti-rabbit IgG H&L (Cy3) preadsorbed. DAPI was used as a nuclear stain. Slides were fixed and imaged using an LSM-710 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany).

TUNEL staining

NP cells in 96-well plates were fixed for 30 min with fresh 4% paraformaldehyde and rinsed three times with PBS. Following incubation (2 min) with 0.1% Triton X-100, an in situ cell death detection kit (Roche, Welwyn Garden City, UK) was used to determine the level of apoptosis. Cells treated with 2 U/mL of DNase were used as positive controls and cells treated without terminal transfer were used as negative controls. Nuclei were counterstained with Hoechst 333425 (2 μ g/mL) for 2 min at room temperature. The apoptotic percentage for each group was determined by the ratio of TUNEL-positive to Hoechst-stained nuclei in each field from at least five fields.

Transmission electron microscopy

Tissues from patients with IDD were fixed in ice-cold 2.5% glutaraldehyde as described in a previous study [51]. Briefly, the IVD tissues were postfixed in 2% osmium tetroxide and block-stained with 2% uranyl acetate. The tissue samples were dehydrated in an acetone series and then embedded in Araldite. Ultra-thin sections were cut and then examined using a TECNAI 10 transmission electron microscope (Hillsboro, OR, USA).

Statistical analysis

Results are presented as the mean $\pm$ SD from three independent experiments performed in triplicate. The Pearson chi-square test was used to analyze the relationship between Nampt expression and clinicopathological features. The Student's *t*-test was used to detect significant differences between two groups. Significant differences between and within multiple groups were examined using one-way ANOVA for repeated measures, followed by Dunnett's Multiple Comparison Test or Bonferroni's Multiple Comparison Test. *p* < 0.05 was considered to indicate a statistically significant result. Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA).

Results

Higher upregulation of Nampt in severe IDD grades compared to mild IDD grades

We assessed the expression levels of Nampt in NP samples of 50 patients with different grades of IDD. QRT-PCR and western blot analysis indicated that Nampt mRNA and protein levels were significantly higher in 26 patients with more severe degeneration (G-IV and G-V) than in 24 patients with mild degeneration (G-II and G-III) (Fig. 1A and B; *P* < 0.01). These results were confirmed by immunostaining of the Nampt protein in NP tissues (Fig. 1C). NP samples with severe degeneration have increased

Fig. 1. Expression of Nampt in IDD tissue samples. (A, B) qRT-PCR and western blot analysis of the expression of Nampt in 50 IDD patients (26 mild Grade II-III, and 24 Grade IV-V, Student's *t*-test: Mann-Whitney test, ***p* < 0.01). (C) Immunostaining of the Nampt protein in 50 IDD samples and quantification by the multiplicative quickscore method. Original magnification, x400.

diaminobenzidine-chromogen staining indicating higher levels of Nampt and more obvious signs of degenerative fibrocartilage, which was confirmed by scoring the percentage of stained cells (Table 2). However, the high or low expression of Nampt was not correlated with age, gender, or disc level. Overall, these results provide strong evidence that the high expression of Nampt associates with the severity of IDD in human NP tissue.

IL-1 β increases Nampt expression and Nampt knockdown inhibits the downregulation of Aggrecan and Collagen II expression induced by IL-1 β

We investigated the influence of IL-1 β on levels of Nampt expression and NP cell degeneration. Expression of Nampt, in NP cells, induced by IL-1 β was measured by qRT-PCR and western blot analysis (Fig. 2A–D). Increased concentrations of IL-1 β and longer incubation times led to significantly increased levels of Nampt at 5–10 ng/mL ($P < 0.05$), reaching the highest levels after 48 h at 10 ng/mL. To further investigate the reduced expression of ECM by IL-1 β , we created a knockdown of Nampt expression by shRNA; three different Nampt shRNA constructs resulted in three levels of Nampt downregulation. The results indicated that shRNA1# expressed Nampt at a lower level than shRNA2# and 3# (Fig. 3A and B). We used the three shRNAs to assess levels of ADAMTS4/5 and MMP-3/13, Aggrecan and Collagen II expression by qRT-PCR and western blots (Fig. 3C–J and Fig. 4). The results indicated that levels of ADAMTS4/5 and MMP-3/13 were significantly reduced, while Aggrecan and Collagen II were significantly increased with the knockdown of Nampt (shRNA1# and 3#) in NP cells incubated with IL-1 β . Aggrecan and Collagen II expression were analyzed by cell immunofluorescence (Fig. 3C–J and Fig. 4). The results indicated that the Nampt shRNA3# has a lower significance to shRNA1#. However, the shRNA2# has no effect on the upregulation of

Table 2. Nampt staining and clinicopathological characteristics of 50 IDD * $p < 0.05$, Chi-square test

Parameters	Nampt		Total	P-value
	Low (%)	High (%)		
Age (years old)				0.564
<50	9 (18.0)	11 (22.0)	20	
≥ 50	16 (32.0)	14 (28.0)	30	
Gender				0.254
Male	13 (26.0)	9 (18.0)	22	
Female	12 (24.0)	16 (32.0)	28	
Disc level				0.303
L3/4	9 (18.0)	7 (14.0)	16	
L4/5	11 (22.0)	8 (16.0)	19	
L5/S1	5 (10.0)	10 (20.0)	15	
MRI Grade				0.024*
G (II–III)	17 (34.0)	9 (18.0)	26	
G (IV–V)	8 (16.0)	16 (32.0)	24

Fig. 2. IL-1 β regulates Nampt expression in NP cells. (A, B) qRT-PCR and western blot analysis of the expression of Nampt in NP cells with indicated concentrations of IL-1 β for 24 h (* $p < 0.05$ vs. 0 ng/mL). (C, D) qRT-PCR and western blot analysis of the expression of Nampt in NP cells induced by IL-1 β (10 ng/mL) for 0, 12, 24, and 48 h (* $p < 0.05$ and ** $p < 0.01$ vs. 0 h).

ADAMTS4/5 and MMP-3/13, and the downregulation of Aggrecan and Collagen II expression induced by IL-1 β (Supplementary Figures 1 and 2 - For all supplemental material see www.karger.com/10.1159/000493843/).

Fig. 3. Knockdown of Nampt decreased ADAMTS4/5 and MMP expression. (A, B) Knockdown of Nampt (shRNA 1#–3#) in NP cells and qRT-PCR and western blots assessment of Nampt expression (* p <0.05, ** p <0.01 and *** p <0.001 vs. scr). (C–J) NP cells were treated with IL-1 β (10 ng/mL) or transfected with scr or shRNA 1# and 3# and co-treated with IL-1 β for 48 h. Untransfected NP cells were used as a control. qRT-PCR and western blot analysis of the expression of ADAMTS4/5 and MMP-3 and -13 in NP cells (** p <0.01 vs. IL-1 β +scr; ** p <0.01 vs. IL-1 β +Nampt shRNA1#; * p <0.05 and ** p <0.01 vs. IL-1 β +Nampt shRNA3#).

Fig. 4. Knockdown of Nampt increased Aggrecan and Collagen II expression. NP cells were treated with IL-1 β (10 ng/mL) or transfected with scr or shRNA 1# or shRNA 3# and co-treated with IL-1 β for 48 h. Untransfected NP cells were used as a control. (A–D) qRT-PCR and western blot analysis of the expression of Aggrecan and Collagen II in NP cells when transfected with shRNA1# (** $p < 0.01$ vs. IL-1 β +scr; ** $p < 0.01$ vs. IL-1 β +Nampt shRNA1#). (E–H) qRT-PCR and western blot analysis of the expression of Aggrecan and Collagen II in NP cells when transfected with shRNA3# (** $p < 0.01$ vs. IL-1 β +scr; * $p < 0.05$ and ** $p < 0.01$ vs. IL-1 β +Nampt shRNA3#).

(I) Immunofluorescence analysis of Aggrecan and Collagen II expression in NP cells.

APO866 induces autophagy by increasing levels of LC3-II and Beclin-1 expression

Nampt inhibitor APO866 was reported to induce autophagy [52]. We used different concentrations of APO866 to treat NP cells and found that cell viability was significantly reduced with 20 nM APO866 (Fig. 5A). MDC, a specific *in vivo* marker for autophagic vacuoles, was used to analyze the extent of autophagy at different concentrations of APO866 (Fig. 5B). Increased concentrations of APO866 led to higher levels of autophagic vacuoles in NP cells. Protein levels of the autophagic markers microtubule-associated protein LC3 and Beclin-1 were assessed in NP cells incubated with different concentrations of APO866 by western blot analysis (Fig. 5C). Beclin-1 expression was increased with increasing concentration of APO866 and the conversion of LC3-I to LC3-II, which is recruited to autophagosomal membranes, was also increased with APO866 concentration. In a time-course experiment, expression of Beclin 1 was at the highest levels after 48 h of incubation with APO866 at concentrations of 10 nmol/L and LC3-I to LC3-II conversion was significantly increased between 24 and 72 h, reaching a peak at 48 h (Fig. 5D). These results were confirmed by visual observations of NP cells containing increased levels of autophagosomal membrane-bound LC3 by immunofluorescence analysis (Fig. 5E and F). In addition, we detected autophagy in GII-III and GIV-V IVD degeneration tissues and found that the higher grade degeneration tissues have more autophagic vacuoles than lower grade tissues (Supplementary Fig. 3).

Fig. 5. APO866 induces NP cells autophagy. (A) CCK-8 assay analysis of cell viability induced by different concentrations of APO866 for 24 h (* $p < 0.05$ and ** $p < 0.01$ vs. 0 nM). (B) MDC assay analysis of autophagy at different concentrations of APO866 for 24 h. (C) Western blot analysis of the expression of LC3 and Beclin-1 in NP cells induced by indicated concentrations of APO866 for 24 h (* $p < 0.05$ and ** $p < 0.01$ vs. 0 nM). (D) Western blot analysis of the expression of LC3 and Beclin-1 in NP cells induced by 10 nmol/L APO866 for 0, 24, 48, 72, and 96 h (* $p < 0.05$ and ** $p < 0.01$, vs. 0 h). (E, F) Immunofluorescence analysis of LC-3 at indicated concentrations of APO866 for 24 h or 10 nM APO866 for 0, 24, 48, 72, and 96 h.

APO866 blocks NP cell degeneration induced by IL-1 β

Next, we also investigated the influence of APO866 on IL-1 β induced Nampt mRNA and protein levels, and NAD levels (Fig. 6A–C). The addition of APO866 made no impact on IL-1 β induced Nampt mRNA and protein levels in NP cells but significantly reduced intracellular levels of NAD in control NP cells and in NP cells that were incubated with IL-1 β ($P < 0.01$) (Fig. 6A–C). mRNA expression and protein levels of ADAMTS4/5, MMP-3/13, Aggrecan and Collagen II were assessed in NP cells induced by IL-1 β with or without APO866 (Fig. 6D–K and

Fig. 6. APO866 effects on ADAMTS4/5 and MMPs expression induced by IL-1 β . NP cells were treated with IL-1 β (10 ng/mL) or APO866 (10 nmol/L) or pretreated with APO866 for 2 h then co-treated with IL-1 β for 48 h. Untransfected NP cells were used as a control. (A, B) qRT-PCR and western blot analysis of the expression of Nampt in NP cells (** $p < 0.01$ vs. IL-1 β and ** $p < 0.01$ vs. IL-1 β +APO866); (C) NAD detection (** $p < 0.01$ vs. IL-1 β , ** $p < 0.01$ vs. APO866 and ** $p < 0.01$ vs. IL-1 β +APO866). (D–K) qRT-PCR and western blot analysis of the expression of ADAMTS4 or 5 and MMP-3 or -13 in NP cells (** $p < 0.01$ vs. IL-1 β , * $p < 0.05$ and ** $p < 0.01$ vs. IL-1 β +APO866).

Fig. 7). Expression levels of ADAMTS4/5 and MMP-3/13 were increased by IL-1 β (Fig. 6D–K) whereas Aggrecan and Collagen II levels were decreased (Fig. 7), we confirmed the effects of IL-1 β and APO866 on Aggrecan and Collagen II expression using cell immunofluorescence (Fig. 7E). The addition of APO866 partially reduced levels of IL-1 β -induced ADAMTS4/5 and MMP-3/13 expression and rescued levels of Aggrecan and Collagen II by IL-1 β treatment. These results indicate that APO866 can inhibit IL-1 β induced levels of ECM degradative-related proteins and restore levels of ECM components possibly through NAD inhibition.

APO866 inhibits IL-1 β induced downregulation of ECM proteins via autophagy and cell apoptosis in degenerated NP cells

To confirm that APO866 induces autophagy in NP cells, an inhibitor of autophagy, 3-MA, was used to assess the autophagic markers Beclin-1 and LC-3 by western blot analysis and immunofluorescence (Fig. 8A and B). We also used nicotinamide mononucleotide (NMN) or recombinant Nampt (rNampt) to detect cell autophagy, the results indicated that NMN and rNampt can inhibit the cell autophagy induced by APO866 (Supplementary Fig. 4). Beclin-1 expression and LC-3 conversion were increased in cells incubated with APO866 with or without IL-1 β but 3-MA inhibited the effects of APO866 induced autophagy.

Fig. 7. APO866 effects on Aggrecan and Collagen II expression induced by IL-1 β . NP cells were treated with IL-1 β (10 ng/mL) or APO866 (10 nmol/L) or pretreated with APO866 for 2 h then co-treated with IL-1 β for 48 h. Untransfected NP cells were used as a control. (A–D) qRT-PCR and western blot analysis of the expression of Aggrecan and Collagen II in NP cells (** p<0.01 vs. IL-1 β , *p<0.05 and **p<0.01 vs. IL-1 β +APO866). (E) Immunofluorescence analysis of Aggrecan and Collagen II expression in NP cells.

Fig. 8. APO866 induces autophagy in NP cells. NP cells were treated with IL-1 β (10 ng/mL) or APO866 (10 nmol/L) alone or pretreated with APO866 for 2 h then co-treated with IL-1 β , or pretreated with APO866 for 2 h then co-treated with 3-MA (10 mmol/L), or pretreated with APO866 for 2 h then co-treated with 3-MA (10 mmol/L) and IL-1 β (10 ng/mL) for 48 h. Untransfected NP cells were used as a control. (A) western blot analysis of the expression of LC3 and Beclin-1 in NP cells (** $p < 0.01$ vs. APO866; ** $p < 0.01$ vs. IL-1 β +APO866; ** $p < 0.01$ vs. APO866+3-MA; ** $p < 0.01$ vs. IL-1 β +APO866+3-MA). (B) Immunofluorescence analysis of LC-3 in NP cells.

Immunofluorescence confirmed that 3-MA reduced levels of autophagosomal membrane-bound LC-3 in NP cells incubated with APO866.

To investigate whether APO866 upregulates expression of ECM proteins via autophagy, we assessed the influence of 3-MA in NP cells, incubated with or without IL-1 β or APO866, on the mRNA and protein levels of Nampt and NAD (Fig. 9A–C) by qRT-PCR and western blot analysis. The mRNA and protein levels of ADAMTS4/5MMP-3/13 (Fig. 9D–K), and Aggrecan and Collagen II were also assessed (Fig. 10A–D). Nampt levels were significantly induced by IL-1 β but remained unaltered by the presence of APO866 or 3-MA. In contrast, NAD was significantly increased by IL-1 β treatment, but was downregulated when treated with APO866; however, this effect was reversed in the presence of 3-MA. Similarly, the influence of APO866 on levels of ADAMTS4/5, MMP-3/13, Aggrecan and Collagen II was inhibited by 3-MA. These results were confirmed in Aggrecan and Collagen II by immunofluorescence (Fig. 10E). The presence of 3-MA prevents APO866 from inhibiting IL-1 β -induced downregulation of the ECM, indicating that APO866 inhibits IL-1 β -induced downregulation of ECM proteins via autophagy. Furthermore, TUNEL analysis indicates that APO866 inhibits IL-1 β induced cell apoptosis in degenerated NP cells; however, 3-MA reverses this effect (Fig. 10F).

The mechanism by which we propose APO866 inhibits IL-1 β induced NP cell degeneration via autophagy is shown in Fig. 11. Briefly, IL-1 β increases Nampt expression and decreases the expression of ECM. The downregulation of ECM induces NP cell degeneration. Nampt inhibitor APO866 can prevent IL-1 β induced ECM degeneration expression via autophagy.

Fig. 9. APO866 upregulates ECM expression in NP cells. NP cells were treated with IL-1 β (10 ng/mL) or APO866 (10 nmol/L) alone or pretreated with APO866 for 2 h then co-treated with IL-1 β , or pretreated with APO866 for 2 h then co-treated with 3-MA (10 mmol/L), or pretreated with APO866 for 2 h then co-treated with 3-MA (10 mmol/L) and IL-1 β (10 ng/mL) for 48 h. Untransfected NP cells were used as a control. (A, B) qRT-PCR and western blot analysis of the expression of Nampt in NP cells (** $p < 0.01$ vs. IL-1 β). (C) analysis of NAD (** $p < 0.01$ vs. IL-1 β ; ** $p < 0.01$ vs. APO866; ** $p < 0.01$ vs. IL-1 β +APO866+3-MA). (D–K) qRT-PCR and western blot analysis of the expression of ADAMTS4 or 5, MMP-3 or -13 in NP cells (** $p < 0.01$ vs. IL-1 β ; ** $p < 0.01$ vs. IL-1 β +APO866; * $p < 0.05$ vs. IL-1 β +APO866+3-MA).

Fig. 10. APO866 inhibits IL-1 β induced downregulating of Aggrecan and Collagen II expression and cell apoptosis. NP cells were treated with IL-1 β (10 ng/mL) or APO866 (10 nmol/L) alone or pretreated with APO866 for 2 h then co-treated with IL-1 β , or pretreated with APO866 for 2 h then co-treated with 3-MA (10 nmol/L), or pretreated with APO866 for 2 h then co-treated with 3-MA (10 mmol/L) and IL-1 β (10 ng/mL) for 48 h. Untransfected NP cells were used as a control. (A–D) qRT-PCR and western blot analysis of the expression of Aggrecan and Collagen II in NP cells (** $p < 0.01$ vs. IL-1 β ; ** $p < 0.01$ vs. IL-1 β +APO866; * $p < 0.05$ and ** $p < 0.01$ vs. IL-1 β +APO866+3-MA). (E) Immunofluorescence analysis of Aggrecan and Collagen II expression in NP cells. (F) TUNEL analysis of cell apoptosis.

Discussion

In recent years, autophagy has been associated with IDD and described as a mechanism by which autophagosomes degrade dysfunctional cellular organelles and excess proteins [51, 53, 54]. In the present study, we have investigated whether autophagy, induced by the inhibition of NAD by APO866, could prevent degradation of the ECM in IDD. We found that mRNA and protein levels of Nampt in NP samples of IDD patients with severe degeneration (G-IV and G-V) were significantly higher than in patients with mild degeneration (G-II and G-III). NP samples with severe degeneration also had more obvious signs of degenerative

fibrocartilage, providing strong evidence that the expression of Nampt increases with the severity of IDD. This finding corresponds with another study that associates Nampt and NAD metabolism with an inflammatory disorder; increased Nampt levels were found in the serum and arthritic paws of mice with collagen-induced arthritis [55]. The authors of the study linked NAD metabolism to inflammatory cytokine secretion by leukocytes and propose that its inhibition may have therapeutic efficacy in immune-mediated inflammatory disorders.

As Nampt expression is known to be induced by IL-1 β , which is thought to mediate ECM degradation [56], we next investigated the influence of IL-1 β on levels of Nampt expression and NP cell degeneration and determined whether this could be reversed by extracellular APO866. We found that expression levels of Nampt in NP cells corresponded to IL-1 β concentration and length of incubation. The addition of APO866 made no impact on IL-1 β induced Nampt mRNA levels but significantly reduced intracellular levels of NAD in control and IL-1 β induced NP cells, indicating that APO866 inhibits NAD biosynthesis through a competitive role. Indeed, structural analysis of Nampt has shown that APO866 binds at the interface of the Nampt dimer to interfere with substrate binding [57].

NAMPT is known to participate in several intra- and extra-cellular functions. Intracellular Nampt (iNampt) plays a critical role in maintaining the activity of NAD-dependent enzymes, exogenous NMN [28]. Extracellular Nampt (eNampt), also known as visfatin, acts as an adipokine and was reported to be associated with inflammatory diseases, such as rheumatoid arthritis (RA) [58, 59]. Levels of eNampt in the synovial fluid of RA patients are positively related to disease severity [60, 61]. The Nampt was upregulated by IL-1 β treatment, and recombinant (rNampt) increased Prostaglandin E2 (PGE2) expression in human chondrocytes from osteoarthritis patients; Moreover, rNampt treatment increased MMP-3 and -13 and ADAMTS-4 and -5 expression in immature mouse articular chondrocytes [62].

To assess the impact of NAD inhibition on proteins involved in ECM degradation and regeneration we measured mRNA expression and protein levels of ADAMTS4/5, MMP-3/13, Aggrecan and Collagen II in NP cells induced by IL-1 β with or without APO866. We found that IL-1 β increased levels of ADAMTS4/5 and MMP-3/13 whereas Aggrecan and Collagen II levels were decreased. However, the addition of APO866, reduced levels of IL-1 β -induced ADAMTS4/5 and MMP-3/13 and returned levels of Aggrecan and Collagen II to those incubated without IL-1 β . Similarly, levels of Aggrecan and Collagen II were not reduced by IL-1 β in NP cells with a Nampt knockdown.

Fig. 11. Schematic model to demonstrate how APO866 inhibits IL-1 β induced NP cell degeneration via autophagy. IL-1 β increases Nampt expression and decreases ECM expression. The downregulation ECM induces NP cell degeneration; Nampt inhibitor APO866 can inhibit IL-1 β induced ECM degeneration expression via autophagy.

Increased concentrations of APO866 led to higher levels of autophagic vacuoles in NP cells and, therefore, LC3 and Beclin-1 were assessed in NP cells incubated with different concentrations of APO866. Beclin-1 expression was increased with increasing concentration of APO866 and the conversion of LC3-I to LC3-II was also increased. These results were confirmed by visual observations of NP cells containing increased levels of autophagosomal membrane-bound LC-3 by immunofluorescence analysis. Furthermore, we also examined IVD degeneration tissues and found that GIV-V tissues have more autophagic vacuoles than GII-GIII degeneration tissues. However, the ability of APO866 to induce autophagy in NP cells was inhibited by 3-MA. Beclin-1 expression and LC-3 conversion were increased in cells incubated with APO866 with or without IL-1 β but not in the presence of 3-MA. Fan et al. reported that NMN can mimic the effects of Nampt on MAPK and NF- κ B activation EMMPRIN/MMP-9 induction and rNampt also activates MAPK and NF- κ B and EMMPRIN/MMP-9 expression in macrophages; However, these effects were inhibited by APO866 treatment [42]. Grozio et al. reported that NMN can reverse Nampt inhibition induced tumor cell death [63]. Sharif et al. reported Nampt inhibition enhances autophagy and breast cancer cell death, however, this effect can be effectively reversed by NMN [64]. In this study, we treated NP cells with NMN or rNampt with or without APO866, and we found that both NMN and rNampt can inhibit the cell autophagy induced by APO866.

Furthermore, Nampt levels that were significantly increased by IL-1 β remained unaltered by the presence of APO866 or 3-MA. In contrast, APO866 inhibited the effects of IL-1 β on levels of NAD but not in the presence 3-MA. In addition, the influence of APO866 on levels of ADAMTS4/5, MMP-3/13, Aggrecan and Collagen II were inhibited by 3-MA. The presence of 3-MA prevents APO866 from inhibiting the IL-1 β -induced downregulation of the ECM, indicating that APO866 inhibits IL-1 β induced downregulation of ECM proteins and cell apoptosis via autophagy.

Many studies view autophagy as a double-edged sword in cell survival and there are contradictory reports on its impact in IDD [65, 66]. Autophagy has been shown to have a positive effect on cell survival by mediating the selective removal of damaged cell components [54, 67]. In addition, autophagy has been shown to instigate a protective effect against oxidative damage to endplate chondrocytes in intervertebral discs as the inhibition of autophagy increased apoptosis [68]. Corresponding with our results, glucosamine was found to activate autophagy in NP cells in a dose-dependent manner and attenuated the decrease of aggrecan to prevent the apoptosis of NP cells induced by IL-1 β , whereas 3-MA partly reversed these effects [69]. Furthermore, the autophagy induced by IL-1 β or H₂O₂ in NP cells was increased by glucosamine. Certainly, the role of autophagy in cell survival and apoptosis in NP cells and IDD warrants further investigation.

This work has some limitations. We were unable to obtain NP samples without IDD to use as a normal control. Therefore, the base level of autophagic markers, Nampt expression, and ECM proteins in undamaged discs could not be discussed. However, our study does distinguish between patients with low-grade and high-grade IDD.

Conclusion

In conclusion, we propose that IL-1 β increases Nampt expression and decreases ECM expression and the downregulation of ECM leads to NP cell degeneration. However, APO866 induces autophagy to protect NP cells by inhibiting IL-1 β -induced cell degeneration and cell apoptosis. Our results indicate that IL-1 β increased ADAMTS4/5 and MMP-3/13 expression and decreased expression of ECM-related proteins. Whereas, knockdown of Nampt expression increased the expression of Aggrecan and Collagen II inhibited by IL-1 β . Furthermore, the autophagic markers, LC3 and Beclin-1, confirmed APO866 induced NP cell autophagy and blocks IL-1 β induction considerably. Nampt inhibitor APO866 can inhibit IL-1 β induced ECM degeneration expression via autophagy. APO866, therefore, may have potential in the alleviation of IDD.

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Disclosure Statement

The authors declare no conflicts of interest.

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