Biochemical and Biophysical Research Communications 441 (2013) 249–255

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Lactoferrin inhibits dexamethasone-induced chondrocyte impairment from osteoarthritic cartilage through up-regulation of extracellular signal-regulated kinase 1/2 and suppression of FASL, FAS, and Caspase 3

Yihui Tu [a,1], Huaming Xue [a,b,1], Wendy Francis [b], Andrew P. Davies [c], Ian Pallister [b], Venkateswarlu Kanamarlapudi [b], Zhidao Xia [b,*]

[a] Department of Orthopaedics, Yangpu District Central Hospital Affiliated to Tongji University School of Medicine, 450 Tengyue Road, Shanghai, China
[b] Institute of Life Science, College of Medicine, Swansea University, Singleton Park, UK
[c] Department of Orthopaedics and Trauma, Moriston Hospital, Swansea, UK

ARTICLE INFO

Article history:
Received 4 October 2013
Available online 17 October 2013

Keywords:
Lactoferrin
Dexamethasone
Osteoarthritis
Chondrocyte
Apoptosis

ABSTRACT

Dexamethasone (Dex) is commonly used for osteoarthritis (OA) with excellent anti-inflammatory and analgesic effect. However, Dex also has many side effects following repeated use over prolonged periods mainly through increasing apoptosis and inhibiting proliferation. Lactoferrin (LF) exerts significantly anabolic effect on many cells and little is known about its effect on OA chondrocytes. Therefore, the aim of this study is to investigate whether LF can inhibit Dex-induced OA chondrocytes apoptosis and explore its possible molecular mechanism involved in. MTT assay was used to determine the optimal concentration of Dex and recombinant human LF (rhLF) on chondrocytes at different time and dose points. Chondrocytes were then stimulated with Dex in the absence or presence of optimal concentration of rhLF. Cell proliferation and viability were evaluated using MTT and LIVE/DEAD assay, respectively. Cell apoptosis was evaluated by multi-parameter apoptosis assay kit using both confocal microscopy and flow cytometry, respectively. The expression of extracellular signal-regulated kinase (ERK), FAS, FASL, and Caspase-3 (CASP3) at the mRNA and protein levels were examined by real-time polymerase chain reaction (PCR) and immunocytochemistry, respectively. The optimal concentration of Dex (25 μg/ml) and rhLF (200 μg/ml) were chosen for the following experiments. rhLF significantly reversed the detrimental effect of Dex on chondrocytes proliferation, viability, and apoptosis. In addition, rhLF significantly prevented Dex-induced down-regulation of ERK and up-regulation of FAS, FASL, and CASP3. These findings demonstrated that rhLF acts as an anabolic effect on chondrocytes through significantly reversing Dex-induced chondrocytes apoptosis. This study may contribute to further investigating the clinical application of LF on OA.

1. Introduction

Osteoarthritis (OA) is an age-related idiopathic disease and has been one of the most common causes of disability in older adults, especially in the population aged over 65 years. Loss of articular cartilage is the major cause of joint dysfunction and disability where chondrocyte apoptosis and proteoglycan depletion are anatomically presented [1]. It is characterized by damage to the extracellular matrix and articular cartilage. In addition, several pro-inflammatory cytokines, such as nitric oxide, tumor necrosis factor, interleukin-1β, and prostaglandin E2 are excessively released. These have been reported to be correlated with the severity of OA and are involved in the progression of the disorder. Inhibition of inflammatory cytokines is especially important to prevent progression of cartilage degeneration at an early stage of OA. Early treatments for OA include oral anti-inflammatory, intra-articular injection of corticosteroids [2,3] or hyaluronic acid [4,5], however, these treatments are mainly for pain relief rather enhancing cartilage regeneration.

Corticosteroids such as glucocorticoids (GCs) are frequently used as powerful anti-inflammatory drugs with analgesic effects in OA, rheumatoid arthritis, and juvenile arthritis. Dex is a widely used GC in clinical treatment, however, at high doses or following repeated use over prolonged periods of time, side effects emerged. It is worthwhile to point out that corticosteroids induce apoptosis in many cell types [6–8]. GC treatment resulted in reduced cell proliferation and viability and induced apoptosis of lymphocytes,

* Corresponding author. Address: Centre for Nanohealth, Institute of Life Science2, College of Medicine, Swansea University, Singleton Park, SA2 8PP, UK.
E-mail address: zhidao.xia@gmail.com (Z. Xia).
[1] These authors contributed equally to the work and be considered as co-first authors.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.047

Table 1
Sequence of primers for the quantitative Real-time PCR experiments.

Gene	Sense	Sequence 5′→3′	Size (bp)
ERK1/2	Forward	ACACATGCTTTGGGTCCTTC	883
	Reverse	AGGAACAGCTCACAGCCCTA	
FASL	Forward	CAGCTCTTCCACCTGCAGAAGG	185
	Reverse	AGATTCCTCAAAATTGATCAGAGAGAG	
FAS	Forward	ATCCGAGCTCTGAGGAGGCGGGTTCATGAAA	205
	Reverse	GGAGGTTCTAGATTCAGGGTCATCCTG	
Caspase 3	Forward	CATGGCCTGTCAGAAAATAC	176
	Reverse	TAACCCGAGTAAGAATGTGC	
GAPDH	Forward	CATCCTGCGTCTGGACCT	480
	Reverse	CATCCTGCGTCTGGACCT	

eosinophils and bone, and cartilage on the growth plate [9–12]. Similarly, *in vivo* and *in vitro* studies have shown GCs increase apoptosis and inhibit proliferation of resting or proliferative chondrocytes in articular cartilage [11]. Chondrocyte apoptosis plays an important role in interfering with cartilage metabolism and may lead to rapid knee arthropathy. Chondrocyte death will also result in poor cartilage matrix turnover because chondrocytes are the only source of matrix synthesis in articular cartilage [13]. Treatments, which can reverse the side effects of corticosteroid therapy, will be invaluable for clinical application.

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein that belongs to the transferrin family. It is a multifunctional factor with significant antimicrobial, anti-oxidation, antitumor, and immunomodulatory activities. Recently, LF has been reported to be an anabolic reagent to osteoblasts [14,15], neurons [16,17], cartilage [15], and fibroblasts [18]. It acts as an anabolic reagent mainly through its powerful proliferative and anti-apoptotic actions, which exceed those of other established anabolic factors including transforming growth factor (TGF)-β, parathyroid hormone (PTH), amylin, and insulin [15,19].

The effect of LF on chondrocyte proliferation and differentiation has been little documented. LF is believed to promote chondrocyte proliferation through the LRP1 and ERK pathways and cartilage differentiation through Sox9 and SMAD 2/2 [20]. It has also been shown that LF up-regulates MAPK, MMP1, and MMP3, and inhibits Aggrecan expression [21].

To date, however, LF has not been tested in OA and little is known about its effect on cartilage and chondrocytes in OA. It was hypothesized that LF as an anabolic reagent has the potential to not only promote proliferation but also reverse apoptosis of HACs. This hypothesis was assessed by studying the effect of LF on chondrocyte apoptosis and proliferation following Dex-induced apoptosis *in vitro*. HACs isolated from OA patients were used for this study. The results show that LF significantly inhibits Dex-induced chondrocyte apoptosis and promotes proliferation and viability of chondrocytes from OA cartilage, and that the effects are regulated by sustained activation of ERK-1/2 and down-regulation of FASL, FAS, and Caspase-3 (CASP3).

2. Materials and methods

2.1. Reagents

Dexamethasone (Dex) and recombinant human lactoferrin (rhLF) were purchased from Sigma–Aldrich (Gillingham, UK). Dex (500 μg/ml) was dissolved in ethanol and rhLF powder (1 mg/ml) was dissolved in phosphate-buffered saline (PBS). Primary antibodies including total ERK-1/2, phosphorylated-ERK1/2 (p-ERK1/2), FASL, FAS, Caspase-3 (CASP3), cleaved-Caspase-3 (cleaved-CASP3), and Northern Lights™ secondary antibody were purchased from R&D Systems Ltd (Abingdon, UK).

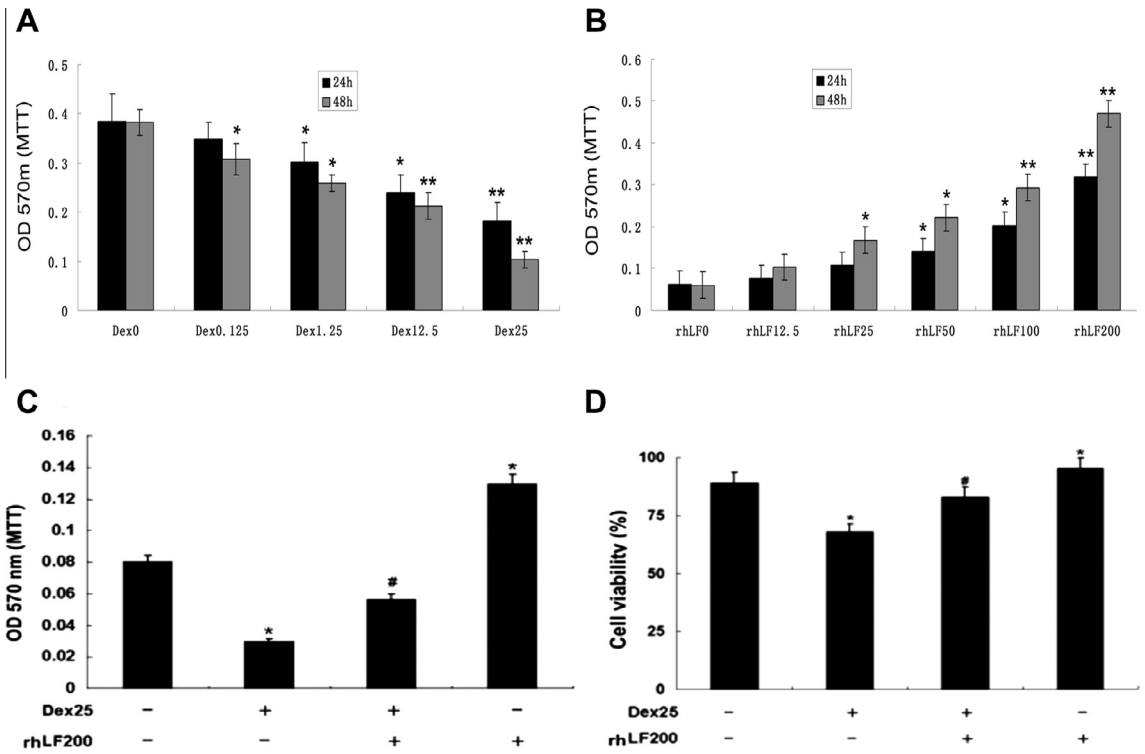

Fig. 1. Effect of Dex and rhLF on HACs activity. The optimal concentration of Dex (A) and rhLF (B) was selected for subsequent experiments as described in the Section 2. (C) Cell proliferation was quantified by MTT assay, and (D) Chondrocytes viability was measured by LIVE/DEAD staining in chondrocytes cultured with Dex alone (25 g/ml) and/or rhLF (200 g/ml). The results represent the mean ± SD of three independent experiments, each yielding similar results ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group, $^{\#}P < 0.05$ compared to Dex alone group).

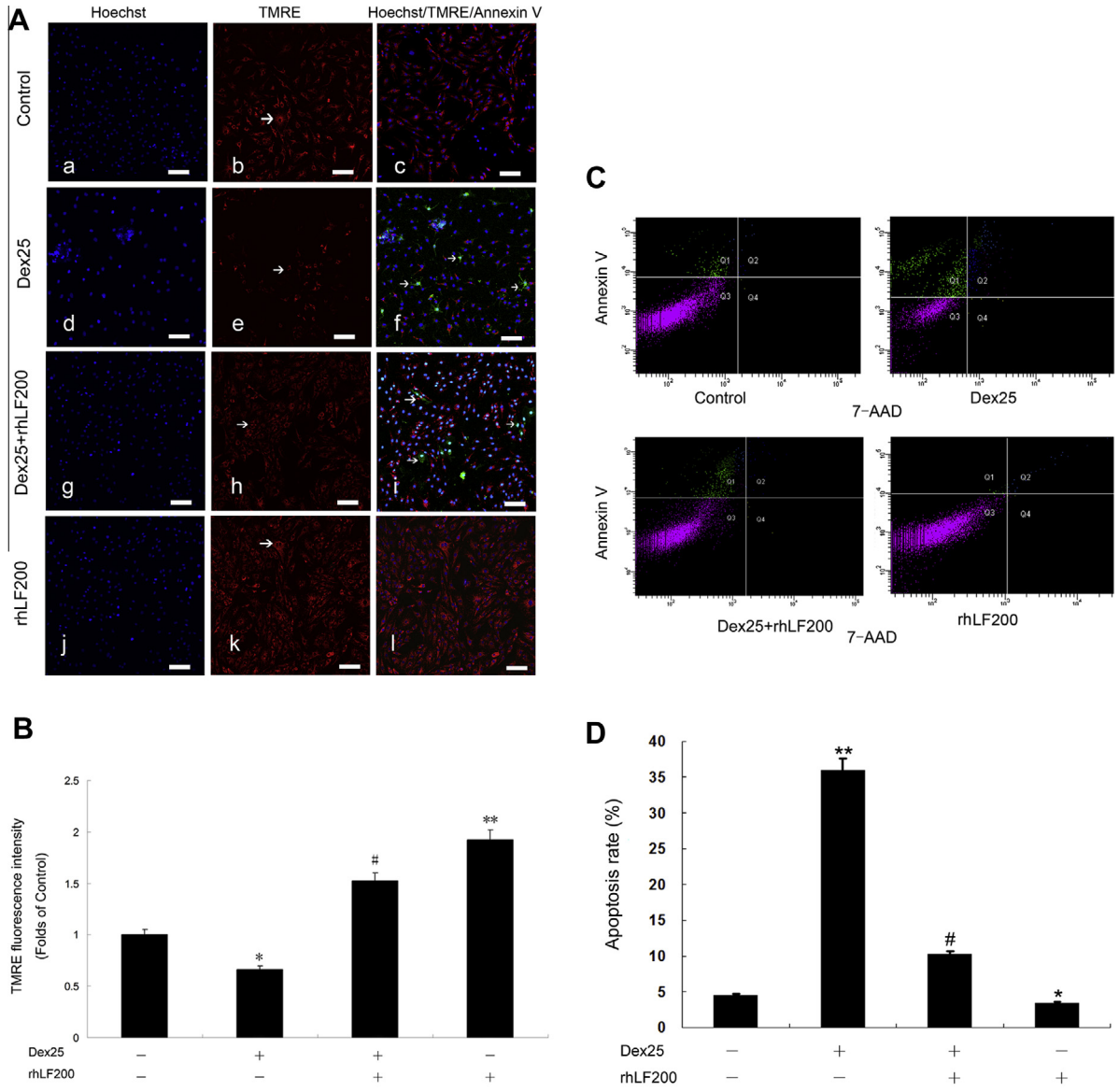

Fig. 2. Effect of rhLF on Dex-induced HACs apoptosis. (A) HACs were treated with Dex alone with or without rhLF for 48 h and the apoptosis was detected with triple staining with multi-parameter apoptosis assay as described in the Section 2. (B) The fluorescence intensity of TMRE was quantified by INCELL 2000 software. (C) The apoptosis was analyzed by flow cytometry, and (D) apoptotic rate of HACs was quantified. The results represent the mean ± SD of three independent experiments, each yielding similar results ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group, $^{\#}P < 0.05$ compared to Dex25 group). (Magnification: 20×). Scale bar = 25 μm (a–l).

2.2. Cell culture

Following full ethical approval from the local research ethics committee (LREC), Eight OA cartilage specimens were collected from patients (61 ± 4 years) undergoing total knee joint replacement surgery at Morriston Hospital, Swansea. HACs were isolated from undamaged femoral condyle and intercondylar fossa of the knee joint as described previously [22]. Human OA articular cartilage was maintained in DMEM/F12 with 10% FBS. Cell viability was assessed by trypan blue dye exclusion. Chondrocytes between passages 1 and 3 were used for analysis. Chondrocytes characterization was performed by morphology, Alcian blue staining and reverse transcriptase-PCR to check the gene expression of collagen II.

2.3. Optimizing dosage of rhLF and Dex

HACs were cultured in either DMEM/F12 with 10% FBS containing Dex at 0, 0.125, 1.25, 12.5, and 25 μg/ml; or DMEM/F12 with 1% FBS containing rhLF at 0, 12.5, 25, 50, 100, and 200 μg/ml for 24 and 48 h. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was then used to select the optimal concentration of Dex and rhLF for treating HACs. The optical absorbance of this colored solution was quantified at 570 nm by a microplate reader (BMG LABTECH, Bucks, UK). The optimal concentration of Dex (25 μg/ml; Dex25) and rhLF (200 μg/ml; rhLF200) and DEMF12 supplemented with 1% FBS were chosen for the following experiments.

2.4. Cell proliferation and viability essays

HACs were treated with Dex25 and rhLF200 in DMEMF12 supplemented with 1% FBS. After 48 h of treatment, cell proliferation was assessed by MTT assay, the OD values correspond to the number of viable cells. In addition, cell viability was assessed by LIVE/DEAD assay. Cells were stained using a LIVE/DEAD stain kit (Life Technologies, Paisley, UK). This kit contains two fluorescent dyes, calcein-AM to stain living cells green and ethidium homodimer-1

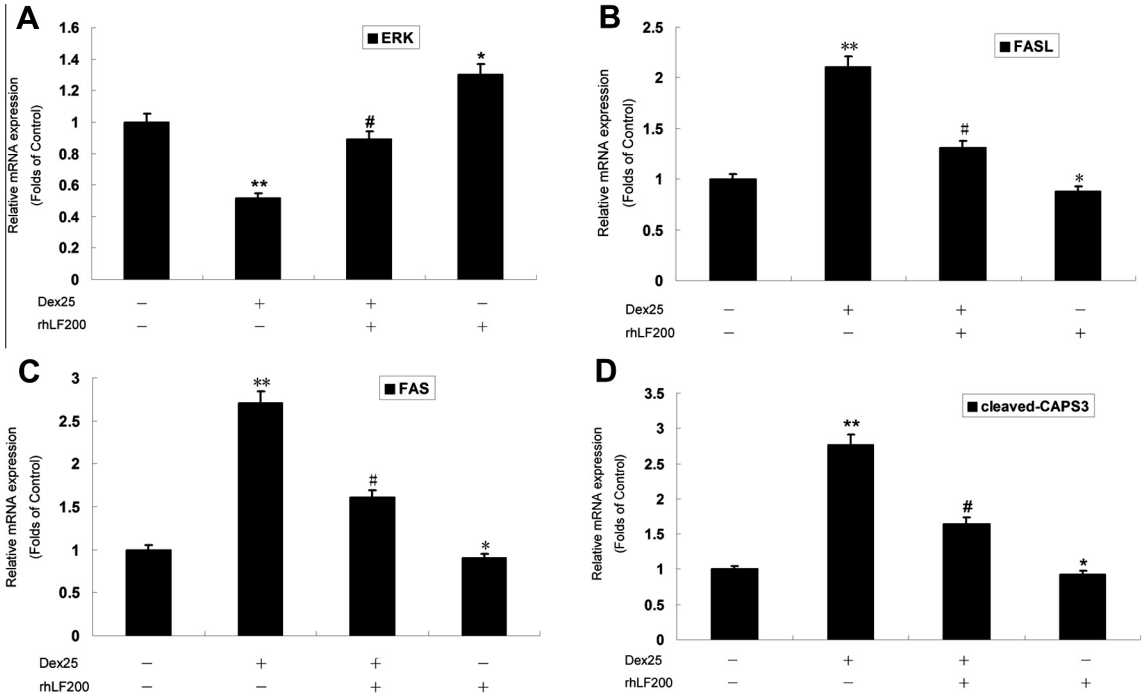

Fig. 3. The effect of rhLF on Dex-induced HAC ERK (A), FASL (B), FAS (C), and CAPS 3 (D) gene expressions. qPCR was performed to detect mRNA levels of target genes. Results are presented as relative changes normalized to GAPDH. The results represent the mean ± SD of three independent experiments, each yielding similar results ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group, $^{\#}P < 0.05$ compared to Dex25 group).

(Ethd-1) to stain damaged or dead cells red. After staining, samples were observed by an epifluorescent microscope (Carl Zeiss, Oberkochen, Germany). For each well, at least five different random fields were examined, and a minimum of 1000 cells were counted to determine the fraction of calcein-AM positive cells vs. Ethd-1 positive cells.

2.5. Multi-parameter apoptosis assay

Cells were cultured in 96-well black plates at 5×10^3 cells/well and treated with Dex25 and/or rhLF200 for 48 h. HACs apoptosis was assessed by the multi-parameter apoptosis assay kit (Bioscience, Cambridge, UK) according to the manufacturer's instruction. This kit employs FITC-conjugated Annexin V as a probe for phosphatidylserine on the outer membrane of apoptotic cells, TMRE as a probe for mitochondrial membrane potential, and Hoechst dye to demonstrate nuclear morphology. The staining was performed and resultant fluorescent stained cells were observed by confocal scanning microscopy (Carl Zeiss, UK).

2.6. Flow cytometry analysis

The apoptotic rate was evaluated using Annexin V-FITC apoptosis detection kit (Bioscience, UK). Briefly, the chondrocytes suspension were mixed with Annexin V-FITC and 7-AAD and incubated for 15 min at room temperature. Samples were analyzed by a BD flow cytometry machine (Becton Dickinson, UK) to identify apoptotic (Annexin V-positive) and necrotic cells (7-AAD-positive).

2.7. Content of mitochondria membrane potential

The lysate of chondrocytes was centrifuged at 12,000 rpm for 10 min at 4 °C, then resuspended and incubated with 50 nM of TMRE (Bioscience, UK) for 20 min at 37 °C. The mixture was stained at room temperature in the dark for 30 min and cells were evaluated by confocal microscopy (excitation wavelength 488 nm, emission wavelength 510 nm). Fluorescence intensity was quantified with the INCELL 2000 software.

2.8. RNA isolation and quantitative real-time RT-PCR (qPCR)

Total cellular RNA was extracted using AllPrep Mini Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instruction. 1 μg RNA was converted to cDNA in 20 μl reactions using high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). For PCR, 2 μl of cDNA was mixed with SYBR green mastermix and relevant primers, and PCR was performed using an iCycler (Bio-Rad, Hertfordshire, UK). The real-time PCR conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 0.15 min, 58 °C for 1 min, and 72 °C for 1 min. The primers used were shown in Table 1. To compare mRNA expression levels, the relative expression of each gene was normalized to GAPDH.

2.9. Immunocytochemical staining and confocal microscope

Cells were seeded into 96 well black plates and treated with Dex25 or rhLF200 or both for 48 h. After being washed twice in cold PBS, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized by washing three times with 0.1% (v/v) Triton X-100, and immunostained with the antibodies overnight at 4 °C. The cells were washed three times with PBS and incubated with a Northern Lights™ antibody (R&D, Abingdon, UK) in the dark at room temperature for 1 h. Cells were washed twice with PBS and incubated with DAPI (Life Technologies, Paisley, UK) at a dilution of 1:1000 of 1 mg/ml for 1 min. The images were visualized and imaged by a laser confocal scanning microscope.

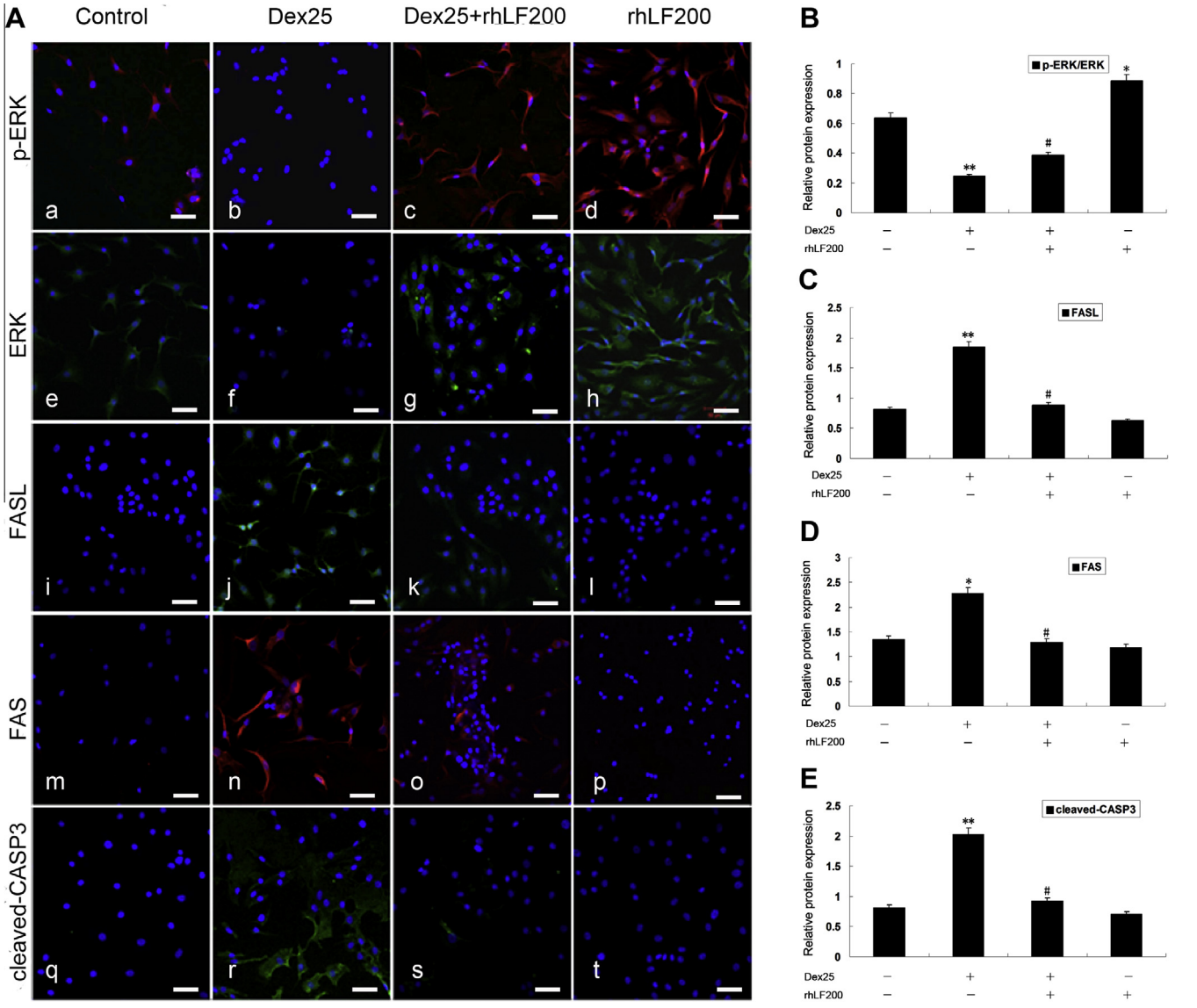

Fig. 4. Effect of hLF on the gene expressions of p-ERK, ERK, FASL, FAS, and cleaved-Caspase 3 (cleaved-Casp3) in Dex induced HACs apoptosis. (A) Micrographs of immunocytochemistry and confocal microscopy illustrate the effect of rhLF on Dex-induced protein expressions. (B–E) The relative protein expression of p-ERK, ERK, FASL, FAS, and cleaved-Caspase 3 were quantified by INCELL 2000 software. The results represent the mean ± SD of three independent experiments, each yielding similar results ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group, $^{\#}P < 0.05$ compared to Dex25 group). (Magnification:40×). Scale bar = 50 μm (a–t).

2.10. Statistics

SPSS 11.5 software was applied for data analysis. Data were presented as the means ± SD of three separate experiments. Significant differences among the mean values of multiple groups were evaluated by one-way analysis of variance (ANOVA) and Student–Newman–Keuls *q*-test. A value of $P < 0.05$ was considered as being significant.

3. Results

3.1. The optimal concentration of Dex and rhLF

We performed MTT assay to select the optimal concentrations of Dex and rhLF fro subsequent experiment. As shown in Fig. 1A, Dex suppressed HAC proliferation in DMEM/F12 with 10% FBS in a dose dependent manner (24 h, $P < 0.01$; 48 h, $P < 0.0001$) and the inhibitory effect in 48 h group was more pronounced than that in 24 h group at each concentration. The effects of Dex were most pronounced at the dose of 25 μg/ml. By contrast, Fig. 1B shows that the rhLF stimulated HACs proliferation in DMEM/F12 with 1% FBS in a dose dependent manner (24 h, $P < 0.05$; 48 h, $P < 0.0001$) and the proliferative effect was more pronounced in 48 h group at each concentration, and the effects were most pronounced at the dose of 200 μg/ml. Based on these tests, Dex at 25 μg/ml and rhLF at 200 μg/ml were selected as the optimal concentrations for further experiments.

3.2. Effect of rhLF on proliferation and viability of HACs

In order to explore the effect of rhLF on HAC proliferation and viability, the effect of Dex25 or rhLF200 or both was assessed using both cell proliferation and viability assays. As expected, Dex25 significantly inhibited HAC proliferation and viability, whereas rhLF200 significantly reversed the inhibitory effect of Dex25 on HACs proliferation and viability (Fig. 1C,D). The viability of cells treated with both Dex25 and rhLF200 was increased to 83% in comparison to 68% (Fig. 1D, $P < 0.01$) in Dex25 alone treated cells. These data indicated that rhLF200 can actually reverse the inhibitory affects of Dex on proliferation and viability of HACs.

3.3. Effect of hLF on Dex-induced HACs apoptosis

To further study the effect of rhLF on HAC apoptosis induced by Dex, a multi-parameter apoptosis assay was used to visualize and quantify HAC apoptosis (Fig. 2A,B). For confocal microscopy analysis, cell nuclei were stained blue by Hoechst (Fig. 2A-a, -d, -g, and -j) and apoptotic cells were stained as Annexin V-positive cells (green)[2], as indicated by arrows in Fig. 2A-f, and -i. Dex25 treatment resulted in highest Annexin V (Fig. 2A-f) staining. This effect was partially rescued by rhLF200 treatment (Fig. 2A-i and -l).

These effects were further confirmed by flow cytometric analysis (Fig. 2B). The ratio of apoptotic HACs was dropped from 36% in Dex25 treatment to 10% in Dex25 and rhLF200 treatment (Fig. 2D, $P < 0.01$). Using highly fluorescent cationic lipophilic dye TMRE, which can reflect the change of mitochondria membrane potential (indicated by white arrows in Fig. 2A-b, -e, -h and –k), we could show that the mitochondrial membrane potential was significantly impaired by Dex and restored significantly by rhLF200 (Fig. 2C, $P < 0.01$).

3.4. Effect of rhLF on mRNA and protein expression levels of ERK, FAS, FASL, and CASP3

To understand the potential mechanism of rhLF in reversing Dex affects on HAC proliferation and apoptosis, cell proliferative, (ERK, Fig. 3A) and apoptotic (FAS, FASL, and CAPS3, Fig. 3B, C, and D, respectively) signals were assessed using real-time PCR. The results showed that rhLF prevented Dex25-induced down-regulation of total ERK1/2 (Fig. 3A) in HACs, and significantly reduced apoptotic gene expression of FAS, FASL, and CAPS3, shown in Fig. 3B, C, and D.

We then sought to examine LF regulation of proliferative (ERK and p-ERK) and apoptotic gene (FASL, FAS, and Cleaved-CAPS3) expression by immunocytochemistry in protein level. As HACs were cultured in 1% FBS, Dex25 completely inhibited both p-ERK (Fig. 4A–b,4B) and ERK (Fig. 4B–f, 4B) expressions and up-regulated expressions of FASL (Fig. 4A-j, 4C), FAS (Fig. 4A-n, 4D) and Cleaved-CAPS3 (Fig. 4A-r, 4E), but these effects were partially reversed by rhLF200 (Fig. 4A-k, -o, and -s, 4B–E).

4. Discussion

Reduced proliferation of cartilage progenitor cells and loss of chondrocytes through apoptosis has not only been an important sign of pathological processes of OA [1], but also a measure of the side effects caused by high dose steroids designed to treat this condition [7]. Anabolic reagents, which can enhance proliferation and prevent HAC apoptosis are highly desired for OA treatment, however, to date there is no approved treatment on the pharmaceutical market with dual effects on HACs.

LF is currently used mainly as an anti-infectious or anti-inflammatory reagent. Its anabolic effects on human skeletal tissue were not identified until 2004 by Cornish and her colleagues [14,15,23]. This current study proves the use of LF as an anabolic reagent for HACs, in which LF not only enhances HAC proliferation, but also inhibits HAC apoptosis induced by Dex.

HACs isolated from OA donors were used in this study. A clinical glucocorticoid Dex, widely used to induce experimental cellular apoptotic response, was chosen to induce HAC apoptosis. This study revealed that Dex significantly suppressed HAC proliferation and induced HAC death, an effect that was more pronounced when culture medium was switched from supplementary 10% to 1% FBS. In the elderly population, not only is the proliferation stage reduced but also cellular responses to serum growth factors are decreased [24]. Therefore the *in vitro* model used in this study is highly reliable and relevant to clinical conditions.

Dex at a concentration of 25 μg/ml resulted in the highest cell death of HACs and was therefore chosen as the optimal dose for this study. This dose is consistent with the reports on Dex-induced cell death in both cartilage and other cell types [10]. The most reported concentration of LF used as an anabolic reagent was 100 μg/ml, which significantly increased cell growth and inhibition of apoptosis of osteoblasts [15,25]. Bovine LF was used in most of these reports. The rhLF used in this study to test the anabolic effect was extracted from recombinant rice, which is not as potent as bovine LF. In our pilot study, it required 200 μg/ml rhLF to match the effect of 100 μg/ml bovine LF (data not shown).

The effect of rhLF on Dex-induced suppression of proliferation and induction of apoptosis of HACs is very consistent throughout this study. LF enhanced HAC proliferation and viability, and inhibited HACs death. These results are demonstrated by MTT, cell viability assay, and FACS analysis and confirmed by qPCR expression of cell proliferative (ERK) and apoptotic (FAS, FASL, and Caspase 3) signaling. The qPCR results are further validated by immunocytochemistry.

ERK signaling molecule promotes cell proliferation by transmitting a variety of extracellular signals [26]. It has been shown previously that Dex suppresses cell proliferation by inhibiting the phosphorylation of ERK [27] whereas LF induces cell proliferation by increasing the phosphorylation of ERK [28]. In our study, Dex and LF not only affected HAC proliferation but also altered the expression of ERK, indicating that these two compounds regulate cell proliferation via altering the ERK signaling cascade. Furthermore, rhLF reversed Dex-induced suppression of chondrocyte proliferation by increasing the expression p-ERK1/2. It is not yet clear how LF reverses Dex-induced inhibition of expression p-ERK.

FAS-mediated death receptor pathway plays a crucial role in Dex-induced cell apoptosis, therefore, we examined whether rhLF prevented Dex-induced chondrocyte apoptosis by modulating FAS-mediated signal molecule expression. Real-time PCR analysis showed that Dex significantly induced mRNA expression of apoptotic genes (FASL and FAS) and rhLF prevented the expression of these factors in HACs. Caspase plays an essential role in most types of apoptosis, including Dex-induced apoptosis. We found that Caspase 3 was activated in Dex-induced apoptosis and that rhLF prevented the activation of this executive Caspase. These results were confirmed and extended at the protein level by immunocytochemistry.

Our study demonstrated that LF is an effective anabolic reagent for HACs through enhancing HAC proliferation and inhibiting HAC apoptosis induced by Dex. The further molecular mechanism needs to be investigated. Anyway, this study will be useful for evaluating the potential of LF for clinical application on OA.

Acknowledgments

This work was supported by funding from National Nature Science Fund (81301566), Shanghai Municipal Science and Technology Commission (134119b1400), Shanghai Municipal Public Health and Family Planning Commission (2013040), and BBSRC UK (to KV), MRC UK (to KV).

References

[1] S. Hashimoto, R.L. Ochs, S. Komiya, M. Lotz, Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis, Arthritis Rheum. 41 (1998) 1632–1638.

[2] C.T. Hepper, J.J. Halvorson, S.T. Duncan, A.J. Gregory, W.R. Dunn, K.P. Spindler, The efficacy and duration of intra-articular corticosteroid injection for knee

[2] For interpretation of color in Fig. 2 , the reader is referred to the web version of this article.

osteoarthritis: a systematic review of level I studies, J. Am. Acad. Orthop. Surg. 17 (2009) 638–646.
[3] G. Habib, A. Safia, The effect of intra-articular injection of betamethasone acetate/betamethasone sodium phosphate on blood glucose levels in controlled diabetic patients with symptomatic osteoarthritis of the knee, Clin. Rheumatol. 28 (2009) 85–87.
[4] V. Strand, H.S. Baraf, P.T. Lavin, S. Lim, H. Hosokawa, A multicenter, randomized controlled trial comparing a single intra-articular injection of Gel-200, a new cross-linked formulation of hyaluronic acid, to phosphate buffered saline for treatment of osteoarthritis of the knee, Osteoarthritis Cartilage 20 (2012) 350–356.
[5] R.R. Bannuru, N.S. Natov, U.R. Dasi, C.H. Schmid, T.E. McAlindon, Therapeutic trajectory following intra-articular hyaluronic acid injection in knee osteoarthritis–meta-analysis, Osteoarthritis Cartilage 19 (2011) 611–619.
[6] G. Silvestrini, P. Ballanti, F.R. Patacchioli, P. Mocetti, R. Di Grezia, B.M. Wedard, L. Angelucci, E. Bonucci, Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone, Bone 26 (2000) 33–42.
[7] F. Nakazawa, H. Matsuno, K. Yudoh, Y. Watanabe, R. Katayama, T. Kimura, Corticosteroid treatment induces chondrocyte apoptosis in an experimental arthritis model and in chondrocyte cultures, Clin. Exp. Rheumatol. 20 (2002) 773–781.
[8] T. Mushtaq, C. Farquharson, E. Seawright, S.F. Ahmed, Glucocorticoid effects on chondrogenesis, differentiation, and apoptosis in the murine ATDC5 chondrocyte cell line, J. Endocrinol. 175 (2002) 705–713.
[9] T.V. Panfilova, A.A. Shtil, E.R. Polosukhina, A.Y. Baryshnikov, B.A. Frolov, Effect of the triterpenoid miliacin on the sensitivity of lymphocytes in the thymus and spleen to dexamethasone-induced apoptosis, Bull. Exp. Biol. Med. 136 (2003) 336–339.
[10] M.A. Hossain, J. Park, S.H. Choi, G. Kim, Dexamethasone induces apoptosis in proliferative canine tendon cells and chondrocytes, Vet. Comp. Orthop. Traumatol. 21 (2008) 337–342.
[11] D. Hong, H.X. Chen, H.Q. Yu, C. Wang, H.T. Deng, Q.Q. Lian, R.S. Ge, Quantitative proteomic analysis of dexamethasone-induced effects on osteoblast differentiation, proliferation, and apoptosis in MC3T3-E1 cells using SILAC, Osteoporos Int. 22 (2011) 2175–2186.
[12] K.L. Sivertson, M.C. Seeds, D.L. Long, K.K. Peachman, D.A. Bass, The differential effect of dexamethasone on granulocyte apoptosis involves stabilization of Mcl-1L in neutrophils but not in eosinophils, Cell Immunol. 246 (2007) 34–45.
[13] T. Aigner, M. Hemmel, D. Neureiter, P.M. Gebhard, G. Zeiler, T. Kirchner, L. McKenna, Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage, Arthritis Rheum. 44 (2001) 1304–1312.
[14] J. Cornish, Lactoferrin promotes bone growth, Biometals 17 (2004) 331–335.
[15] J. Cornish, K.E. Callon, D. Naot, K.P. Palmano, T. Banovic, U. Bava, M. Watson, J.M. Lin, P.C. Tong, Q. Chen, V.A. Chan, H.E. Reid, N. Fazzalari, H.M. Baker, E.N. Baker, N.W. Haggarty, A.B. Grey, I.R. Reid, Lactoferrin is a potent regulator of bone cell activity and increases bone formation *in vivo*, Endocrinology 145 (2004) 4366–4374.
[16] C. Fillebeen, M.M. Ruchoux, V. Mitchell, S. Vincent, M. Benaissa, A. Pierce, Lactoferrin is synthesized by activated microglia in the human substantia nigra and its synthesis by the human microglial CHME cell line is upregulated by tumor necrosis factor alpha or 1-methyl-4-phenylpyridinium treatment, Brain Res. Mol. Brain Res. 96 (2001) 103–113.
[17] Y. Yu, Z. Pang, W. Lu, Q. Yin, H. Gao, X. Jiang, Self-assembled polymersomes conjugated with lactoferrin as novel drug carrier for brain delivery, Pharm. Res. 29 (2012) 83–96.
[18] L. Tang, T. Cui, J.J. Wu, W. Liu-Mares, N. Huang, J. Li, A rice-derived recombinant human lactoferrin stimulates fibroblast proliferation, migration, and sustains cell survival, Wound Repair Regen. 18 (2010) 123–131.
[19] D. Naot, A. Grey, I.R. Reid, J. Cornish, Lactoferrin – a novel bone growth factor, Clin. Med. Res. 3 (2005) 93–101.
[20] Y. Takayama, K. Mizumachi, Inhibitory effect of lactoferrin on hypertrophic differentiation of ATDC5 mouse chondroprogenitor cells, Biometals 23 (2010) 477–484.
[21] N. Brandl, A. Zemann, I. Kaupe, S. Marlovits, P. Huettinger, H. Goldenberg, M. Huettinger, Signal transduction and metabolism in chondrocytes is modulated by lactoferrin, Osteoarthritis Cartilage 18 (2010) 117–125.
[22] Z. Xia, X. Duan, D. Murray, J.T. Triffitt, A.J. Price, A method of isolating viable chondrocytes with proliferative capacity from cryopreserved human articular cartilage, Cell Tissue Bank. 14 (2012) 23–29.
[23] J. Cornish, D. Naot, Lactoferrin as an effector molecule in the skeleton, Biometals 23 (2010) 425–430.
[24] P.A. Guerne, F. Blanco, A. Kaelin, A. Desgeorges, M. Lotz, Growth factor responsiveness of human articular chondrocytes in aging and development, Arthritis Rheum. 38 (1995) 960–968.
[25] A. Grey, Q. Zhu, M. Watson, K. Callon, J. Cornish, Lactoferrin potently inhibits osteoblast apoptosis, via an LRP1-independent pathway, Mol. Cell. Endocrinol. 251 (2006) 96–102.
[26] J. Troppmair, J.T. Bruder, H. Munoz, P.A. Lloyd, J. Kyriakis, P. Banerjee, J. Avruch, U.R. Rapp, Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation, J. Biol. Chem. 269 (1994) 7030–7035.
[27] Q.Z. Gao, J.J. Lu, Z.D. Liu, H. Zhang, S.M. Wang, H. Xu, Dexamethasone suppresses DU145 cell proliferation and cell cycle through inhibition of the extracellular signal-regulated kinase 1/2 pathway and cyclin D1 expression, Asian J. Androl. 10 (2008) 635–641.
[28] R. Jiang, V. Lopez, S.L. Kelleher, B. Lonnerdal, Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in Caco-2 cells, J. Cell. Physiol. 226 (2011) 3022–3031.