Humanin prevents undesired apoptosis of chondrocytes without interfering with the anti-inflammatory effect of dexamethasone in collagen-induced arthritis

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Abstract

Objective

Prolonged use of glucocorticoids (GCs) for treatment of inflammatory and autoimmune conditions may have several negative side effects, such as impaired bone growth which has been linked to increased apoptosis in growth plate chondrocytes. It has recently been shown that humanin, a small mitochondrial derived peptide, rescues growth plate chondrocytes from GC-induced apoptosis. Our aim was to study if a synthetic analogue of humanin, [Gly14]-HNG (HNG), can be safely used to prevent GC-induced toxicity in growth plate chondrocytes without interfering with the desired anti-inflammatory effect in an in vivo model of arthritis.

Methods

Arthritis was induced in DBA/1 mice by collagen type II in complete Freund's adjuvant and the animals were treated with Dexamethasone (Dexa) (0.25 mg/kg/day) with or without HNG (100 µg/kg/day) for 14 days. The animals were observed daily for the presence of arthritis including signs of erythema and swelling of the joints. The paws were scored based on the severity of the swelling. After termination, histological scoring was performed of all paws. Chondrocyte apoptosis and proliferation were analysed by TUNEL assay and PCNA staining, respectively.

Results

We found that HNG treatment in combination with Dexa protected from Dexa-induced chondrocyte apoptosis in both articular and growth plate cartilage. Furthermore, based on clinical and histology scoring analyses, HNG did not interfere with the desired anti-inflammatory effect of Dexa.

Conclusion

Our results suggest that the combination of HNG and GCs may provide a new treatment strategy in conditions of chronic inflammation, which could potentially prevent bone growth impairment.

Key words

humanin, dexamethasone, apoptosis, chondrocyte, collagen type II induced arthritis

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Introduction

Chronic inflammatory conditions during childhood are commonly associated with growth retardation, which mainly is a result of high levels of pro-inflammatory cytokines and long-term glucocorticoid (GC) treatment, affecting the sensitive regulation of growth plate chondrocytes (1-4). Longitudinal bone growth is a complex and tightly regulated process that occurs within the growth plate, a thin layer of chondrocytes at different stages of differentiation. Pro-inflammatory cytokines such as interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) interact directly with growth plate chondrocytes impairing longitudinal bone growth (5, 6). Despite a high degree of therapeutic efficacy in reducing inflammation, it is well known from studies *in vitro* and *in vivo* that GCs may have severe adverse effects. One of the most common side effects is impaired bone growth, which is linked to systemic as well as local effects at the growth plate level (7, 8). To date, no specific treatment is available which may prevent bone growth impairment in GC-treated children with chronic inflammatory conditions. Consequently, there is an unmet need for new treatment strategies addressing bone growth rescue.

GC-induced apoptosis in growth plate chondrocytes is mediated by suppression of the PI3K-Akt signalling pathway and activation of the caspase cascade where Bax, a key mediator of intrinsic apoptosis, plays an important role (9). Previous studies have shown that the mitochondrial derived peptide humanin may rescue from Dexa-induced bone growth retardation by inhibiting the activation of Bax and thereby preventing cell death (10). Humanin-Gly14 (HNG), a humanin analogue with enhanced potency, has been shown to have therapeutic potential in Alzheimer disease, diabetes, and stroke (11-14). We have previously shown that HNG can rescue from growth retardation induced by the anti-cancer drug bortezomib and GC treatment in mice (15, 16) Interestingly, this study also showed that HNG in itself have anti-inflammatory effects by suppressing cytokine levels in LPS challenged human monocyte-derived macrophages (16). However, these protective effects of HNG have so far been studied only in normal mice and human macrophages and the effects of HNG in a disease model of chronic inflammation have not yet been clarified.

Based on these previous findings, we hypothesised that HNG can be used in combination with Dexa without interfering with the desired anti-inflammatory effects of GCs and that HNG may prevent GC-induced chondrocyte toxicity in a model of chronic inflammation. To test this hypothesis, the therapeutic effect of Dexa and HNG in combination was investigated in collagen type II induced arthritis (CIA) in DBA/1 mice, which is the most widely used model of rheumatoid arthritis sharing many features with the human disease (17).

Materials and methods

Induction and of collagen type II Arthritis

All experimental procedures were approved by the Stockholm North Ethical Committee, Sweden (ethical permit no. N 464/12). Female DBA/1 mice, 18-20g (approximately 8 weeks old), were purchased from Taconic and housed in specific pathogen-free animal facilities at the Karolinska University Hospital, Solna, Sweden. The mice were housed five animals per cage and had free access to water and standard chow. A 12-h light/dark circle was maintained at all time. Under isoflurane anesthesia, mice were injected subcutaneously at the base of the tail with type II collagen (CII) prepared from bovine nasal cartilage emulsified in Freund's complete adjuvant. Each mouse received 100 μg of CII and 300 μg of *Mycobacterium tuberculosis* in 100 μL of emulsion. On day 28 after the first injection, the mice received a booster injection of 100 μg CII in 100 μL Freund's incomplete adjuvant. Mice that did not develop any signs of arthritis within 14 days after the booster injection were excluded from the study. Figure 1 illustrates in detail the experimental set up.

Clinical evaluation of arthritis

The mice were observed daily for the

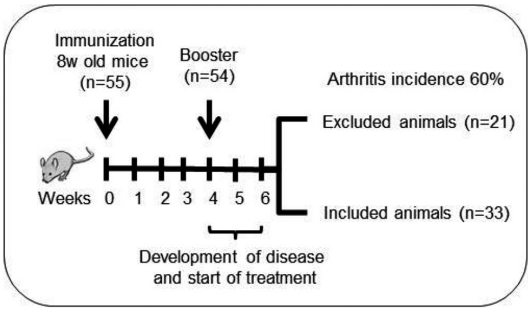
presence of arthritis including signs of erythema and swelling of the joints by a well-established protocol described previously (18). Briefly, the interphalangeal joints of the digits, the metacarpophalangeal joints and wrist in the forepaws and the metatarsophalangeal joints and ankle joints in the hind-paws were each considered as one category of joints. Individual paws were scored on a scale of 0–3 as follows: 0 = no signs of arthritis, 1 = one type of joint affected, 2 = two types of joints affected, and 3 = the entire paw affected. Thus, the maximal score for each animal is 12, but mice reaching a total score of 9 were sacrificed due to ethical restrictions; therefore, score 9 represents the highest possible score allowed in this study. Clinical evaluation of arthritis was performed by three observers blinded to the identity of the animals.

Establishment of treatment protocol

The onset of disease, demonstrated by the clinical arthritis score, occurred within 14 days after the booster injection (Fig. 1). The animals were randomly assigned a treatment regime when the animals reached a total score of 3. In a pre-study, three different concentrations of Dexa (SigmaAldrich) (1, 0.25 and 0.1 mg/kg) were administered by intraperitoneal (ip) injections to arthritic mice for 21 consecutive days. Treatment with 1 mg/kg of Dexa showed a lower clinical score compared with vehicle ($p<0.001$) while 0.1 mg/kg Dexa showed a trend towards lower scores compared with vehicle, however not statistically significant (Supplementary Fig. 1). Based on these findings, a dose of 0.25 mg/kg of Dexa ($p<0.01$ compared with vehicle) and a treatment period of 14 days was used in subsequent experiments.

The animals in the main study were treated with daily ip injections of 1) Dexa (SigmaAldrich) (0.25 mg/kg/day) dissolved in sterile sodium chloride (NaCl), 2) HNG (Genescript) (100 μg/kg/day) dissolved in NaCl, 3), a combination of Dexa and HNG, or 4) vehicle (NaCl). After 14 days of treatment, the animals were sacrificed by CO_2, blood collected by heart punctuation and serum stored at -80°C until analysed.

Fig. 1. Experimental design, time points of induction and treatment and number of animals included in the main study.



Paws and hind limbs were dissected for histopathological analysis.

Tissue collection and preparation

Paws and femur from all animals were dissected and fixed in 4% formaldehyde for 24 hrs followed by decalcification in EDTA buffer for 3–4 weeks before dehydration and paraffin embedding. Serial sections (5 μm thick) were stained with hematoxylin-eosin and Safranin O-Fast green.

Histological scoring of arthritis

All paws were analysed and the joints were scored for arthritis severity according to a previously described protocol (18). Briefly, cell infiltration and cartilage destruction were scored on a scale of 0 (no infiltration/no abnormalities) to 3 (maximal cell infiltration/completely destroyed joint) by two observers blinded to the treatment. A mean score of each parameter was calculated for the four paws of each animal.

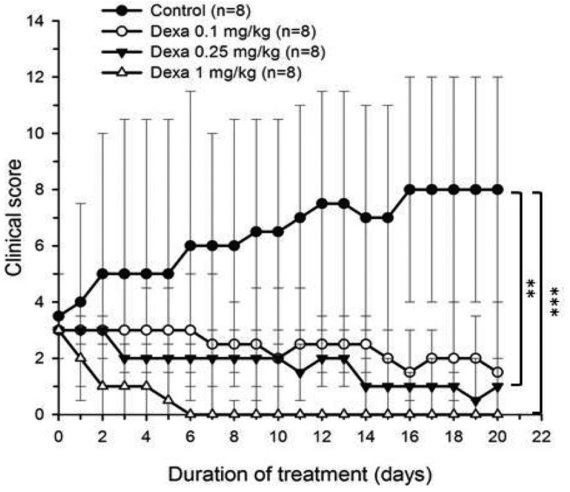
Cytometric bead assay

Cytokines in serum samples were analysed using cytometric bead assay (CBA) flexbeads (BD Biosciences, Pharmingen, San Diego, CA, USA). In mouse sera detection of TNF, IL-1α, IL-1β, IL6, KC, IFN-γ and MCP-1 was performed according to the manufacturer's instructions on a CYAN flow cytometer and analysed with FCAP Array software (BD v. 3.1).

TUNEL analysis in femur growth plate and articular cartilage

Apoptotic cell death by DNA fragmentation in chondrocytes was studied by terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labelling (TUNEL). Serial sections of femur (n=5 bones per group) were stained with a TUNEL kit (QIA33, TdT-FragEL, Calbiochem), according to the manufacturer's instructions. Images covering the whole growth plate as well as the articular cartilage were analysed under a fluorescence microscope (a Nikon E800 fitted with an Olympus DP70 camera). All TUNEL positive cells (Alexa-546, Invitrogen) were counted and divided by the total number of cells (DAPI stained) to achieve the percentage of TUNEL posi-

Supplementary Fig. 1. Clinical scores from the pre-study where animals where treated with three different doses of Dexa (0.1, 0.25 and 1 mg/kg) for 21 consecutive days.



tive cells. For the digital cell counting Image J software (NIH) was used.

PCNA staining of femur growth plate

Cell proliferation in the growth plate cartilage was assessed by analysing proliferating cell nuclear antigen (PCNA) expression in serial sections of femur growth plates (n=5 bones per group) as described previously (19). Briefly, after deparaffinisation and rehydration, antigen retrieval was performed in sodium citrate buffer (10 mM pH 6.0). The sections were blocked with 3% bovine serum albumin (BSA) before incubation overnight at 4°C with the primary rabbit anti-PCNA antibody (1:100, Abcam 18197). Sections were incubated for 1 hour at RT with secondary goat anti-rabbit IgG-HRP antibody (1:300, Santa Cruz sc-2004) followed by incubation with an avidin-peroxidase complex (Vectastain ABC-kit PK-6100) and visualised with 3,3' diaminobenzidine (DAB) (Dako K3468) development for 3 minutes. Thereafter sections were counterstained with Alcian blue, dehydrated and coverslipped. Images were captured by a Nikon Eclipse E800 light microscope connected to a digital camera (Hamamatsu C4742-95, Hamamatsu City, Japan) with a digital colour camera system (Olympus DP70). Using the Image J software, the number of PCNA positive cells was determined by one observer blinded to the treatment.

Cell cultures and analysis of apoptosis

The RCJ3.1C5.18 rat chondrocytic cell line was used and cultured as previously described (20). Briefly, after 4 days in culture when the cells had reached confluency, fresh MEM alpha with 50 µg/ml ascorbic acid and10nM β-glycerophosphate was added, before the cells were treated with Dexa (30µM) with/without HNG (10nM) for 72h. Apoptosis was determined by quantification of cytoplasmic histon-associated DNA fragments by photometric enzyme immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics) according to manufacturer's instructions.

Statistical analysis

Statistical analysis of the clinical score is based on area under the curve for each individual animal. The values are median clinical score and the error bars represent interquartile range. For the histological scoring, a mean value was calculated for each animal and values are presented as mean ± SD. For differences between several groups, parametric one-way analysis of variance (ANOVA) followed by Holm-Sidak test, or the non-parametric Kruskal-Wallis ANOVA on Ranks followed by Dunn's multiple comparison test was applied. TUNEL and PCNA quantification values are presented as mean±SD. Analyses were performed using the SigmaPlot software (Systat Software Inc., IL, USA) ***p<0.001, **p<0.01 and *p<0.05 were considered statistically significant.

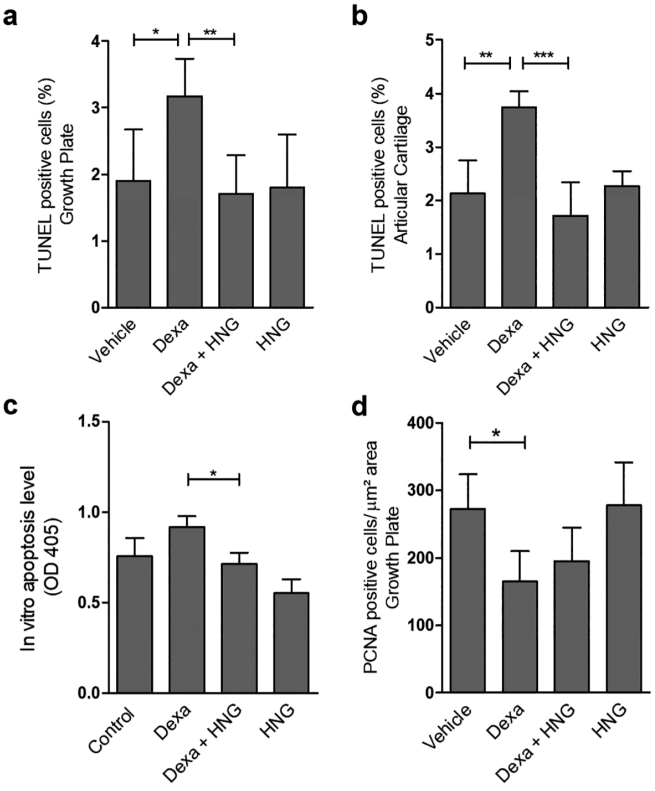


Fig. 2. Effect of HNG on GC-induced apoptosis and proliferation. TUNEL analysis detecting apoptotic chondrocytes in femur growth plate and articular cartilage from CIA mice. Quantification of TUNEL positive cells in growth plate (n=5) (**A**) and articular cartilage (n=5) (**B**). Quantification of apoptosis in RCJ3.18 chondrocytes treated with Dexa (30µM) with/without HNG (10nM) for 72h (n=3) (**C**). Quantification of PCNA positive area in the growth plate (n=5) (**D**). *p<0.05, **p<0.01, ***p<0.001.

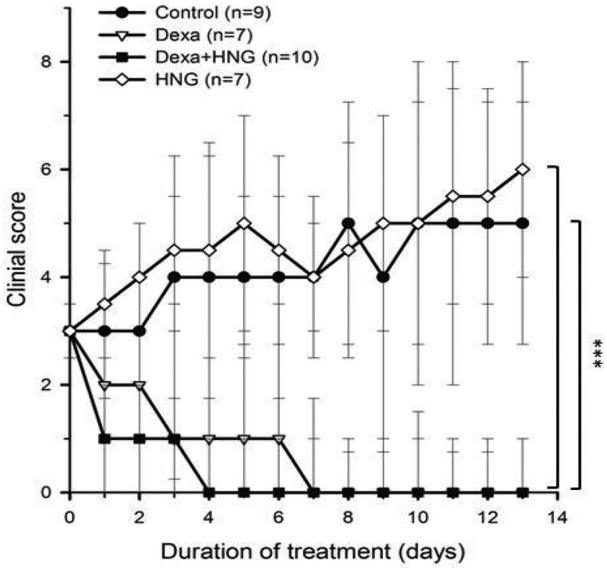


Fig. 3. HNG does not interfere with the desired anti-inflammatory effect of Dexa. DBA/1 mice induced with collagen type II arthritis were treated for 14 days with Dexa, with or without HNG, starting from when the animals reached a clinical score of 3. The development of arthritis was significantly attenuated with treatment with Dexa and Dexa in combination with HNG, compared with the vehicle treated animals. ***p<0.001.

Results

Humanin suppressed Dexa-induced chondrocyte apoptosis in arthritic mice

To study if HNG may rescue from Dexa-

induced chondrocyte apoptosis under inflammatory conditions, TUNEL staining was performed in sections of the femur from arthritic mice. In growth plate cartilage, increased chondrocyte apoptosis was detected in Dexa treated animals when compared to the vehicle group (Fig. 2A; $p<0.05$). Interestingly, humanin prevented this effect and less apoptotic cells were detected in animals treated with a combination of HNG and Dexa when compared to Dexa alone (Fig. 2A; $p<0.01$). Similarly, Dexa treatment increased chondrocyte apoptosis in articular cartilage (Fig. 2B; $p<0.01$), and when HNG was administered in combination with Dexa, this effect was completely abolished (Fig. 2B; $p<0.001$). The protective effect of HNG was also verified in cell cultures of RCJ3.1C5.18 chondrocytes, where Dexa-induced apoptosis could be rescued by co-treatment with HNG (Fig. 2C). PCNA staining of femur growth plates showed decreased chondrocyte proliferation in Dexa treated mice compared to vehicle (Fig. 2D; $p<0.05$) and HNG was not capable to significantly rescue from this effect (Fig. 2D).

Humanin did not interfere with the anti-inflammatory effect of Dexa

Daily treatment with Dexa for 14 consecutive days effectively reduced the clinical arthritis score when compared to the vehicle group (Fig. 3; $p<0.001$). When HNG was administered in combination with Dexa, the clinical arthritis score was effectively reduced (Fig. 3; $p<0.001$) in a similar way as in mice treated with Dexa alone. Treatment with HNG alone did not affect the clinical severity of arthritis when compared to the vehicle group. In accordance with the clinical scoring, histopathological analysis demonstrated increased cell infiltration and joint destruction in untreated animals compared to the Dexa treated group with significantly reduced histological scores (Fig. 4A-B; $p<0.05$). Treatment with HNG in combination with Dexa decreased the histological scores similarly as in animals treated with Dexa alone (Fig. 4A-B) while HNG alone had no effect on the scores. Serum levels of TNF, IL-1α, IL-1β, IL6, KC, IFN-γ and MCP-1 were analysed with cytometric bead array CBA, but levels were low or below detection range for all cytokines (data not shown).

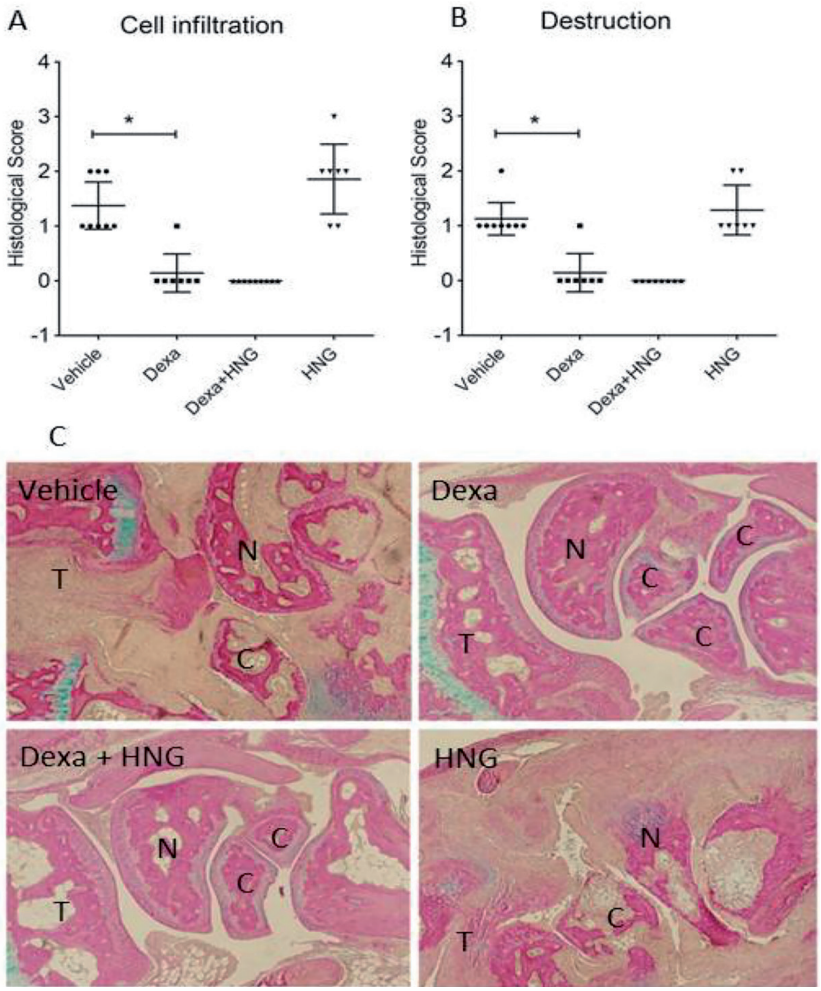


Fig. 4. Histological score. Cell infiltration (**A**) and joint destruction (**B**) was analysed in sections from paws (n=7-9). Representative micrographs (**C**) illustrating the tarsal area of the hind paws from one animal from each treatment group. Navicular bone (N), cuneiform bones (C), talus bone (T). *$p<0.05$

Discussion

Herein, we showed that systemic treatment with HNG, a potent analogue of the mitochondrial DNA encoded peptide humanin, can rescue from Dexa-induced chondrocyte apoptosis in a disease model of experimental arthritis. The chondrocyte rescuing effect was demonstrated in both articular and growth plate cartilage as well as in cell cultures *in vitro* suggesting that HNG can be used to prevent toxic effects of GCs in both these tissues. Moreover, and importantly our data demonstrated that HNG treatment did not interfere with the desired anti-inflammatory effect of Dexa.

Recently, HNG was reported to rescue from Dexa-induced apoptosis in growth plate chondrocytes in treated mice as well as in cultures of human growth plate chondrocytes (16). Furthermore, HNG was shown to prevent apoptosis in other pathological conditions including neurodegeneration and cardiac injury (21-23). Our present data demonstrated that HNG is capable of rescuing chondrocytes from Dexa-induced apoptosis also in a disease model of chronic inflammation. It is important to emphasise that we did this observation not only in growth plate chondrocytes but also in articular cartilage, which widens the potential application of our findings. We think these data may open for the clinical development of a novel strategy combining a humanin analogue with GCs when treating conditions of chronic inflammation.

We found that Dexa treatment not only effectively suppressed inflammation in CIA but also caused growth plate and articular chondrocytes to undergo undesired apoptosis. This finding is of particular interest as it has not been reported if GCs given to suppress inflammation also may trigger undesired toxic effects in bone and cartilage. Our results clearly indicated that GC treatment enhanced chondrocyte apoptosis although systemic inflammation was suppressed. nflammation is known to trigger undesired apoptosis, which may cause bone growth impairment and articular cartilage degradation (6). As GCs are known to suppress inflammation, one could therefore hypothesise that GC treatment would also suppress the apoptotic activity. However, our data showed the opposite suggesting that GC-induced chondrocyte apoptosis is independent from the immunosuppressive effect.

Concomitantly, we observed that chondrocyte proliferation was suppressed by Dexa treatment when studied in the femur growth plate cartilage of arthritic mice. These results confirm previous reports where Dexa treatment was found to suppress chondrocyte proliferation in normal mice (16, 24). GC-induced suppression of chondrocyte proliferation has earlier been linked to impaired bone growth (25). When combining HNG and Dexa treatment, we found chondrocyte proliferation to still be decreased although the effect was less pronounced than in arthritic animals given Dexa alone. HNG alone did not affect chondrocyte proliferation in this experimental model of chronic inflammation. Our present data confirmed recently published data in normal mice where HNG was reported to partially rescue growth plate chondrocytes from Dexa-induced suppression of cell proliferation (16).

We here present *in vivo* data obtained from an experimental disease model of arthritis showing that HNG does not interfere with the desired anti-inflammatory effect of Dexa, an effect previously only reported in cultured cells and LPS-challenged normal mice (16). Studies of both inflammatory cell infiltration and joint destruction confirmed that HNG does not interfere with the beneficial effects of Dexa treatment in the arthritis model. These data suggest that the combination of HNG and Dexa may offer a novel approach to rescue bone growth when treating conditions of chronic inflammation.

Previous studies have demonstrated that HNG has an anti-inflammatory effect by suppressing TNF-α and IL-6 levels in normal mice challenged to LPS (16, 26). In contrast, no anti-inflammatory effect of HNG could be confirmed in the CIA model used in this study. This may be due to several reasons. Firstly, the penetrance of HNG into the joint is unknown, emphasising the importance of performing pharmacokinetic studies and dose titrations to clarify the anti-inflammatory effect of HNG in conditions of chronic inflammation. Secondly, in CIA, the mice develop a severe form of arthritis with erythema and swelling of the joints and it is possible that the HNG concentration used in this study was not sufficient to have an anti-inflammatory effect on its own. The experimental CIA model used is the golden standard mouse model when studying arthritis (17). However, as the animals were immunised at an age of 8 weeks, meaning it was not possible to study effects on longitudinal bone growth as their growth already had reached a plateau at time of disease onset. Due to this limitation of the model, our aim was restricted to clarifying whether HNG could be used in combination with Dexa without interfering with the desired anti-inflammatory effect.

Conclusion

In conclusion, the present study showed that systemic treatment with the humanin analogue HNG rescued growth plate and articular chondrocytes from Dexa-induced cell death in an experimental mouse model of arthritis. Importantly, HNG did not interfere with the desired anti-inflammatory effect of Dexa suggesting that exogenous HNG in combination with GCs could represent a new treatment strategy to prevent toxic effects of GCs in the growth plate and articular cartilage. Further studies are needed to clarify the anti-inflammatory and growth rescuing effects of HNG in conditions of chronic inflammation.

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