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Altered TNF-α response by Aconibal® and methotrexate in a lipopolysaccharide-induced setting of inflammatory conditions: Potential on a synergistic combination

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ABSTRACT

Ethnopharmacological relevance: *Aconitum carmichaelii* (AC) is a common herbal medicine used as anti-inflammatory and analgesic agent in Eastern Asia. In Korea, a commercial processed AC (Aconibal®) is traditionally used to treat the symptoms of spondylosis deformans and rheumatic pain.

Aim of study: Rheumatoid arthritis (RA) is systemic and autoimmune disease characterized by chronic inflammation. Methotrexate (MTX) is often the first-line therapy for RA. If MTX monotherapy is ineffective or RA is initially severe, adding a tumor necrosis factor alpha (TNF-α) inhibitor to the treatment can be beneficial. However, its inhibitory effects on RA when combined with MTX are unknown. Therefore, we investigated the stable modulation of and synergistic to additive effect on TNF-α using AC combined with MTX (AMC).

Materials and methods: An inflammatory response mimicking RA was induced in the mouse macrophage cell line Raw 264.7 using interferon-γ or lipopolysaccharide (LPS). We predicted that AC and MTX at a 3:1 ratio would have synergistic therapeutic effects and this was determined using the Chou-Talalay method of median effect analysis and CalcuSyn software. We analyzed the profiles of various inflammatory cytokine-related proteins using Search tool for retrieval of interacting genes and Kyoto Encyclopedia of Genes and Genomes.

Results: The expression levels of selected inflammatory immune mediators such as interleukin (IL)-6, IL-1α, chemokine ligand 5, granulocyte-colony stimulating factor, nitric oxide synthase, and cyclooxygenase were reduced via regulation of the mitogen-activated protein kinase signaling pathway. AMC inhibited the levels of matrix metalloproteinases-1 and −3 in the human synovial cell line SW982.

Conclusions: Our data show for the first time the potential beneficial effects of AMC in RA management.

1. Introduction

Rheumatoid arthritis (RA) is a widely prevalent, systemic autoimmune disease characterized by inflammation, hyperplasia of the synovium, and macrophage and lymphocyte infiltration (Kaplan, 2013). It leads to erosion of articular cartilage and progressing joints (Karsdal et al., 2011). Methotrexate (MTX) is the standard disease-modifying anti-rheumatic drug (DMARD) used for the management of RA (Karsdal et al., 2011). Generally, MTX improves the signs and symptoms of RA and slows the progression of joint destruction in RA patients (St Clair et al., 2004). However, many patients fail to achieve stable response to MTX treatments (Pavelka et al., 2012). Finally, MTX is often used combination with other conventional synthetic DMARDs to improve treatment outcomes (O'dell et al., 1996). The use of biologic DMARDs, which are also anti-tumor necrosis factor (TNF)-α agents, with or without MTX is recommended for managing early RA with high disease activity and poor prognosis (O'dell et al., 1996). Approved biological DMARDs such as inhibitors of TNF-α (Humira, Cimzia, Enbrel, Simponi, and Remicade), IL-1 receptor (Kineret), and (IL)-6 receptor (Actemra) (Šenolt et al., 2009).

TNF-α is a pleiotropic function of proinflammatory cytokine secreted by macrophages in the synovial membrane and lining layer of cartilage-pannus junction (Feldmann et al., 1996; Kinne et al., 2000). It plays a critical role in the mediation of synovitis, articular matrix

Abbreviations: AC, *Aconitum carmichaelii*; AMC, AC (Aconibal®) and methotrexate; Cox-2, Cyclooxygenase-2; DMSO, Dimethyl sulfoxide; ERK, Extracellular signal-regulated kinase; GCSF, Granulocyte-colony stimulating factor; HRP, Horseradish peroxidase; IFN, Interferon; IL, Interleukin; iNOS, Inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; MIP, Macrophage inflammatory protein; MMP, Matrix metalloproteinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MFDS, Ministry of Food and Drug Safety; MTX, Methotrexate; NO, Nitric oxide; PGE2, Prostaglandin E2; RA, Rheumatoid arthritis; SDF, Stromal cell-derived factor; sTNF-R, Soluble TNF-receptor; STRING, Search Tool for the Retrieval of Interacting Genes; TNF-α, Tumor necrosis factor alpha

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degradation, and bone erosion in RA (McInnes and Schett, 2007). Anti-TNF-α antibodies and TNF-α receptor constructs have shown remarkable efficacies in acute diseases and in suppressing radiographic progression (Palladino et al., 2003). Mounting evidence suggests that TNF-α activation is implicated in the RA pathogenesis (Palladino et al., 2003). Moreover, TNF-α regulates the production of many cytokines such as IL-1β and interferon (IFN)-γ (Vazquez et al., 2015). It also stimulates cartilage and bone resorption and inhibits the syntheses of articular collagen and proteoglycan (Zhen and Cao, 2014). Inhibition of TNF-α in a transgenic animal model of inflammatory arthritis remarkably suppressed joint inflammation (Li and Schwarz, 2003). To check pathogenic cytokine production, MAPK pathways have been investigated via their signaling enzymes and their downstream effector pathways (Huh et al., 2012). Collectively, these studies indicate that TNF-α may be a novel therapeutic target in the management of RA.

Aconitum carmichaelii (AC) is a common herbal medicine in Eastern Asia (Zhou et al., 2015). It has several pharmacological activities, which include anti-inflammatory, analgesic, anti-tumor, hypoglycemic, hypolipidemic, and anti-aging effects (Nyirimigabo et al., 2015; Zhou et al., 2015). It also has effects on energy metabolism and the immune system (Zhou et al., 2015). Additionally, it has protective effects in the kidney and liver. In Korea, a commercial processed AC (Aconibal®) is used to treat the symptoms of spondylosis deformans and rheumatic pain (Park et al., 2016). However, no studies have been conducted on its role in combination therapy of MTX and TNF-α inhibition.

In this study, the stable modulation of and synergistic to additive effect on TNF-α were investigated using AC and MTX (AMC) as a combination therapy. In addition, the inhibitory effects of AMC on inflammatory cytokine responses induced by lipopolysaccharide (LPS) were assessed 1) in vitro systemic inflammatory setting by macrophage and 2) knee articular inflammation-mimicked setting by human synovial cells. The possible mechanisms involved in the above effects were also evaluated by assessing the impact of AMC on the mitogen-activated protein kinase (MAPK) signaling pathway.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS), were purchased from Gibco (MD, USA). MTT, IFN-γ, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Rabbit anti-Cox-2 was obtained from Thermo (Cambridge, UK). Rabbit anti-iNOS was obtained from Santa Cruz (Cambridge, UK). Anti-rabbit-horseradish peroxidase (HRP) secondary antibody was purchased from Assay Designs (Ann Arbor, USA). All other reagents used were of guaranteed or analytical grade.

2.2. Experimental drug material information

Excluding the excipient, the processed AC powder (code: #4535; batch number: 1509) was kindly supplied by HanPoong Pharmaceutical Co., Ltd. (Seoul, Korea), and a voucher sample (KIOM-ACO) was deposited at the herbarium of the Korea Institute of Oriental Medicine (Daejeon, Korea). Benzoyl aconine (37 mg/g) was used as quality control index compound and in a purity test, aconitine (0.0018 mg/g) of the processed AC (Aconibal®) was analyzed and characterized according to the Korean herbal pharmacopoeia. According to the quality control report from HanPoong Pharmaceutical Company, the prerequisite information of AC, such as, visual check, comparison of standard drug, mass deviation test and disintegration test were clearly stated in compliance with Korea Ministry of Food and Drug Safety (MFDS). Unfortunately, the method of extraction and preparation of the final formulation could not be stated here according to the confidentiality maintenance agreement by HanPoong Pharmaceutical Co., Ltd.

2.3. Cell culture

Raw 264.7 cell, mouse macrophage cell line, was obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea). SW982 cell, human synovial cell line, was obtained from the America Type Culture Collection (ATCC; VA, USA). Cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in condition of 95% air and 5% CO_2 at 37 °C. All experiments were conducted 12 h after the cells had been seeded on the 96 and 24-well plates at densities of 1×10^4 and 1.2×10^4 cells/well, respectively.

2.4. Measurement of cytotoxicity

The cells were treated simultaneously with AC or MTX at concentrations of 1–1000 μg/mL for 24 h by the experimental stimuli of LPS at 100 ng/mL and IFN-γ at 100 μg/mL, and then incubated with 1 mg/mL MTT for 3 h. The medium was aspirated carefully from the wells and the formazan dye was eluted using DMSO. The absorbance was measured using a spectrophotometer (Versamax microplate reader, Molecular Device, Sunnyvale, CA, USA) at a wavelength of 570 nm and expressed as a percentage of the control.

2.5. Measurement of the expression levels of inflammatory cytokines, RANTES, IL-6, IL-1, prostaglandin E2 (PGE2), fractalkine, Cox-2, iNOS, and matrix metalloproteinases (MMPs), and evaluation of MAPK signaling

MAPK signaling and the levels of inflammatory cytokines, RANTES, IL-6, IL-1, PGE2, fractalkine, Cox-2, iNOS, and MMPs were quantified using commercial and enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. In addition, western blotting was performed according to previously published methods (Park et al., 2016).

To detect Cox-2 and iNOS proteins, cells were lysed and the lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10–15% gel). Next, the proteins were transferred onto membranes, which were then incubated with 5% skim milk in Tris-buffered saline with Tween for 1 h. Afterwards, the membranes were incubated with primary antibodies (1:1000 dilutions) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were detected using an ECL detection kit, and an LAS-4000 mini system (Fujifilm Corporation, Tokyo, Japan) was used for visualization.

2.6. Analysis of the effects of AMC

Possible interactions between AC and MTX were evaluated using the isobologram method and the median effect method described by Chou and Talalay (Chou and Talalay, 1984). In the isobologram method, the obtained doses were noted and a line of additivity was drawn to connect both data points. An extended combinatory effect (synergism, antagonism, or additivity) of AC and MTX was determined by the median effect analysis described by Chou Talalay using CalcuSyn software (version 1.0; BIOSOFT, Cambridge, UK). In this method, the interpretation of combination index (CI) values was as follows: < 1, synergism; 1, additive effect; and > 1, antagonism.

2.7. Data analysis

Search Tool for the Retrieval of Interacting Genes (STRING) was used to construct a network model showing protein interactions based on known and predicted protein-protein interactions. In addition, details of protein ontology and functional relationships were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG).