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Anti-citrullinated protein antibodies suppress let-7a expression in monocytes from patients with rheumatoid arthritis and facilitate the inflammatory responses in rheumatoid arthritis

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ABSTRACT

We hypothesized that anti-citrullinated protein antibodies (ACPAs) could affect the expression of miRNAs in monocytes and contribute to the inflammatory responses in rheumatoid arthritis (RA). The expression profiles of 270 human miRNAs, co-cultured with ACPAs or human immunoglobulin G (IgG), were analyzed using real-time polymerase chain reaction. Ten miRNAs exhibited differential expression in U937 cells after co-cultured with ACPAs compared with human IgG. The expression levels of these miRNAs were investigated in monocytes from 21 ACPA-positive RA patients and 13 controls. Among these miRNAs, the expression levels of let-7a was decreased in monocytes from ACPA-positive RA patients. The expression levels of let-7a showed a negative correlation with positivity of rheumatoid factor in patients sampled. We found that transfection of U937 cells with let-7a mimic suppressed K-Ras protein expression. In the ACPA-mediated signaling pathway, transfection of U937 cells with let-7a mimic suppressed the ACPA-enhanced phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), and the expression and secretion of interleukin (IL)-1β. In conclusion, ACPA-mediated decreased let-7a expression in monocytes from ACPA-positive RA patients. Decreased let-7a expression was associated with the positivity of RF in ACPA-positive RA patients. The decreased expression of let-7a could facilitate the inflammatory pathway via enhanced ACPA-mediated phosphorylation of ERK1/2 and JNK and increased expression of IL-1β through an increase in the expression of Ras proteins.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovial joints and its pathogenesis involves multiple immunological, environmental, and genetic factors (Firestein, 2003; Cooles and Isaacs, 2011). Recent studies indicated that ACPAs could directly contribute to the pathogenesis of RA. Our previous studies showed that ACPAs could activate c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) in monocytes and leads the activation of nuclear factor (NF)-κB and secretion of tumor necrosis factor (TNF)-α (Lu et al., 2010, 2013a). In addition, ACPAs could also induce macrophages to produce TNF-α via dual engagement of Fcγ receptor and Toll-like receptor 4 (Clavel et al., 2008; Sokolove et al., 2011) and activate the complement system (Trouw et al., 2009). Furthermore, ACPAs could lead to bone destruction in patients with RA through enhancing the differentiation of osteoclast precursors into mature bone resorbing cells (Harre et al., 2012).

MicroRNAs (miRNAs), which are small, non-coding RNA molecules, can play a crucial role in regulating both the innate and adaptive immune responses. Aberrantly expressed miRNAs are known to participate in the pathogenesis of RA (Duroux-Richard et al., 2012). However, the effect of ACPAs on the expression of miRNAs in monocytes and its contribution to the inflammatory

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response in RA has not been investigated. We hypothesized that ACPAs can influence miRNAs expression in monocytes and thereby contribute to the inflammatory response in patient with RA. Therefore, we used a step-by-step strategy to search for aberrantly expressed miRNAs in RA monocytes mediated by ACPAs. First, the expression profile of miRNAs mediated by ACPAs was identified using U937 cells. The expression levels of these miRNAs were measured in monocytes from ACPA-positive RA patients and healthy controls. In addition, functional aspects of the aberrantly expressed miRNAs were explored and correlated with clinical parameters of RA. Finally, we investigated the biological functions of the specific miRNA in a transfection study.

2. Material and methods

2.1. Patients with RA and controls

Twenty-one ACPA-positive RA patients who have established RA defined according to the American College of Rheumatology 1987 revised criteria (Arnett et al., 1988) and 13 healthy controls were recruited for this study. The whole study was approved by the institutional review board of Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taiwan (No. B10203024). All participants provided their informed consent prior to enrollment. Blood samples were collected at least 12 h after the last dosage of immunosuppressants to minimize any drug effects. The demographic and clinical characteristics including age, sex, positivity and titers of rheumatoid factor (RF), titers of ACPAs, C-reactive protein (CRP), and medication use of the patients and controls were recorded.

2.2. Purification of ACPAs using pooled sera from ACPA-positive RA patients

Serum samples from ACPA-positive RA patients containing high concentration of ACPAs (>340 IU/ml), detected using an ELISA kit (Pharmacia Diagnostics AB, Uppsala, Sweden), were pooled. The pooled sera were then purified by affinity chromatography using the first generation synthetic cyclic citrullinated peptide (CCP) antigen (Schellekens et al., 2000). The purification process was performed using fast protein liquid chromatography (FPLC) with an ÄKTA purifier 10 (GE Healthcare, Little Chalfont, UK) as previously described (Lu et al., 2013a). The amount of ACPA used is based on the antigen-binding activity measured by the ELISA kit. The antigen-binding activity of the affinity-purified ACPAs at a protein concentration of 1 μg/ml was equal to 16 IU/ml. Therefore, to be comparable with a protein concentration of 120 IU/ml ACPAs, 7.5 μg/ml human polyclonal immunoglobulin G (IgG) was used as control. The endotoxin levels in purified ACPAs were less than <0.5 EU/ml, measured using *Limulus* amebocyte cell lysate assay (Lonza, Basel, Switzerland).

2.3. U937 cells pretreated with ACPAs

Purchased U937 cells (5×10^6) (American Type Culture Collection, Manassas, VA, USA) were incubated with 120 IU/ml ACPAs or 7.5 μg/ml human IgG (Sigma–Aldrich, St. Louis, MO, USA) in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing heat-inactivated fetal bovine serum (10%), L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 mg/ml) for 48 h. Each treatment was repeated three times.

2.4. Isolation of RNA

Total RNA (including miRNAs) was extracted from purified monocytes or U937 cells using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The RNA concentrations were quantified with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Assessment of miRNAs expression by real-time polymerase chain reaction

All miRNAs in the extracted total RNA (500 ng) were converted to corresponding cDNAs in a one-step reverse transcription (RT) reaction and the expression levels of miRNA were quantified by real-time polymerase chain reaction (PCR) using an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) as previously described (Lu et al., 2013b; Lai et al., 2013). Relative expression levels of miRNA were defined by the following equation: (39-threshold cycle [Ct] after adjusted by the expression of U6 small nuclear RNA [snRNA]).

2.6. Isolation of monocytes from ACPA-positive RA patients

Monocytes were isolated from heparinized venous blood obtained from ACPA-positive RA patients and healthy controls by negative selection using Dynabeads Untouched Human Monocytes kit (Life Technologies, Carlsbad, CA, USA) as previously described (Lu et al., 2013a).

2.7. Transfection of miRNA mimic into U937 cells

U937 cells (2×10^6/ml) were electroporated with 1 μg of scrambled oligonucleotides (as a control measure) or specific miRNA mimics (Ambion, Austin, TX, USA) using the Gene Pulser MXcell electroporation system (Bio-Rad Laboratories, Hercules, USA) with a setting of 250 V, 300 μF, and 1000 Ω according to a previous study (Jordan et al., 2008). Levels of miRNAs, mRNA, or proteins expressed in U937 cells transfected with let-7a mimics or scrambled oligonucleotides were analyzed after culture for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

2.8. Measurement of mRNA expression levels by real-time PCR

The mRNA expression levels of interleukin (IL)-1β, IL-6, IL-10, and TNF-α were quantified by real-time PCR using a one step RT-PCR kit (TaKaRa, Shiga, Japan) with an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) using the primers and method according to previous studies (Bechara et al., 2008; Lu et al., 2014).

2.9. Western blotting of cell lysates

U937 cell lysate (80–100 μg) was electrophoresed and transferred to a polyvinylidene difluoride (PVDF) sheet (Sigma–Aldrich). The membranes were non-specifically blocked in 1% skim milk solution and then incubated with the primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Primary antibodies were rabbit monoclonal anti-JNK, anti-phospho-JNK (Thr183/Tyr185), anti-p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2 (all from Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-KRAS antibodies (from ABcam, Cambridge, UK), rabbit monoclonal anti-c-Jun, anti-phospho-c-Jun (Ser63), anti-IL-1β, anti-CREB, anti-phospho-CREB (Ser133) antibodies and anti-β-actin antibodies as an internal control (Sigma–Aldrich); and as secondary antibodies, HRP-conjugated goat-anti-mouse IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and goat anti-rabbit IgG antibodies (Cell Signaling Technology) were utilized. Complexes formed were identified visually via enhanced chemiluminescence (ECL) detection system (GE