様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Study of cartilage exosomes released under mechanical stimulation and their application to cartilage regeneration

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研究成果の概要（和文）：エキソソームは細胞によって分泌される小胞で，そこに様々タンパク質、メッセンジャーRNAやマイクロRNAが含まれている．エキソソームは細胞の信号伝達に重要な役割を果たしている．生体内の関節では，静水圧力は軟骨細胞が感じる主な力学刺激であり，軟骨分化に重大な影響を及ぼす．本研究の目的は分泌されるエキソソームに対する静水圧力の影響を調べることだった．
一年目，沈殿方法でエキソソームの抽出を最適化した．二年目は，未分化軟骨細胞において分化した軟骨細胞によって分泌されたエキソソームの効果を調べた．最後に静水圧力を負荷した細胞のエキソソームを抽出し，含まれているマイクロRNAをマイクロアレイ技術で検出した．

研究成果の学術的意義や社会的意義
エキソソームの研究は比較的に新しい分野なので，本研究の成果でエキソソームに関する知識を深めることができた．特に，in vitro で培養細胞が分泌するエキソソームがとても少なくて，in vitro での研究はまだ珍しい．しかも，細胞に対する力学刺激の影響を調べる分野であるメカノバイオロジーの中で，静水圧力に注目する研究が少なくて，本研究は新しい結果につながると思われる．
軟骨細胞において過大静水圧力を負荷して，変形性関節症の細胞レベルの症状を再現できるので，本研究の成果で変形性関節症の理解や治療につながることを期待できる．

研究成果の概要（英文）：Exosomes are small vesicles released by cells and that contain various proteins, messenger RNAs and micro-RNAs. Those vesicles play a crucial role in cell signaling. Hydrostatic pressure (HP) is one of the main mechanical stimuli sensed by cartilage cells during joint loading in vivo and is known to affect the differentiation of cartilage. The purpose of the project was to understand how HP affects the production of cartilage exosomes.
During the first year of the project, we have optimized the method for extracting exosomes by a precipitation method. During the second year, the effects of exosomes isolated from differentiated chondrocytes cells on un-differentiated chondrocyte progenitors cells were investigated. During the third year, the micro-RNAs present in exosomes isolated from cells under pressure were identified by microarray technology.

研究分野：細胞生物学

キーワード：エキソソーム　軟骨　静水圧　細胞シグナル伝達

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

During normal physical activity, compressive stresses generated in human articular cartilage range from 0 to several MPa, reaching around 20 MPa during more intense activities [1]. Due to the high water content of cartilage (about 70%), around 3/4 of the compressive load is sustained by the interstitial fluid [2]. As a result, it is assumed that one of the main physical stimuli sensed by chondrocytes embedded in the cartilage matrix is hydrostatic pressure (HP). At the cellular level, moderate HP is known to promote cartilage differentiation by increasing the expression of cartilage matrix proteins such as aggrecan and type 2 collagen, coded by the Acan and Col2a1 genes. Conversely, excessive HP has the opposite, detrimental effects on cartilage and chondrocytes, with increased apoptosis and decreased extracellular matrix synthesis [3], changes all typical of osteoarthritic cartilage. Therefore, understanding how physiological and excessive HP exert their beneficial and detrimental effects on chondrocytes may be a key to grasp cartilage homeostasis and the pathogenesis of osteoarthritis.

Exosomes are membrane-derived vesicles around 40 to 150 nm in diameter produced by most types of cells, and carry lipids, proteins, messenger RNAs and non-coding RNAs (such as micro-RNAs) between cells as a way of intercellular signaling. Recently, exosomes have gained attention as they have been found to play essential roles in development, organogenesis and wound healing [4]. Many studies have also shown their great potential in regenerative medicine [5]. The role of exosomes in cartilage development and disease, however, has not been investigated. It is quite possible, for instance, that, in a pro-chondrogenic environment, chondrocytes release exosomes that promote differentiation, while in a pathological setting like osteoarthritis, exosomes that promote inflammation and matrix degradation may be produced. Therefore, due to its essential role as one of the main physical stimuli acting on cartilage, we focused on the effects of HP on the production of exosomes by cartilage precursor cells.

２．研究の目的

The purpose of the project was to understand how HP affects the production of cartilage exosomes and characterize the contents of exosomes produced by chondrocytes under HP. We also intended to test whether the findings could be applied to tissue engineering to promote the differentiation of chondrocytes in vitro using exosomes from differentiated cells.

３．研究の方法

(1) Cell culture:

The mouse chondrocyte progenitor cell line ATDC5 was maintained in DMEM/F12 + 5% fetal bovine serum (FBS). During experiments involving exosome isolation, exosome-depleted serum was used (ThermoFisher).

(2) Exosome isolation:

Exosomes were isolated from culture medium using the miRCURY Exosome Cells/Urine/CSF Kit (Qiagen) according to the manufacturer's instructions.

(3) Exosome quantification:

Exosomes were quantified using the FluoroCet exosome quantitation kit (System Biosciences) according to the manufacturer's instructions.

(4) Gene expression analysis:

To assess the effect of pressure or exosomes on cartilage marker expression, gene expression of the cartilage markers Acan, Col2a1 and Sox9 was measured by real-time PCR. The results were normalized to the expression of the reference gene Rpl13a.

(5) Exosome RNA characterization:

Exosome RNA was extracted using the Exiqon miRCURY RNA Isolation Kit (Cells and Plant). The quality of the extracted RNA was assessed using a Bioanalyzer (Agilent) with a Small RNA Chip. RNA of good enough quality was then used for microarray analysis using the SurePrint G3 miRNA Microarray Kit, 8 × 60K (G4872A), the miRNA Complete Labeling and Hybridization Kit (5190-0456) and a G2505C Microarray Scanner System (all from Agilent).

４．研究成果

(1) Exosome-free medium does not affect the response of ATDC5 cells to pressure

Normal FBS contains many bovine exosomes, which would make it impossible to analyze the much fewer exosomes released by the cells in the culture medium. We therefore used exosome-depleted FBS for most experiments. To check whether this FBS affected the response of ATDC5 cells to pressure, we performed PCR on Acan, Col2a1 and Sox9, three genes, which are known to be down-regulated by pressure. We found that the response of ATDC5 cells to 1 or 24h of pressurization under 25MPa down-regulated all genes in both media containing normal and exosome-depleted serum (Fig. 1).

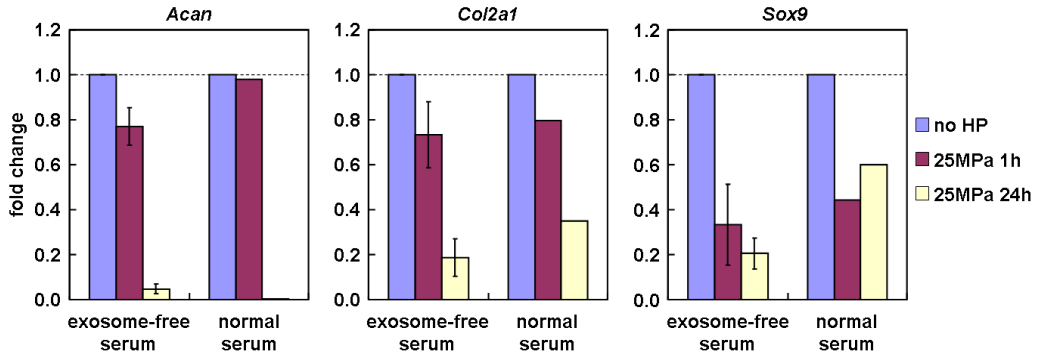

Fig. 1: expression of Acan, Col2a1 and Sox9 in ATDC5 cells pressurized for 0, 1 or 24h under 25MPa in culture medium containing normal serum and exosome-free serum. The graph shows the mean +/- Std Dev of three experiments.

(2) Effect of pressure on the production of exosomes

In order to determine whether pressure affected the production of exosomes, ATDC5 cells were pressurized for up to 24h and the amount of exosomes produced per cell was quantified. Quantification showed no significant difference (ANOVA p>0.5) in the number of exosomes produced per cell between pressurized and un-pressurized cells (Fig. 2).

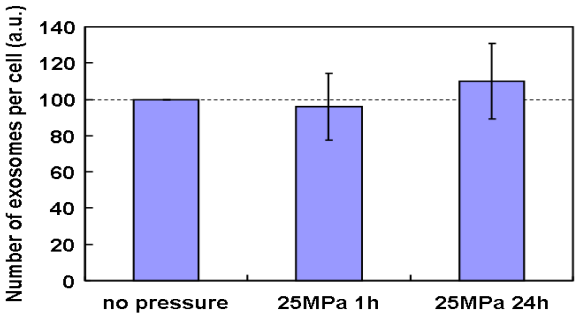

Fig.2: number of exosomes released in the medium over 24h by ATDC5 cells pressurized for 0, 1 or 24h under 25MPa, normalized to the cell number (a.u.). The graph shows the mean +/- Std Dev of three experiments.

(3) Effect of exosomes on the differentiation of ATDC5 cells in culture

In order to investigate whether exosomes produced from differentiating chondrocytes could trigger differentiation in progenitor cells, ATDC5 cells were differentiated by rotation culture, leading to aggregate formation, or monolayer culture in the presence of insulin, both protocols inducing differentiation. Exosomes were collected from the medium and used to stimulate non-differentiated ATDC5 cells. Real-time PCR showed a slight increase in Acan, Col2a1 and Sox9 expression after two days, but this was not significant (Fig. 3).

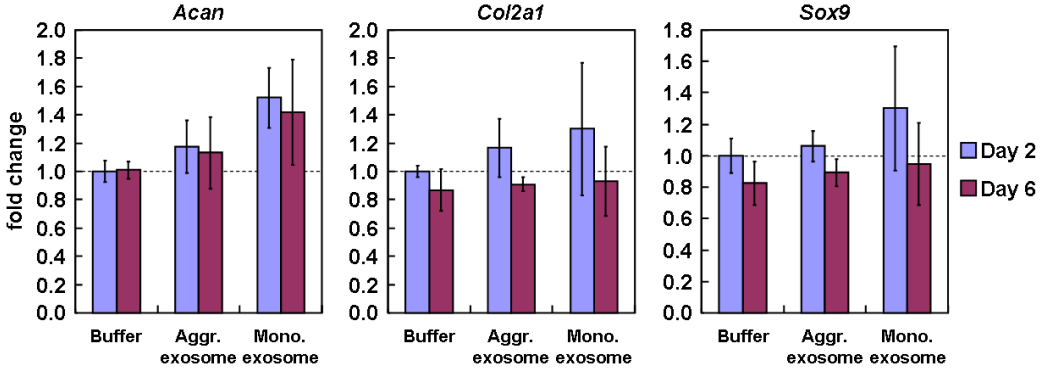

Fig. 3: *expression of Acan, Col2a1 and Sox9 in ATDC5 cells cultured in medium supplemented*

with or empty exosome resuspension buffer, exosomes from differentiated cell aggregates (Aggr.) or monolayers (Mono.) for 2 or 6 days. The graph shows the mean +/- Std Dev of three experiments

(4) Characterization of microRNAs contained in exosomes

In order to characterize exosomes produced under pressure, ATDC5 cells were pressurized for up to 24h under 25MPa and the exosomes were purified. RNA was then extracted from the exosomes and gel electrophoresis was performed using a Bioanalyzer. The electropherogram shows that most of the RNA is short and more than 50% consists of micro-RNA (Fig. 4). We therefore decided to identify the micro-RNAs contained in the samples. Micro-RNA microarray analysis was therefore performed. Many micro-RNAs were identified, including several involved in inflammation. This is in accordance with our previous study showing that high HP modulates the expression of many mRNAs involved in inflammation [6].

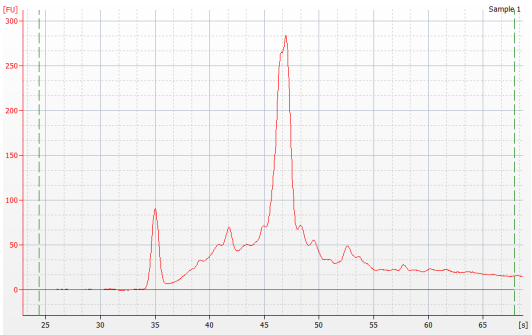

Fig. 4: typical electropherogram obtained from the exosome RNA samples. The first peak is an internal marker.

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〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　0件／うちオープンアクセス　2件）

１．著者名 Montagne Kevin, Onuma Yasuko, Ito Yuzuru, Aiki Yasuhiko, Furukawa Katsuko and Ushida Takashi	４．巻 12
２．論文標題 High hydrostatic pressure induces pro-osteoarthritic changes in cartilage precursor cells: a transcriptome analysis	５．発行年 2017年
３．雑誌名 PLOS One	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1371/journal.pone.0183226	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Kim Jeonghyun, Montagne Kevin, Nemoto Hidetoshi, Ushida Takashi and Furukawa Katsuko	４．巻 12
２．論文標題 Hypergravity down-regulates c-fos gene expression via ROCK/Rho-GTP and the PI3K signaling pathway in murine ATDC5 chondroprogenitor cells	５．発行年 2017年
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オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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