Biochemical and Biophysical Research Communications 421 (2012) 418–424

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

A selective estrogen receptor modulator inhibits tumor necrosis factor-α-induced apoptosis through the ERK1/2 signaling pathway in human chondrocytes

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ARTICLE INFO

Article history:
Received 17 March 2012
Available online 29 March 2012

Keywords:
Chondrocyte
Apoptosis
Selective estrogen receptor modulators
ERK1/2
TNF-α

ABSTRACT

Tumor necrosis factor α (TNF-α) is a pleiotropic cytokine mediating inflammatory as well as cell death activities, and is thought to induce chondrocytic chondrolysis in inflammatory and degenerative joint diseases. Selective estrogen receptor modulators (SERMs), such as raloxifene, which are commonly used in clinical settings act as estrogen agonists or antagonists. It is assumed that estrogens have a potential role in cartilage protection; however, the precise molecular mechanism for the protective effects of estrogens is unclear. This study was designed to examine whether raloxifene inhibits TNF-α-induced apoptosis in human chondrocytes and to clarify the mechanisms involved. We also investigated the signaling pathways responsible for the anti-apoptotic effect of raloxifene. Apoptosis in chondrocytes was determined by DNA fragmentation assay and caspase-3 activation. Raloxifene significantly inhibited TNF-α-induced caspase-3 activation and cell DNA fragmentation levels in chondrocytes. The inhibitory effect of raloxifene was abolished by the estrogen receptor antagonist ICI 182,780. Extracellular signal-regulated kinase 1/2 (ERK1/2) regulates apoptosis, acting as an apoptotic or anti-apoptotic signal. TNF-α-induced apoptosis was significantly enhanced by the ERK1/2 pathway inhibitor PD98059. Raloxifene stimulated a further increase in ERK1/2 phosphorylation in TNF-α-treated chondrocytes. Furthermore, the anti-apoptotic effects of raloxifene were inhibited by PD98059. In addition, the anti-apoptotic effects of raloxifene were completely abolished in ERK1/2 siRNA-treated chondrocytes. These results suggest that raloxifene prevents caspase-3-dependent apoptosis induced by TNF-α in human chondrocytes by activating estrogen receptors and the ERK1/2 signaling pathway.

1. Introduction

Chondrocytes are the only cell type in articular cartilage [1] and play a pivotal role in articular cartilage remodeling and maintenance by controlling matrix synthesis and degradation [2]. It has been suggested that tumor necrosis factor α (TNF-α) produced from chondrocytes or infiltrating cells induce chondrocytic chondrolysis in both inflammatory and degenerative joint diseases. The observed number of chondrocytes synthesizing TNF-α is more in osteoarthritis (OA) cartilage [3–5]. Rheumatoid arthritis (RA) is often associated with the destruction of articular cartilage, and TNF-α is produced by the neutrophils and monocytes/macrophages infiltrated into the inflammatory sites [4,6,7]. Studies using cultured chondrocytes or chondrocyte-like cell lines have demonstrated the existence of functional TNF-α receptors [8,9] and the cellular pleiotropic effects of TNF-α.

A remarkable gender difference is observed in the development of OA. Among the multiple physiopathological mechanisms involved in OA, those related to sex hormone control, especially those involving estrogens, have attracted much attention [10]. In contrast to other tissues such as the endometrium, breast, brain, and non-joint bone, it was traditionally thought that joint tissues were non-responsive to estrogens or an estrogen deficit. However, an interest in estrogens has been generated by the large proportion of postmenopausal women with OA and the complexity of their role in this disease. Indeed, considerable efforts have been made

Abbreviations: TNF-α, tumor necrosis factor α; ERK1/2, extracellular signal-regulated kinase1/2; SERMs, selective estrogen receptor modulators; ERs, estrogen receptors.

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http://dx.doi.org/10.1016/j.bbrc.2012.03.111

to understand the potential role of estrogens in the biology of joint tissues as well as in the development and progression of OA, which has led to a better understanding of the effects of estrogen on joint tissues and cartilage in particular [10–12]. Additionally, the development of a cartilage-protective drug is very important in the treatment of degenerative joint diseases.

Selective estrogen receptor modulators (SERMs), such as raloxifene, are commonly used to treat osteoporosis in clinical settings. Interestingly, drugs in this class show estrogen-antagonistic effects on mammary glands and the uterus, whereas they show estrogen-agonistic effects on bone and other tissues [13]. Thus, these drugs are expected to overcome the adverse effects of conventional hormone replacement therapy. However, the precise molecular mechanism for the effects of raloxifene is unclear. There are no reports indicating the protective effects of raloxifene against pro-inflammatory cytokines such as TNF-α in chondrocytes as well as the mechanism involved.

In the present study, we examined whether raloxifene inhibits TNF-α-induced apoptosis in human chondrocytes, and investigated the signaling pathways responsible for the anti-apoptotic effect of raloxifene in human chondrocytes.

2. Materials and methods

2.1. Materials

Raloxifene, 17β-estradiol (E2), ICI 182,780, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). The MEK1 inhibitor PD98059, antibodies against caspase-3 detecting full length caspase-3 (35 kDa) and cleaved-caspase-3 (17 kDa), and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA, USA). Fetal bovine serum (FBS) was obtained from PAA (Morningside, QLD, Australia). Polyvinylidenedifluoride (PVDF) membranes were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, England). Fast Western Blot Kits and SuperSignal West Picochemiluminescence reagents were obtained from Thermo Scientific (Waltham, MA, USA). The Cell Death Detection ELISA Plus kit and Cell Proliferation Kit I (MTT) were purchased from Roche (Mannheim, Germany).

2.2. Cell culture

HCS-2/8 cells, a chondrocytic cell line established from human chondrosarcoma [14], were grown in DMEM supplemented with 10% FBS, penicillin G sodium (100 U/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 g/ml). The cells were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2. The HCS-2/8 cell line has a phenotype similar to that of normal chondrocytes. In all experiments, HCS-2/8 cells were used at passages 5–7 and plated at a concentration of 10×10^4 cells/ml. DMSO was used as a solvent for raloxifene, E2, ICI 182,780, and the MEK1 inhibitor PD98059.

Normal human knee chondrocytes in frozen vials were purchased (NHAC-Kn, lot no. 8F3341) from Lonza (Walkersville, MD, USA). The cells were handled according to the manufacturer's protocol. The seeding density for attachment was 10,000 cells/cm^2, and the culture medium used was CGM BulletKit (Lonza). The cells were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2.

2.3. Apoptosis assay

Cell apoptosis was quantified by DNA fragmentation, using the Cell Death Detection ELISA Plus kit (Roche). Briefly, cells were seeded and grown to 70–80% confluence. After a 4-h serum starvation, cells were exposed to medium containing different concentrations of test compounds. After 24 h, cells were lysed in 200 μl lysis buffer, and 20 μl of the supernatant was subjected to a reaction with 80 μl of anti-DNA immunocomplex conjugated with peroxidase which binds to nucleosomal DNA and anti-histone-biotin which interacts with streptavidin-coated wells in a microtiter plate for 2 h. At the end of the incubation, 100 μl of substrate was added, and color development was quantified at 405 nm wavelength.

2.4. Cell viability assay

Cell viability was determined by MTT assay, using the Cell Proliferation Kit I (MTT) (Roche). Briefly, cells were seeded in 96-well plates and grown to 60–70% confluence. After a 4-h serum starvation period, cells were exposed to medium containing different concentrations of test compounds. After 24 h, 10 μl of MTT labeling reagent was added to each well and the cells were incubated at 37 °C for 4 h. Solubilization solution (100 μl) was added to each well. After an overnight incubation, the solubilized mixture was measured by reading absorbance at 550 nm wavelength on a microplate reader.

2.5. Small interfering RNA (siRNA) and electroporation

siRNA against p44/42 MAPK (Erk1/2) (SignalSilence®) was obtained from Cell Signaling Technology. HCS-2/8 cells were mixed with siRNAs and added to 2-mm gap cuvettes. Cells were electroporated at 175 V for 5 ms using a NEPA21 electroporator (Nepa Gene, Chiba, Japan), transferred to a 12-well tissue culture plate with 2 ml medium, and incubated for 24 h. Knocked-down effects were evaluated by quantitative RT-PCR.

2.6. Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real time RT-PCR was carried out using the LightCycler System with FastStart Master PlusSYBR Green I (Roche, Indianapolis, IN, USA). ERK1/2 and GAPDH primers were designed and synthesized by Takara Bio (Shiga, Japan) and Nihon Gene Research Laboratories (Miyagi, Japan).

2.7. Western blotting

Signal transduction and production of apoptosis-related molecules were evaluated by Western blotting. After a brief treatment with reagents, the cells were washed twice in ice-cold PBS and lysed. Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes. These membranes were blocked with 5% skimmed milk in TBS for 1 h prior to hybridization with primary antibodies. After hybridization with secondary antibodies, proteins were detected by the chemiluminescence method. A densitometric analysis was performed using an image scanner and analyzing software (CS Analyzer 2.0). The data were normalized to β-actin for phospho-ERK1/2 (Thr202/Tyr204).

2.8. Statistical analysis

Values were expressed as mean ± SD. Statistical comparisons were performed with the SPSS 19.0 version of Windows software. All data were analyzed using an unpaired *t*-test. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Effect of raloxifene on TNF-α-induced HCS-2/8 cell apoptosis and cell viability

First, we investigated the effect of raloxifene on TNF-α-induced apoptosis in HCS-2/8 cells. According to basic amount examinations, 10 ng/mL TNF-α was used in this experiment (data not shown). DNA fragmentation measurements revealed that raloxifene significantly inhibited HCS-2/8 cell apoptosis in a concentration-dependent manner (0.01–10 μmol/L) (Fig. 1A). To determine the percentage of apoptotic cells, we also investigated the effect of raloxifene on the viability of HCS-2/8 cells exposed to TNF-α. HCS-2/8 cell death induced by TNF-α was abrogated in the presence of raloxifene, suggesting that raloxifene inhibited HCS-2/8 cell apoptosis induced by TNF-α (Fig. 1B).

Caspase-3, a proenzyme, is a key executioner of apoptosis. It is cleaved to yield the p17 active fragment upon a cell death stimulus [15]. The p17 fragment cleaves the inhibitor of the endonuclease caspase-activated DNase, leading to caspase-activated DNase activation and thus, resulting in DNA damage [16]. We performed Western blotting to investigate whether caspase-3 activation was involved in TNF-α-induced apoptosis in HCS-2/8 cells. We found that the cleaved caspase-3 subunit p17 manifested after treating

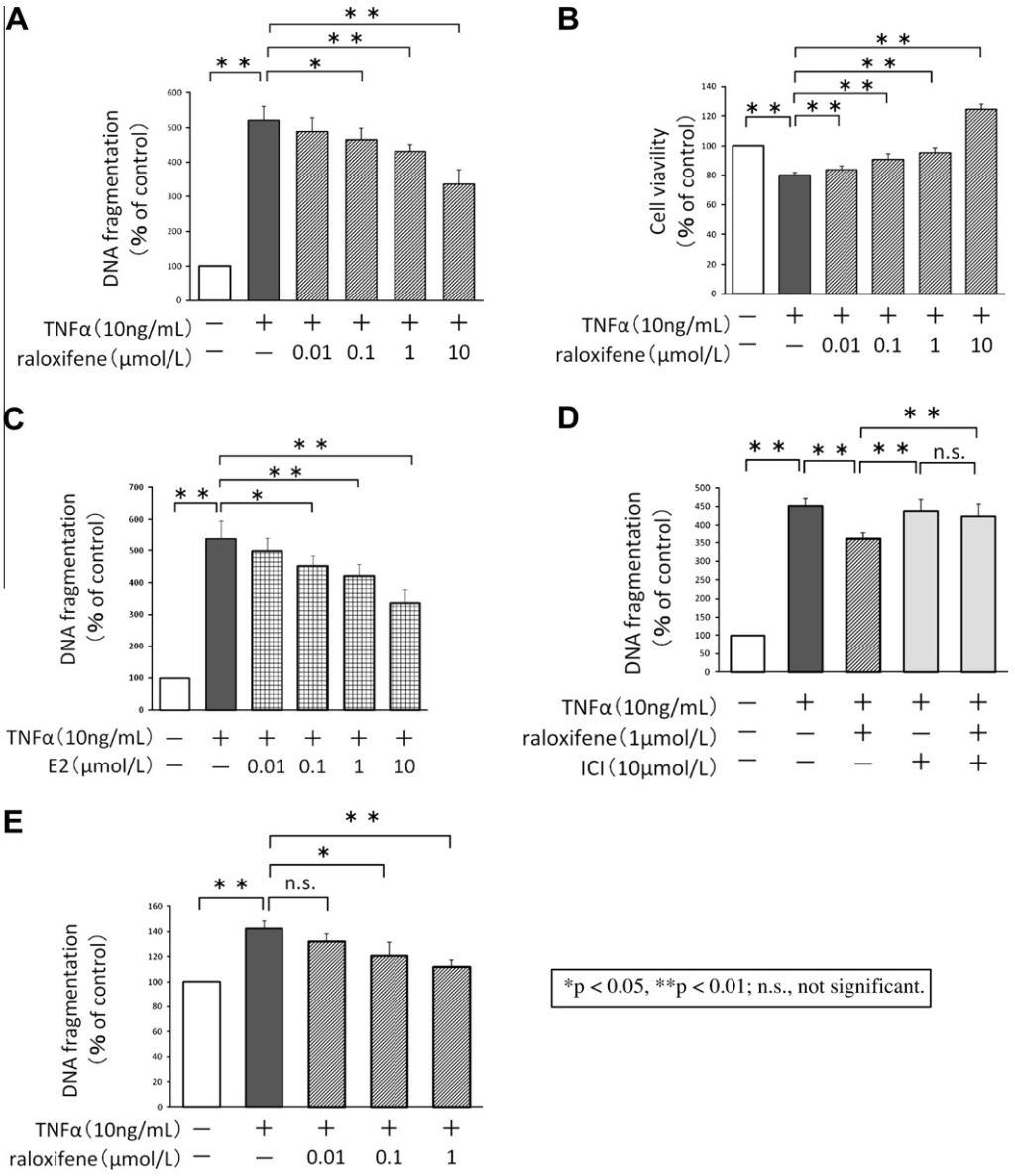

Fig. 1. Effect of raloxifene on tumor necrosis factor (TNF)-α-induced apoptosis and cell viability. (A), (C), (D), and (E): DNA fragmentation measurements: HCS-2/8 cells were starved and exposed to TNF-α (10 ng/mL) for 24 h, at 70–80% confluence. Various concentrations of raloxifene (0.01–10 μmol/L) (A) or E2 (0.01–10 μmol/L) (C) were added to the culture medium simultaneously with TNF-α stimulation for the apoptosis assay. In the estrogen receptor antagonist experiment (D), cells were pretreated with ICI 182,780 (10 μmol/L) for 1 h before adding raloxifene (1 μmol/L). Cell apoptosis was evaluated by DNA fragmentation. (E) NHAC cells were starved and exposed to TNF-α (10 ng/mL) for 24 h at 70–80% confluence. Various concentrations of raloxifene (0.01–1 μmol/L) were added to the culture medium simultaneously with TNF-α stimulation for the apoptosis assay. Data are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 6$). (B): Cell viability assay: HCS-2/8 cells were serum starved and exposed to TNF-α (10 ng/mL) for 24 h, at 70–80% confluence. Various concentrations of raloxifene (0.01–10 μmol/L) were added to the culture medium simultaneously with TNF-α stimulation for the viability assay. The MTT assay was performed after 24 h. Data are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 6$).

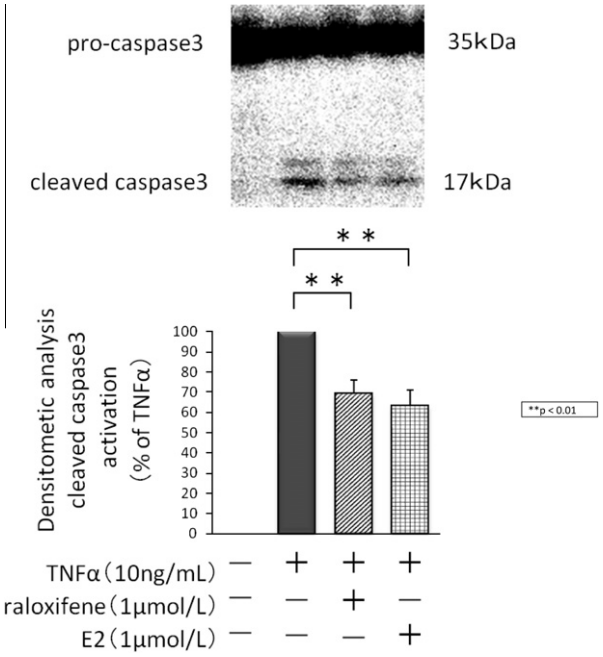

Fig. 2. Involvement of the caspase-3pathway in the anti-apoptotic action of raloxifene. HCS-2/8 cells were starved at 70–80% confluence. Cells were stimulated with 10 ng/mL of tumor necrosis factor (TNF)-α and 1 μmol/L of raloxifene, simultaneously, for 24 h to determine cleaved-caspase-3 levels by Western blot. Antibodies against caspase-3 detecting endogenous levels of full-length caspase-3 (35 kDa) and the large caspase-3 fragment resulting from cleavage (17 kDa) were used. Representative blots and quantitative data evaluated by densitometry are shown. The data are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 4$).

the cells with 10 ng/mL of TNF-α for 24 h. Raloxifene (1 μmol/L) significantly decreased cleaved caspase-3 (p17) compared to TNF-α (Fig.2). These results suggest that caspases-3 activation is involved in TNF-α-induced HCS-2/8 cell apoptosis, and that the inhibitory effect of raloxifene on TNF-α-induced apoptosis in HCS-2/8 cells is caspase-3-dependent. The endogenous ligand for estrogen receptors (ERs), 17β-estradiol (E2), also significantly inhibited TNF-α-induced cell DNA fragmentation (Fig. 1C) and cleaved caspase-3 (p17) levels (Fig.2). The ER antagonist ICI 182,780 significantly inhibited the anti-apoptotic effects by raloxifene (Fig. 1D). These results indicate that raloxifene suppressed TNF-α induced chondrocyte apoptosis, and that the anti-apoptotic activity of raloxifene was mediated by ERs through a caspase-3-dependent mechanism.

3.2. Activation of p38 MAPK, JNK, ERK1/2, and Akt induced by TNF-α

The MAP-kinase and the PI3-kinase/Akt signaling pathways regulate apoptosis [17]. To determine which signal pathways are activated by TNF-α-induced apoptosis, we performed Western blotting using a specific antibody against the phosphorylated form of kinases. Phosphorylation levels of p38 MAPK, JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), and Akt (Ser473) increased after exposure to TNF-α, without any significant changes in total protein levels. Maximum phosphorylation was observed at 30 min for p38 MAPK and Akt (data not shown), at 10 min for JNK (data not shown), and at 15 min for ERK1/2 (Fig. 3A). To determine whether the activation of p38 MAPK, JNK, ERK1/2, or Akt could act as apoptotic or anti-apoptotic signals, we examined the effects of inhibitors on cell DNA fragmentation. The p38 MAPK inhibitor SB203580 and the JNK inhibitor SP600125 significantly decreased TNF-α-induced HCS-2/8 cell apoptosis (data not shown), whereas the PI3-kinase inhibitor wortmannin and the MEK1 (the immediate upstream regulator of ERK) inhibitor PD98059 significantly enhanced apoptosis (Fig. 3B). These results indicate that p38 MAPK and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of HCS-2/8 cell apoptosis.

3.3. Involvement of the MEK/ERK1/2 pathway in the anti-apoptotic action of raloxifene

We examined the effects of raloxifene on the phosphorylation levels of p38 MAPK, JNK, ERK1/2, and Akt. Based on time–response experiments, cells were stimulated with TNF-α (10 ng/mL) for 30 min to examine p38 MAPK and Akt activity, 10 min to examine JNK activity, and 15 min to examine ERK1/2 activity. Raloxifene significantly enhanced ERK1/2 phosphorylation (Fig. 4A). However, no changes in phosphorylation levels of Akt, p38 MAPK, or JNK were induced by raloxifene (data not shown). To investigate the role of ERK1/2 in the anti-apoptotic effect of raloxifene, we examined the effects of PD98059 on DNA fragmentation. The anti-apoptotic effects of raloxifene were abrogated by PD98059 (Fig. 4B), whereas PD98059 alone did not induce HCS-2/8 cell apoptosis (Fig. 3B).

To further explore the effect of raloxifene on TNF-α-induced apoptosis in chondrocytes, we elucidated the role of ERK1/2 in

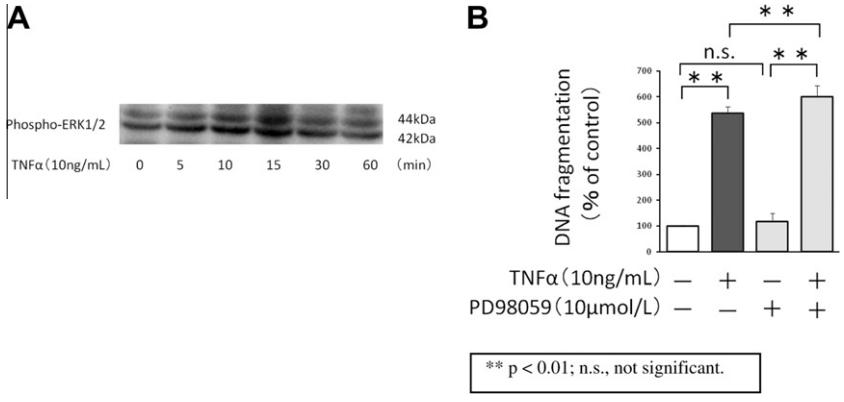

Fig. 3. Activation of ERK1/2 induced by tumor necrosis factor (TNF)-α. (A) Starved HCS-2/8 cells were stimulated with TNF-α (10 ng/mL) and harvested at the times indicated for Western blot analysis. Antibodies against phospho-ERK1/2 (Thr202/Tyr204) were used. (B) In the inhibitor experiment, starved cells were stimulated with the MEK1 inhibitor PD98059for 1 h before adding TNF-α (10 ng/mL). After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation. Values are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 6$).

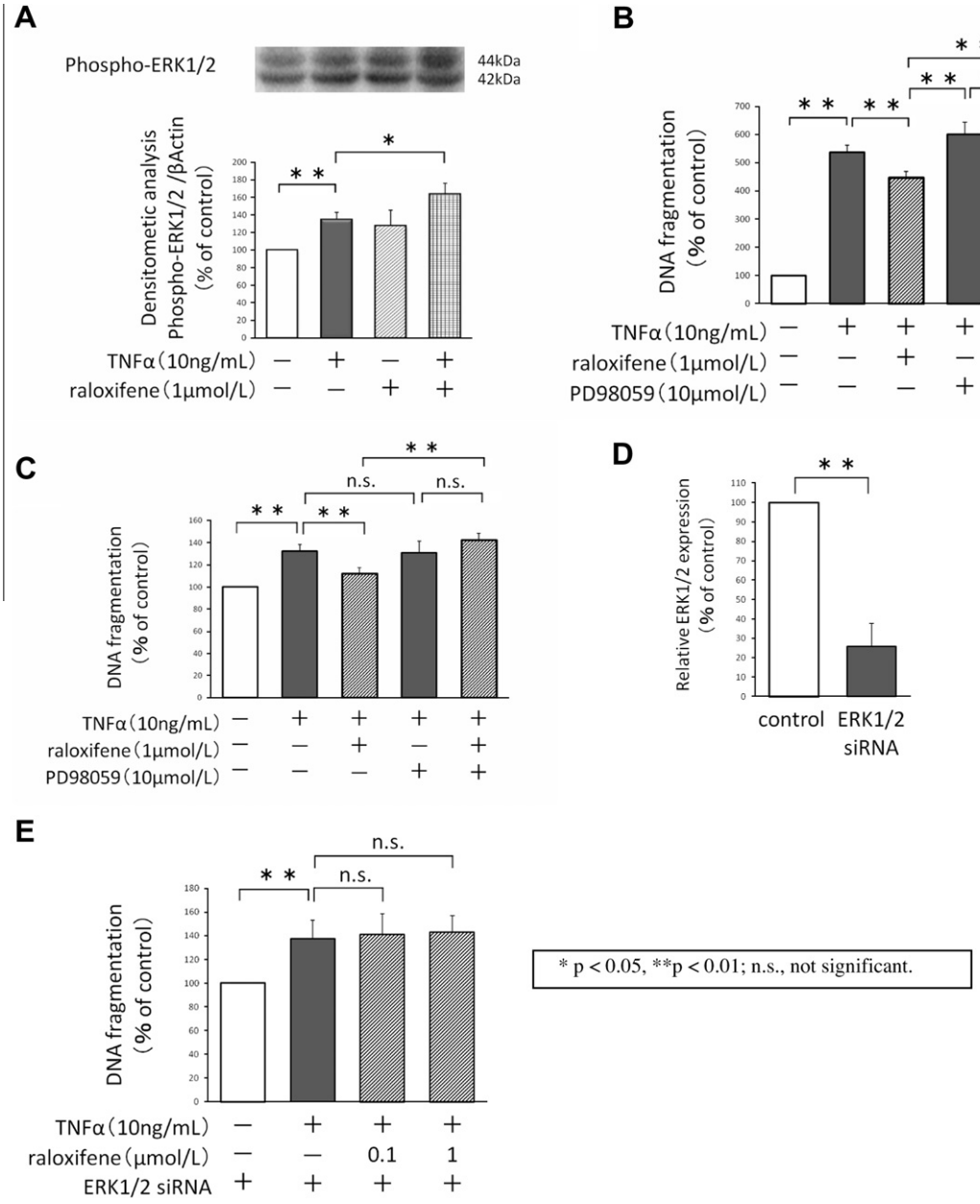

Fig. 4. Involvement of the MEK/ERK1/2 pathway in the anti-apoptotic action of raloxifene. (A) Starved HCS-2/8 cells were stimulated with raloxifene (1 μmol/L) and TNF-α (10 ng/mL) for 15 min to determine ERK1/2 (Thr202/Tyr204) activity. Cells were harvested, lysed, and used for Western blot analysis. ERK1/2 activities were measured. Representative blots and quantitative data evaluated by densitometry are shown. The data for phospho-ERK1/2 were normalized to β-actin. Data are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 4$). (B) HCS-2/8 cells and (C) NHAC cells were starved and exposed to TNF-α (10 ng/mL) for 24 h at 70–80% confluence. In the MEK1 inhibitor PD98059 experiment, cells were pretreated with PD98059 for 1 h before adding raloxifene (1 μmol/L) and TNF-α (10 ng/mL). After 24 h of incubation, cell apoptosis was evaluated by DNA fragmentation. Values are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 6$). (D) and (E): Inhibition of ERK1/2 expression by siRNA: NHAC cells were electroporated with 500 nm siRNA targeted against ERK1/2 (ERK1/2 siRNA). ERK1/2 siRNA-treated NHAC-Kn cells were cultured for 24 h, (D) and starved cells were stimulated with TNF-α (10 ng/mL). After 15 min, total RNA was extracted and reverse transcription was performed. Relative expression of ERK1/2 was determined by RT-PCR and normalized against GAPDH. Data are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 4$). (E) Starved cells were stimulated with raloxifene (1 μmol/L) and TNF-α (10 ng/mL). After 24 h of incubation, cell apoptosis was evaluated by DNA fragmentation. Values are expressed as mean ± SD. Differences with a p value of <0.05 were considered statistically significant ($n = 6$).

the anti-apoptotic effects of raloxifene in NHAC-Kn cells. DNA fragmentation measurements revealed that raloxifene significantly inhibited NHAC-Kn cell apoptosis in a concentration-dependent manner (0.01–1 μmol/L) (Fig. 1E). The anti-apoptotic effects of raloxifene were abrogated by PD98059 (Fig. 4C). These results indicate that the anti-apoptotic effect of raloxifene was mediated through selective activation of the survival signal, MEK/ERK1/2 pathway in human chondrocytes.

3.4. siRNA targeting ERK1/2 specifically inhibits the anti-apoptotic effects of raloxifene

Furthermore, we examined the role of ERK1/2 in the anti-apoptotic effects of raloxifene in HCS-2/8 and NHAC-Kn cells using RNAi method. We electroporated HCS-2/8 and NHAC-Kn cells with siRNA against ERK1/2 and evaluated the knocked-down effects

by quantitative RT-PCR. We observed that ERK1/2 siRNA-treated HCS-2/8 and NHAC-Kn cells reduced the expression level of ERK1/2 by 78% (data not shown) and 74% (Fig. 4D), respectively. To investigate the role of ERK1/2 in the anti-apoptotic effects of raloxifene, we examined the effects of siRNA targeting ERK1/2 on DNA fragmentation. The anti-apoptotic effects of raloxifene were abrogated in ERK1/2 siRNA-treated HCS-2/8 (data not shown) and NHAC-Kn cells (Fig. 4E). These results indicate that the anti-apoptotic effects of raloxifene were mediated by selective activation of the MEK/ERK1/2 survival signal pathway in human chondrocytes.

4. Discussion

Our study is the first to demonstrate that raloxifene prevents caspase-3-dependent apoptosis induced by TNF-α in human chondrocytes by activating ERs and the ERK1/2 signaling pathway.

Increasing evidence indicates that estrogens play a relevant role in maintaining the homeostasis of articular tissues and, hence, the joint itself [18]. The dramatic rise in OA prevalence among postmenopausal women [19,20], which is associated with the presence of ERs in joint tissues [21–24], suggests a link between OA and loss of ovarian function. This association indicates a potential role of estrogens in protection against OA development. Recent *in vitro*, *in vivo*, genetic, and clinical studies have shed further light on these issues [18]. However, no reports have indicated that estrogens and SERMs have anti-apoptotic effects in chondrocytes which would clarify the molecular and cellular mechanisms involved.

To determine whether the anti-apoptotic effects of raloxifene are mediated by ERs in HCS-2/8 cells, we examined the effects of the specific ER antagonist ICI 182,780. The anti-apoptotic effects of raloxifene were abolished by ICI 182,780. In addition, 17β-estradiol (E2), an endogenous ligand for ERs, significantly inhibited apoptosis in HCS-2/8 cells. These observations suggest that raloxifene acts as an ER agonist in chondrocytic cells and is thus responsible for chondrocytic cell survival.

The caspase-3-dependent pathway is involved in the apoptotic process [15,16]. Several lines of evidence suggest that caspase-3 crucially controls cellular changes related to TNF-α-induced apoptosis [25–27]. Furthermore, TNF-α activates multiple signal transduction pathways, such as PI3-kinase/Akt [28] or mitogen-activated protein (MAP) kinase including ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK [17]. Recent studies support the idea that TNF-α-induced apoptosis is apparently regulated by a balance between the death signaling and survival signaling kinases [17,29]. ERK1/2 signaling is involved in both protection [30,31] and induction [32,33] of apoptosis. In our study, TNF-α-induced apoptosis was significantly enhanced by the MEK1/ERK1/2 inhibitor PD98059 in HCS-2/8 cells. Thus, ERK1/2 acted as a survival signal during HCS-2/8 cell apoptosis. Subsequently, we investigated the signaling pathways responsible for the anti-apoptotic effect of raloxifene and found that raloxifene enhanced the increase in ERK1/2 phosphorylation levels. Furthermore, the anti-apoptotic effect of raloxifene was completely abolished in HCS-2/8 and NHAC-Kn cells in the presence of PD98059. In addition, the anti-apoptotic effects of raloxifene were completely abolished in ERK1/2 siRNA-treated HCS-2/8 and NHAC-Kn cells. We demonstrated that the anti-apoptotic effect of raloxifene is mediated through enhanced ERK1/2 signaling in HCS-2/8 and NHAC-Kn cells. Therefore, increased ERK1/2 phosphorylation induced by raloxifene plays a beneficial role in human chondrocyte apoptosis. However, we demonstrated only a part of the molecular and cellular mechanism by which raloxifene exerts its effects in human chondrocytes. Further investigations are required for a better understanding of the action of raloxifene in human chondrocytes.

In summary, our data have demonstrated that raloxifene prevents TNF-α-induced caspase-3-dependent apoptosis in human chondrocytes by activating the ERs and the ERK1/2 signaling pathway. Thus, raloxifene has a protective effect on human chondrocytes. We consider that raloxifene may become a new specific estrogen ligand which is used as a potential therapeutic drug to treat OA and RA.

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