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Original article

GC/TOF-MS-based metabolomic profiling in cultured fibroblast-like synoviocytes from rheumatoid arthritis

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ABSTRACT

Objectives: Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by synovial inflammation and hyperplasia. Fibroblast-like synoviocytes (FLS) in RA exhibit a tumor cell-like aggressive phenotype. Thus, gas chromatography/time-of-flight-mass spectrometry (GC/TOF-MS) was employed to identify the characteristic metabolic profiling of RA FLS.
Methods: Metabolite profiling of RA FLS and osteoarthritis (OA) FLS was performed using GC/TOF-MS in conjunction with statistical analyses. We performed metabolite set enrichment analysis to establish which pathways are affected.
Results: A total of 129 metabolites were identified. A principal component analysis and hierarchical clustering analysis demonstrated clear differentiation of the metabolic profiling between RA FLS and OA FLS. The levels of 35 metabolites that belonged to the amines, fatty acids, phosphates, and organic acids class were significantly increased in RA FLS compared to those in OA FLS. Also, the levels of 26 metabolites that belonged to the amino acids, sugars, and sugar alcohols class were significantly decreased in RA FLS compared to those in OA FLS. The sugar metabolism (glycolysis and pentose phosphate pathway) and amino acid metabolism (tyrosine and catecholamine biosynthesis, and protein biosynthesis) were severely disturbed in RA FLS compared to those in OA FLS.
Conclusions: Our metabolic results suggested that the alteration of sugar metabolism, lipolysis, and amino acid metabolism in RA FLS is related to synovial hyperplasia and inflammation. This is the first metabolomic study to determine metabolic changes characteristic of RA FLS, which will provide valuable information to gain in-depth insights into the pathogenesis of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune joint disease characterized by inflammation in the synovial lining and synovial hyperplasia, which leads to joint destruction and disability [1]. The synovium in RA transforms from a quiescent, relatively acellular structure to a hyperplastic, invasive tissue. The hyperplastic synovial tissue, called pannus, invades and erodes contiguous cartilage and bone in RA [2]. The pannus, considered as a locally invasive tumor, is comprised of many cell types, including T cells, B cells, and fibroblast-like synoviocytes (FLS). FLS are unique cells that populate the intima lining of the synovium and become a prominent component of the destructive pannus in RA [2]. RA FLS resemble immature, transformed fibroblasts and differ in many ways from normal FLS and osteoarthritis (OA) FLS [3]. The characteristic of RA FLS is to exhibits a "tumor-like" aggressive phenotype including resistant apoptosis, anchorage independence, loss of contact inhibition, and a pattern of oligoclonal expansion [1–7]. The hyperplastic synovial tissue may be explained by an increased rate of proliferation or by a decreased rate of apoptosis of FLS [8]. RA FLS are expected to require much of the metabolic energy when induced into proliferating via resistant apoptosis and the loss of contact inhibition.

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Information in metabolic pathways is highly dynamic and represents the current physiological or pathological states of individual cells that result from the interactions between genes and the environment [9]. The metabolomic approach enables the identification of changes in the metabolic pattern and the elucidation of variations in phenotypes imposed by perturbations such as gene modification or environmental stimuli [10,11]. Recently, metabolomics has been used to study the metabolic changes that occur in cancer cells and FLS obtained from Dupuytren's disease or RA [12–15]. Although the pathogenic behaviors of RA FLS have been studied extensively, the metabolic alterations of their transformed phenotype in RA remain to be elucidated. Furthermore, while the transformed FLS in RA may undergo a metabolic alteration similar to that observed in cancer cells, there have been no published reports of non-targeted metabolomic studies of RA FLS.

Because immortalized mammalian RA FLS lines are not yet available for scientific research, primary FLS cultures have been employed. Actually, primary FLS cultures have been widely used to understand the pathogenesis and to evaluate novel therapeutic targets in RA. Also, the use of cell lines in metabolomics has the advantage of easy control and interpretation without possible confounding factors compared to the use of other human subjects [16].

A systematic characterization of the metabolic pathways in transformed RA FLS is currently lacking. Thus, we screened the intracellular metabolites in the RA FLS, which worked as key effector cells in RA, and examined the metabolite profiling of RA FLS using gas chromatography/time-of-flight-mass spectrometry (GC/TOF-MS) to characterize the metabolic profile of RA FLS compared to that of OA FLS.

2. Methods

2.1. Isolation and culture of fibroblast-like synoviocytes obtained from RA and OA

Our experimental protocol was reviewed and approved by the Samsung Medical Center institutional review board and signed consent was obtained from each patient included in this study. This study was conducted in accordance with the principles expressed in the Helsinki Declaration.

According to the American College of Rheumatology criteria [17], patients were classified as having RA. Synovial tissue was obtained from RA or OA of 3 patients at the time of an arthroscopic wrist synovectomy or total knee joint replacement. The clinical characteristics of the patients are depicted in Appendix A, Table S2 [See the supplementary material associated with this article online].

Synovial tissue was carefully excised without the adjacent adipose/connective tissue and digested overnight with 5 mg/mL type IV collagenase (Sigma, Poole, UK) and 150 μg/mL type I DNase (Sigma, Poole, UK), and was then separated from the undigested tissue by unit gravity sedimentation. After the suspended cells were collected into fresh tubes, the cells were harvested by centrifugation at 500 g (relative centrifuge force) for 10 min. The pellet was then washed twice with Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS). Next, the resuspended cells were plated at a concentration of 2×10^6 cells/mL in a total volume of 1 mL/200 mm^2 in T-25 culture flasks. Following overnight incubation, non-adherent cells were removed by replenishment with fresh culture medium, and the attached cells were cultured in DMEM with 10% FBS and 50 units/mL penicillin, 50 mg/mL streptomycin, and 0.025 mg/mL amphotericin B until 90% confluent growth. We used primary cultured cells at three to five passages for the metabolomic investigations.

2.2. Metabolite sample preparation of fibroblast-like synoviocytes

For the metabolic profiling of FLS, the sample preparation was performed with a modification to the previous studies [18,19]. After 5 mL of phosphate-buffered saline (PBS) buffer added to the cell culture medium, adherent FLS were gently detached using a rubber tripped cell scraper. To obtain metabolites from detached FLS, 1 mL of cell suspension was collected by vacuum-filtering using a nylon membrane filter (0.45-μm pore size; 30 mm diameter; Whatman, Piscataway, NJ, USA) and washed with 2 mL of PBS buffer at room temperature. The cells on the membrane were immersed in 10 mL WiPM (water/2-propanol/methanol, 2:2:5, v/v/v) and then the extraction mixture was frozen by immersion in liquid N_2. After being thawed, the extraction mixture was vortexed for 3 min. The supernatant was collected after centrifugation at 16,100 g for 5 min at 4 °C, and was then concentrated to dryness using a vacuum concentrator (Labcono, Kansas City, MO, USA). The entire metabolite extraction procedures were performed in duplicate for each FLS. All extracts were stored at −80 °C until derivatization and GC/TOF-MS analysis.

2.3. Gas chromatography/time-of-flight-mass spectrometry experiments

The dried samples were derivatized with 5 μL of methoxyamine hydrochloride in pyridine (40 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 90 min at 30 °C, followed by the addition of 45 μL of N-methyl-N-trimethylsilyl trifluoroacetamide (Fluka, Buchs, Switzerland) for 30 min at 37 °C, and finally, we added a mixture of fatty acid methyl esters to the samples.

The derivatized metabolites were analyzed by an Agilent 7890B GC (Agilent Technologies, Wilmington, DE, USA) coupled to a Pegasus HT time-of-flight-mass spectrometer (TOF-MS; Leco, St. Joseph, MI, USA) with electron impact at 70 eV by recoding in the mass range of 85 to 500 m/z at an acquisition rate of 17 spectra/s. A 1-μL aliquot of metabolite samples was injected directly into the GC in splitless mode. An RTX-5Sil MS column (30 m × 0.25 mm, 0.25 μm of film thickness; Restek, Bellefonte, PA, USA) and an additional 10-m-long integrated guard column were used for the separation of metabolites. The initial column temperature was 50 °C for 5 min, followed by a linear ramp to 320 °C at a rate of 20 °C/min. The temperatures of the injector, transfer line, and ion source were 250 °C, 280 °C, and 250 °C, respectively.

2.4. Statistical analyses

The data obtained from the GC/TOF-MS were preprocessed using Chroma TOF software (ver. 4.50; Leco). Next, we used BinBase, an in-house database, for further processing of the preprocessed data [20]. The processed data were then normalized by the abundance of the sum of the identified metabolites. For the statistical analysis of the processed data, we conducted univariate and multivariate statistical analyses using Statistica (ver. 7.1; StatSoft, Tulsa, OK, USA) and MultiExperiment Viewer (Dana-Farber Cancer Institute, Boston, MA, USA) [21,22]. To establish which pathways are affected in RA FLS, we conducted metabolite set enrichment analysis (MSEA) using MetaboAnalyst [23].

3. Results

3.1. Overview of metabolite profiles of cultured FLS

To investigate the metabolic profile characteristics of RA FLS, the metabolites obtained from cultured FLS from RA and OA were analyzed by GC/TOF-MS and over 1000 unique m/z values with retention times were detected. After deconvolution and alignment