Chemico-Biological Interactions 230 (2015) 58–70

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

A novel therapeutic approach targeting rheumatoid arthritis by combined administration of morin, a dietary flavanol and non-steroidal anti-inflammatory drug indomethacin with reference to pro-inflammatory cytokines, inflammatory enzymes, RANKL and transcription factors

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ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form 22 January 2015
Accepted 10 February 2015
Available online 16 February 2015

Keywords:
Morin
Indomethacin
Pro-inflammatory cytokines
Inflammatory mediators

ABSTRACT

The present study was designed to assess the combined efficacy of morin, a dietary flavanol and non-steroidal anti-inflammatory drug indomethacin against adjuvant-induced arthritis in rats, an experimental model for rheumatoid arthritis. Arthritis was induced by intradermal injection of complete freund's adjuvant (0.1 ml) into the right hind paw of the Wistar albino rats. Morin (30 mg/kg b.wt), indomethacin (3 mg/kg b.wt) and combination of morin and indomethacin were administered intraperitoneally (from 11th to 20th day) after adjuvant injection. We have found that the activities/levels of lysosomal acid hydrolases (acid phosphatase, β-galactosidase, N-acetyl glucosaminidase and cathepsin-D), glycoproteins (hexose and hexosamine), urinary constituents (hydroxyproline and glycosaminoglycans), reactive oxygen species (LPO and NO), elastase, inflammatory mediators (TNF-α, IL-1β, MCP-1, VEGF and PGE2) and paw edema were significantly increased in arthritic rats compared to controls. Whereas, the anti-oxidant status (SOD, CAT, GPx, glutathione, and ceruloplasmin), body weight and bone collagen was found to be decreased. The mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-17, IL-6 and MCP-1), inflammatory enzymes (iNOS and COX-2), RANKL, and transcription factors (NF-kB p65 and AP-1) was found upregulated in the ankle joints of arthritic rats in qRT-PCR analysis. In addition, the increased protein expression of NF-kB p65 and COX-2 was also detected by immunohistochemical analysis. On the other hand, the above said imbalances were regulated back effectively to near normal as evidenced by the histopathological and radiological analysis on combined treatment with morin and indomethacin. Our study indicates that the combination therapy was more effective than either single drug alone in suppressing the pathogenesis of RA.

1. Introduction

Rheumatoid arthritis (RA) is one of the commonest autoimmune disorders characterized by joint involvement leading to disability, deformity, morbidity and mortality [1]. This disease strikes approximately 1% of the adult population and the most prominent clinical symptoms are swollen, red and pain joints. Although the case of RA is yet obscure, the pathogenic mechanisms driving the disease are better seen. Activated immune cells, such as T and B lymphocytes and macrophages infiltrate the affected synovial tissues, and boost the release of pro-inflammatory cytokines, including tumor necrosis factor, interleukin-1 and interleukin-6, which contribute to synovial inflammation, pannus formation and joint destruction in RA. The pro-inflammatory cytokines and chemokines further attract leucocytes from blood vessels in the joints and stimulate these leucocytes to migrate to synovium to develop synovitis. In addition, activated T cells in the synovial joint result in the departure of large amounts of RANKL (receptor activator of nuclear factor-kB ligand) which in turn stimulates macrophages to differentiate into mature osteoclasts within pathological joints, where mature osteoclasts resorb bone resulting in bone destruction [2]. The most noticeable characteristic of RA is the progressive demolition of articular cartilage and bone, which

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http://dx.doi.org/10.1016/j.cbi.2015.02.007
0009-2797/

is coordinated by activating RA fibroblast like synoviocytes, a major cell type accounting for the thickened lining layer as well for pannus formation in synovium. Fibroblast like synoviocytes share many characteristics with tumor cells with up regulated expression and production of pro-inflammatory cytokines, matrix metalloproteinases (MMPs), prostaglandin E2 (PGE2), and cyclooxygenase-2 (COX-2) which further promote inflammation, hyperplasia and cartilage destruction [3]. NF-kB is a pleiotropic transcription factor that acts as a significant part in modulating the expression of pro-inflammatory cytokine (TNF-α, IL-1β, IL-6 and IL-17) genes involved in immune responses, which are all closely connected with the pathogenesis of RA. The inflammatory gene products further activate transcription factor NF-kB and leads to the expression of inflammatory mediators, including chemokines, matrix metalloproteinases and adhesion molecules, which contributes to the pathogenesis of RA by stimulating bone erosion and systemic bone loss [4]. Many studies have validated a role of reactive oxygen species in the pathogenesis of rheumatoid arthritis. It is known that ROS can function as a second messenger to activate nuclear factor kappa-B, which orchestrates the expression of a spectrum of genes involved in the inflammatory response [5]. Nitric oxide (NO), a short lived signaling molecule plays an important role in the pathophysiology of various inflammatory diseases including RA. The inflamed joint in RA is the predominant source of NO and several cell types like osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils and endothelial are found to generate NO in the inflamed synovium [6]. Numerous investigators are found to correlate between serum nitrite concentration and RA disease activity or radiological progression [7]. It has likewise been reported that reduction in the production of NO via suppressing or inhibiting inducible nitric oxide synthase (iNOS) decreased disease activity in experimental RA [8]. Since, it is evident that pro-inflammatory cytokines, inflammatory enzymes, inflammatory mediators (prostaglandin E2 and cyclooxygenase-2) and reactive oxygen species are highly expressed in rheumatoid joint and play key role in the pathophysiology of RA. Therefore, targeting the intracellular signaling pathways, cytokines and other inflammatory mediators which are responsible for the pathogenesis of RA would be beneficial for the dominance and the management of rheumatoid arthritis.

Though, significant therapeutic advances have improved the lives of patients with RA, the difficulty is hardly settled. As for many years, for the treatment of rheumatoid arthritis, non-steroidal anti-inflammatory drugs, steroidal agents, disease modifying anti-rheumatic drugs and immunosuppressant are used generally. Among these drugs, non-steroidal anti-inflammatory drugs are normally prescribed by the clinicians due to their clinical efficacy. Nevertheless, long term administration of high dosages has various side effects, including gastrointestinal disorders and renal morbidity, which limit their utility in the treatment of RA [9]. In sight of these limitations, it is necessary to extend the search to develop safe preventive and therapeutic agents for RA. The combination of NSAIDs with natural products is an alternative for the enhancement of anti-inflammatory and anti-arthritic effects, without side effects like gastric injury. In this respect, an approach has been made to treat RA with a natural dietary compound having potent antioxidant activity in combination with NSAIDs.

Flavonoids are polyphenolic compounds widely available in plant kingdoms, dietary factors, and beverages and they are regularly consumed in a healthy diet in many countries [10]. Flavonoids are found to possess many interesting biological properties including anticancer, antimicrobial, anti-inflammatory, immunomodulatory and antithrombotic activities. Among these biological activities, many investigations have repeatedly demonstrated that different flavonoid molecules (rutin and quercetin) and flavonols (hesperidin and hesperitin) exhibit anti-inflammatory functions in acute and chronic inflammatory animal models [11]. Morin (2′,4′,3,5,7-pentahydroxyflavonoid) is one such dietary bioflavonoid (light yellow pigment) found in guava leaves, onion, apples, and Moraceae group which is used as dietary factors and herbal medicines [12]. It exhibits many biological activities including antioxidant, cytoprotection, antimutagenesis, antidiabetic and anticarcinogenic effects [13]. In addition, the anti-inflammatory properties of morin are also reported. For instance, morin inhibited the level of IL-1β in chronic experimental colitis in rats and suppressed the production of NO and PGE2 in lipopolysaccharide (LPS)-induced RAW 264.7 cells [14–15]. Recently, it has been reported that morin inhibited the production of NO and PGE2 and suppressed the expression of iNOS and COX-2 in human articular chondrocytes *in vitro* as well as in osteoarthritic animal models [16].

Morin was also found to inhibit lipoxygenase-1, inducible nitric oxide synthase, inflammatory cytokines and cyclo-oxygenase expression in activated immune cells like macrophages and mast cells [17–19]). It has also been shown to modulate the transcription factor NF-kB activation via the ERK and p38 MAPKs signaling pathways by its reactive oxygen species scavenging activity [20]. Moreover, it has been reported that morin administration for prolonged period has not shown any toxicity in experimental animals even at higher dosages [21]. Considering its anti-inflammatory potential and lack of reports on its mechanism of anti-inflammatory action against rheumatoid arthritis, the present study was designed to investigate the anti-arthritic efficacy of morin with or without indomethacin, a NSAID in adjuvant-induced arthritis in rats with reference to oxidative stress, inflammatory markers, bone degrading enzymes, pro-inflammatory cytokines, RANKL, and transcription factor NF-kB and AP-1.

2. Materials and methods

2.1. Drug and chemicals

Complete freund's adjuvant (CFA), Morin, N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide, and thiobarbituric acid (TBA) were purchased from Sigma Aldrich, St. Louis, USA. Indomethacin was purchased from Micro Labs Limited, Bangalore, India. Antibodies were purchased from Cell Signalling Technology, Massachusetts, USA. All other reagents and solvents used were of analytical grade obtained from local commercial sources.

2.2. Animals

Wistar young albino rats of either sex (130–160 g) were selected, since it is well established that young rats are susceptible to the development of arthritis and there is no difference in incidence between males and females. They were housed in the Animal House of VIT University at an ambient temperature of 25 ± 2 °C with standard laboratory conditions 12 ± 1 h day and night rhythm throughout the experimental period. They received free access to standard rodent pellet diet and water ad libitum. The experimental protocol was carried in conformity with the Institutional Animal Ethical Committee (IAEC), VIT University, India. The animals were treated and cared in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

2.3. Experimental design

Rats were divided into six groups, each comprising six animals.

Group I: control rats administered vehicle alone (sterile saline) for 10 days.