THE ROLE OF ENDOTHELIN-1 FOR ECTOPIC BONE FORMATION IN THE SPINAL LIGAMENTS.

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Introduction

Ossification of the posterior longitudinal ligament (OPLL) of the spine is characterized by ectopic bone formation progressive in the spinal ligaments. Mechanical stress has been suggested to play an important role in the progression of OPLL. However, the mechanism by which mechanical stress promotes the ossification of ligaments in OPLL is still unknown. We reported that the transcriptome analysis with cDNA microarray about between mechanical stress and ossification of ligaments and endothelin-1 (ET-1) was found to be upregulated in OPLL cells by cyclic stretch. However, we think OPLL cells had a hyper-responsiveness to ET-1 compared with non-OPLL cells, we examined the role of ET-1 in the development of OPLL.

Materials and Methods

1. Loading of mechanical stress on human spinal ligament cells

Spinal ligaments were harvested aseptically from OPLL patients and non-OPLL patients during surgery. Ligament cells were cultured by the explant method. Fifth-passage cells were placed on a 3.5 x 4.0 cm² deformable silicon chamber coated with 0.1% gelatin (IWAKI GLASS, Japan) at a density of 10,000 cells/cm². After cultures reached confluence, cells were incubated in DMEM supplemented with 1% FBS for 24 hours and then subjected to motor driven computer controlled uniaxial sinusoidal cycle stretch by using a four-point bending apparatus (Scholertec Corp., Osaka, Japan), in 120% peak to peak, at 0.5 Hz for 0, 3, 6, 9 hours.

2. Transcriptome analysis with cDNA microarray

Total RNA was extracted from OPLL and non-OPLL cells with an RNeasy Kit (Qiagen, USA) according to the manufacturer’s protocol. Two µg of total RNA was reverse transcribed into cDNA using T2 Labeling and Detection Kit (PerkinElmer Life Sciences, Inc. USA). Then, we performed transcriptome analysis using Agilent Human1 cDNA Microarray Kit (Agilent, USA).

3. RT-PCR

The mRNA expressions of ET-1, endothelin-2 (ET-2), endothelin receptor subtype A (ET-A) and B (ET-B) were quantified by RT-PCR.

4. Treatment of OPLL and non-OPLL cells with ET-1

OPLL and non-OPLL cells were treated with 10 to 100 µM of ET-1 for 9 hours. To investigate the effects of ET-1 in OPLL cells, expression of ALP and prostaglandin I2 (PGI2) mRNA were analyzed by RT-PCR. Then we used ET-A blocker (compound A) and ET-B blocker (BQ-788) to investigate about ET-1/ET receptor pathways.

5. ELISA assay

ET-1 released from OPLL and non-OPLL cells into culture media for 9 hour-cycle stretch was assessed by an ET-1-specific enzyme-linked immunosorbent assay (ELISA; R&D systems, USA).

6. Mineralization assay

OPLL cells and non-OPLL cells were plated at 2.5x10⁶ cells/35 mm gelatin-coated dish and maintained in DMEM supplemented with 10% FBS. On confluence, designated day 0, cells were then exposed to 1) fresh medium, 2) fresh medium + 100 nM ET-1 or 3) osteogenic medium containing DMEM supplemented with 10% FBS, 50 µg/mL of ascorbic acid, and 5 mM β-glycerophosphate, replacing medium for 3-4 days. Cells were processed on 0, 4 and 8 weeks and alizarin red assay (Sigma Chemical, St.Louis, MO, USA) was performed to determine mineralization.

Results

1. In 18,000 genes on microarray, ET-1 as well as ossification markers such as alkaline phosphatase, osteocalcin, IG-F-2, TGF-β, CTGF, VEGF and bone morphogenetic factor-2, -4, prostaglandin I2 (PGI2) synthase, PGI2 receptor, we reported their relation to OPLL previously, increased significantly.

2. In OPLL cells, the ET-1 mRNA was highly expressed by mechanical stress in a time-dependent manner for 9 hours. ET-A mRNA was highly expressed in OPLL cells but did not show any change by mechanical stress. ET-2 and ET-B mRNA were slightly expressed and ET-B mRNA tended to be decreased by mechanical stress. In non-OPLL cells, the expression level of their mRNA was as well as in OPLL cells, but mechanical stress did not change the level.

3. In OPLL cells, ET-1 increased the mRNA expression of ALP in a dose-dependent manner, but significantly in 100 nM. In non-OPLL cells, the expression level of their mRNA was as well as in OPLL cells, but mechanical stress did not change the level.

4. Released ET-1 in OPLL cells after 9 hours cycle stretch in creased to non-stretched about 5 times, but in non-OPLL cells mechanical stress did not change the level.

5. Even in normal medium, OPLL cells exhibit mineralization slightly, but in osteogenic medium ossification significantly progressed. ET-1 can also induce ossification in OPLL cell cultures. On the other hand, in non-OPLL cell cultures any calcification was not observed neither in osteogenic medium nor in the presence of 100 nM ET-1.

Discussion

Endothelin (ET), originally isolated from cultured porcine aortic endothelial cells, is a potent vasconstricting peptide with 21 amino acid residues. Three distinct members of the ET family, namely, ET-1, -2, -3, have been identified through cloning. ET-A receptors are selective for ET-1 and ET-2, whereas ET-B receptors bind ET-1, ET-2 and ET-3 with equal potency. ET-1 has diverse biological actions such as stimulation of nitric oxide and PGI2 release from endothelial cells, and modulation of hormone secretion. In addition to them, ET-1 has been elucidated to be a regulator of bone metabolism. In this study, cyclic stretch-induced increases in ALP and ET-1 mRNA were found by cDNA microarray and confirmed by RT-PCR and mineralization assay in OPLL cell but not in non-OPLL cells. ET-1 release from OPLL cells was also stimulated by cyclic stretch. ET-1 addition upregulated ALP expression. From the above results and our previous report, it is suggested that OPLL cells are already transformed into osteoblastic cells and mechanical stress promotes development and progression of ossification in OPLL cells through ET-1/ET receptor system and PGI2.

References


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Fig. 1 Scheme of signal trnsduction