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Leukotriene D4 induces cellular senescence in osteoblasts

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ABSTRACT

Aging is associated with the development of osteoporosis, in which cellular senescence in osteoblasts plays a key role. Leukotriene D4 (LTD4), an important cysteinyl leukotriene (cysLT), is a powerful pro-inflammatory mediator formed from arachidonic acid. However, little information regarding the effects of LTD4 on the pathogenesis of osteoporosis has been reported before. In the present study, we defined the physiological roles of LTD4 in cellular senescence in osteoblasts. Our results indicate that LTD4 treatment decreased the expression of SIRT1 in a dose-dependent manner in MC3T3-E1 osteoblastic cells. Additionally, LTD4 significantly increased the expression of p53, p21 and plasminogen activator inhibitor-1 (PAI-1). LTD4 was also found to elevate the activity of β-galactosidase (SA-β-Gal) but to prevent BrdU incorporation. Our results indicate that cysteinyl leukotriene receptor 1 (cysLT1R) could be detected in MC3T3-E1 osteoblastic cells at both the mRNA and protein levels. However, cysLT2R was not expressed in these cells. Interestingly, we found that knockdown of cysLT1R or use of the selective cysLT1R antagonist montelukast abolished the LTD4-induced reduction in SIRT1 and increase in p53, p21, and PAI-1. Notably, knockdown of cysLT1R by transfection with cysLT1R siRNA or treatment with montelukast attenuated the LTD4-induced increase in SA-β-Gal activity. Our study shows for the first time that LTD4 has a significant impact on cellular senescence in osteoblasts.

1. Introduction

Aging is associated with reduced bone density and increased bone loss [1]. Old age has been found to be the most important risk factor for the development of osteoporosis [2]. Induction of cellular senescence in osteoblasts plays a key role in the pathogenesis of age-related bone loss [3]. Senescent cells remain metabolically active but enter a state of permanent growth arrest [4]. Senescent cells exhibit a senescence-associated secretory phenotype (SASP), which causes them to secrete a variety of inflammatory cytokines as well as other soluble and insoluble factors. Furthermore, a senescence-associated increase in β-galactosidase (β-Gal) activity is an important biomarker of cellular senescence. The regulation of cellular senescence is complex. The type III histone/protein deacetylase SIRT1 plays a central role in regulating cellular senescence [5]. SIRT1 prevents growth arrest and senescence by negatively regulating the expression and activity of p53, a central transcriptional factor in the regulation of cellular senescence and the cell cycle. PAI-1 and p21 are two important senescence genes. p21 protein mediates the central activating pathways of senescence via p53 [6].

Leukotriene D4 (LTD4), an important member of the cysteinyl leukotriene (cysLT) family, is a powerful pro-inflammatory mediator formed from arachidonic acid through the action of 5-lipoxygenase (5-LOX) [7]. CysLTs mediate their biological actions through two currently identified G-protein coupled receptors (GPCR): the cysLT type 1 receptor (cysLT1R) and type 2 receptor (cysLT2R) [8]. CysLT1R has a higher affinity for LTD4 than cysLT2R [9]. LTD4 has been associated with the pathogenesis of several inflammatory disorders, such as asthma, allergies, atherosclerosis, and inflammatory bowel disease [10,11]. In addition, cysLT1R has been reported to mediate a wide range of pro-inflammatory effects including the activation and migration of leukocytes [12]. The licensed cysLT1R antagonist montelukast is approved by the Food and Drug Administration (FDA) for use in the treatment of asthma and allergic rhinitis by blocking the action of LTD4 on cysLT1R without inhibiting cysLT2R-mediated effects. In addition to inhibiting the cysLT1R-mediated bronchoconstrictor element of asthma, montelukast exerts its anti-inflammatory activity throughout various types of tissues and cells [13]. However, little information regarding the effects of LTD4 on the pathogenesis of osteoporosis has

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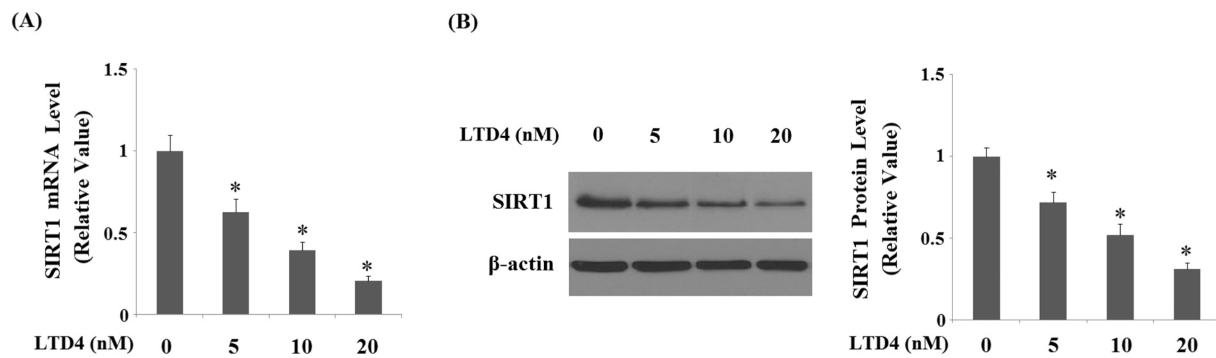

Fig. 1. Leukotriene D4 (LTD4) inhibits expression of SIRT1 in murine MC3T3-E1 osteoblastic cells in a dose-dependent manner. (A) Murine MC3T3-E1 osteoblastic cells were treated with LTD4 (5, 10, 20 nM) for 12 h. Expression of SIRT1 at the mRNA level was determined by real-time PCR analysis; (B) Murine MC3T3-E1 osteoblastic cells were treated with LTD4 (5, 10, 20 nM) for 24 h. Expression of SIRT1 at the protein level was determined by western blot analysis (*, $P < 0.001$ vs. previous column group, n = 4–6).

been reported before. In the current study, we defined the physiological roles of LTD4 in cellular senescence in osteoblasts.

2. Materials and methods

2.1. Cell culture and treatment

The osteoblastic cell line MC3T3-E1 (subclone 4) was purchased from ATCC, USA. Cells were cultured in α-MEM medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were initially cultured in six-well plates at the density of 6×10^4 cells/well and incubated at 37 °C in a 5% CO_2 incubator. Cells were treated with LTD4 (5, 10, 20 nM) [14,15] as indicated in Fig. 1 for 12 h or 24 h. MC3T3-E1 cells were transfected with nonspecific or cysLT1R-specific siRNA (50 nM, Dharmacon) using Lipofectamine RNAiMAX (Life Technologies, USA) in accordance with the manual protocols.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total intracellular RNA was isolated from cultured MC3T3-E1 osteoblastic cells using an RNAeasy mini-kit (Qiagen, USA) and treated with RNase-free DNase (Invitrogen, USA) in accordance with the manual instructions. An equal amount of purified RNA (1 μg) was used to synthesize cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Synthesized cDNA was used for RT-PCR with a commercial RT-PCR kit (Thermofisher Scientific, USA) to evaluate the expression of cysLT1R and cysLT2R in MC3T3-E1 osteoblastic cells.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total intracellular RNA was isolated from cultured MC3T3-E1 osteoblastic cells using an RNAeasy mini-kit (Qiagen, USA) and treated with RNase-free DNase (Invitrogen, USA) in accordance with the manual instructions. An equal amount of purified RNA (1 μg) was used to synthesize cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Synthesized cDNA was used for real-time PCR analysis with SYBR GREEN PCR Master Mix (Applied Biosystems, USA). Target gene expression was normalized to the housekeeping gene GAPDH using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot analysis

Cells were lysed with lysis buffer (Cell Signaling Technology, USA) supplemented with a protease and phosphatase inhibitor cocktail (Sigma–Aldrich, USA). After centrifugation at 4 °C for 10 min at $10000 \times g$, protein content was determined by bicinchoninic acid assay (Sigma–Aldrich, USA). Equal amounts of proteins (20 μg) in supernatants were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% BSA and incubated with specific primary antibodies overnight at 4 °C. Membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Dako, USA) for 1 h at room temperature. Finally, membranes with immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Healthcare, USA).

2.5. Assays for senescence-associated phenotypes

Cellular senescence was assayed as previously described [16]. After the indicated treatment, cells were washed 3 times with PBS and fixed with 0.4% paraformaldehyde. Cells were stained in X-Gal solution for 16 h at 37 °C, then visualized under a light microscope and assessed for SA-β-Gal activity.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

After the indicated treatment, MC3T3-E1 osteoblastic cells were probed with 10 μM BrdU (Roche, USA) for 2 h, fixed with 4% paraformaldehyde for 1 h, and immunostained with anti-BrdU antibody (Roche, USA) followed by staining with TRITC-conjugated goat anti-mouse IgG (Life Technologies, USA) for 1 h at room temperature. Then cells were counter-stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI). Staining was visualized under an Axiovert 200M fluorescent microscope (Carl Zeiss, Germany). At least 200 nuclei were counted. The percentage of BrdU positive nuclei over the total number of nuclei was calculated.

2.7. Statistical analysis

All experiments were conducted at least three times. Experimental data are expressed means ± standard error of the mean (SEM). Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

We investigated SIRT1 expression levels in response to LTD4 treatment. Murine MC3T3-E1 osteoblastic cells were treated with LTD4 (5, 10, 20 nM) as indicated in Fig. 1 for 12 h or 24 h. Interestingly, LTD4 significantly reduced the expression of SIRT1 in a concentration-dependent manner at both the mRNA levels (Fig. 1A) and protein levels (Fig. 1B).

SIRT1 is an important regulator of cellular senescence. Therefore, we investigated the expression of senescence markers in MC3T3-E1 cells in response to LTD4 treatment. Our results indicate that LTD4 treatment significantly up-regulated the expression of p53 at both the

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