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Research paper

Atractylodes macrocephala Koidz stimulates intestinal epithelial cell migration through a polyamine dependent mechanism

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ABSTRACT

Ethnopharmacological relevance: *Atractylodes macrocephala* Koidz (AMK), a valuable traditional Chinese herbal medicine, has been widely used in clinical practice for treating patients with disorders of the digestive system. AMK has shown noteworthy promoting effect on improving gastrointestinal function and immunity, which might represent a promising candidate for the treatment of intestinal mucosa injury. The aim of this study was to investigate the efficacy of AMK on intestinal mucosal restitution and the underlying mechanisms via intestinal epithelial (IEC-6) cell migration model.

Materials and methods: A cell migration model of IEC-6 cells was induced by a single-edge razor blade along the diameter of the cell layers in six-well polystyrene plates. After wounding, the cells were grown in control cultures and in cultures containing spermidine (5 μM, SPD, reference drug), alpha-difluoromethylornithine (2.5 mM, DFMO, polyamine inhibitor), AMK (50, 100, and 200 mg/L), DFMO plus SPD and DFMO plus AMK for 12 h. The polyamines content was detected by high-performance liquid chromatography (HPLC) with pre-column derivatization. The Rho mRNAs expression levels were assessed by Q-RT-PCR. The Rho and non-muscle myosin II proteins expression levels were analyzed by Western blot. The formation and distribution of non-muscle myosin II stress fibers were monitored with immunostaining techniques using specific antibodies and observed by confocal microscopy. Cell migration assay was carried out using inverted microscope and the Image-Pro Plus software. All of these indexes were used to evaluate the effectiveness of AMK.

Results: (1) Treatment with AMK caused significant increases in cellular polyamines content and Rho mRNAs and proteins expression levels, as compared to control group. Furthermore, AMK exposure increased non-muscle myosin II protein expression levels and formation of non-muscle myosin II stress fibers, and resulted in an acceleration of cell migration in IEC-6 cells. (2) Depletion of cellular polyamines by DFMO resulted in a decrease of cellular polyamines levels, Rho mRNAs and proteins expression, non-muscle myosin II protein formation and distribution, thereby inhibiting IEC-6 cell migration. AMK not only reversed the inhibitory effects of DFMO on the polyamines content, Rho mRNAs and proteins expression, non-muscle myosin II protein formation and distribution, but also restored cell migration to control levels.

Conclusions: The results obtained from this study revealed that AMK significantly stimulates the migration of IEC-6 cells through a polyamine dependent mechanism, which could accelerate the healing of intestinal injury. These findings suggest the potential value of AMK in curing intestinal diseases characterized by injury and ineffective repair of the intestinal mucosa in clinical practice.

1. Introduction

Damage to the gastrointestinal epithelium can result from infection (ulcer), chemical agents (alcohol or other drugs), stress, or mechanical forces (stretching), and immediate repair is required to restore the epithelial barrier against luminal antigens. Restitution of superficial wounds in the gastrointestinal mucosa occurs by sloughing off the damaged epithelial cells and migration of

Abbreviations: AMK, *Atractylodes macrocephala* Koidz; PUT, putrescine; SPD, spermidine; SPE, spermine; TCM, traditional Chinese medicine; ODC, ornithine decarboxylase; UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease

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remaining viable cells from areas adjacent to, or just beneath, the injured surface to cover the wounded area in vivo (Rao et al., 1999). This early rapid mucosal reepithelialization is a consequence of epithelial cell migration into the damaged area rather than epithelial cell proliferation and absolutely requires cellular polyamines (McCormack et al., 1993; Gao et al., 2013).

Eukaryotic cells contain and synthesize the polyamines spermidine and spermine and their precursor, putrescine. The first rate-limiting step in polyamine synthesis is the production of putrescine from the amino acid ornithine. This reaction is catalyzed by ornithine decarboxylase (ODC), ODC can be inhibited by α-difluoromethylornithine (DFMO), which binds covalently and irreversibly to the activated enzyme (Johnson and McCormack, 1999). Increasing evidence has demonstrated that the cellular polyamines, either synthesized endogenously or supplied luminally, play an important role in the regulation of mucosal restitution and are essential for the maintenance of gastrointestinal mucosal integrity. Polyamines accelerate early mucosal restitution of gastric and duodenal mucosal stress erosions in vivo (McCormack et al., 1993) and IEC-6 cell migration over a denuded area in vitro (Wang et al., 1996).

The coordinated movement of epithelial cells is a complex process that depends on the cytoskeleton. Changes in both the distribution and formation of the cytoskeleton alter the adhesion, spreading, and motility of cells. Recently, the regulation of cytoskeletal rearrangements required for directed cell migration has become focused on the Rho family of guanine nucleotide triphosphate (GTP)-binding proteins including RhoA, Rac1, and Cdc42 (Hanna and El-Sibai, 2013; Thumkeo et al., 2013). Increasing evidence indicates that activated Rho proteins interact with cellular target proteins or effectors to regulate a signal transduction pathway linking surface receptors to the formation of actomyosin stress fibers and focal adhesions (Rao et al., 2001; Murali and Rajalingam, 2014). The activation and expression of RhoA result in the formation of actomyosin stress fibers by initiating myosin light chain phosphorylation. On the other hand, activation of Rac promotes de novo actin polymerization at the cell periphery to form lamellipodial extensions and membrane ruffles, and activation of Cdc42 results in actin polymerization to form filopodia or microspikes (Rao et al., 2001). In IEC-6 cells, Cdc42, Rac1, and RhoA work in concert with each other, creating a complex regulatory network for the regulation of the actin cytoskeleton, and consequently cell migration (Ray et al., 2003).

It is generally accepted that non-muscle myosin II acts downstream of Rho in the regulation of the early phase of intestinal epithelial restitution. Non-muscle myosin II is an important cellular motor molecule within the epithelial cells of the intestinal mucosa, it is implicated in the lamella and lamellipodia of migrating cells, and non-muscle myosin II is thought to play a major role in the regulation of cell shape and the direction and rate of migration (Wolfenson et al., 2009). When cells were depleted of polyamines with DFMO, there was a noticeable absence of actin stress fibers and an increase in the density of the actin cortex (Ray et al., 2002).

The rhizome of *Atractylodes macrocephala* Koidz (AMK, “Baizhu” in Chinese), which belongs to the Compositae family, is one of the most popular traditional Chinese medicinal herbs and has been widely used for thousands of years. It was first recorded in the Chinese book “Prescriptions for Fifty-two Diseases” written in Warring States period (476–221 BC). AMK has long been used to treat splenic hypofunction with inappetence, abdominal distension and diarrhea, dizziness and palpitation due to retention of phlegm and fluid, edema, spontaneous sweating, and threatened abortion. With regard to the chemical constituents of processed AMK, the major ingredients are eudesmane-type sesquiterpenoids, i.e., atractylon and atractylenolides I, II, and III, which are closely related to pharmacological functions of AMK (Li and Yang, 2014), including the gastroprotective activity (Wang et al., 2010), anti-inflammatory activity (Dong et al., 2008; Wang et al., 2009), stimulating effects on immune system (Lee et al., 2007), and anti-carcinogenic activity (Kang et al., 2011).

We recently demonstrated that AMK could stimulate the migration of intestinal epithelial cells through polyamine-mediated signaling pathway (Song et al., 2014). AMK increased polyamines content both in normal and polyamine-dificient IEC-6 cells. However, the precise mechanisms at the molecular level by which AMK mediates polyamine-dependent cell migration after wounding remain to be elucidated. AMK was demonstrated to exhibit significant efficacy in the treatment of gastrointestinal disorders as recorded by the Chinese Pharmacopoeia (China Pharmacopoeia Committee, 2010) as well as some previous studies of our laboratory (Hu et al., 2011; Wen et al., 2012; Song et al., 2014) and others (Liu et al., 2008). Therefore, the present study was designed to investigate the efficacy of AMK on intestinal mucosal restitution and the underlying mechanisms via IEC-6 cell migration model. In the current investigation, we proposed to determine the beneficial role of AMK in the cellular pathway leading to increased cell migration by raised Rho and non-muscle myosin II expression following polyamines elevation during restitution in intestinal epithelial cells. First, we examined the effects of AMK on the changes in the rate of cell migration when it was added to normal and polyamine-depleted IEC-6 cells after wounding. Second, we were particularly interested in the involvement of GTP-Rho and non-muscle myosin II expression in the mechanisms responsible for AMK-stimulated IEC-6 cell migration, since previous studies (Wang et al., 1996; Ray et al., 2003) have demonstrated that GTP-Rho and non-muscle myosin II are absolutely required for stimulation of IEC-6 cell migration. Third, we determined whether treatment with AMK would affect the cellular formation and distribution of non-muscle myosin II stress fibers.

2. Materials and methods

2.1. Cell lines, chemicals, and biochemicals

The IEC-6 cell line, which derived from normal rat small intestine, was purchased from American Type Culture Collection (ATCC) at passage 14 (Lot. 58541019). Dulbecco's modified Eagle's medium (DMEM, Lot. 8112144), fetal bovine serum (FBS, Lot. 1027810), and Penicillin Streptomycin (Pen Strep, Lot. 1116265) were purchased from Life technologies (Eugene, Oregon, USA). Insulin (Lot. 11070-73-8), Putrescine (PUT, Lot. BCBH3135V), Spermidine (SPD, Lot. BCBH7695V), Spermine (SPE, Lot. BCBG0222V), and alpha-difluoromethylornithine (DFMO, Lot. #039K4712V) were purchased from Sigma (St. Louis, MO, USA). RNAiso Plus reagent (Lot. A7603-1), PrimeScript™ RT Master Mix (Lot. AK2301), and SYBR Premix Ex Taq™ II (Lot. AK2901) were obtained from TaKaRa (Dalian, China). Mouse anti-beta actin (Lot. GR111289-1), Mouse anti-Rac1 (Lot. ab33186), Mouse anti-Cdc42 (Lot. GR116439-1), Rabbit anti-RhoA (Lot. GR503-6), Rabbit anti-non-muscle myosin II (Lot. GR144491-1) antibodies, Goat polyclonal secondary antibody to Rabbit IgG (HRP, Lot. GR1010882-1), Goat polyclonal secondary antibody to mouse IgG (HRP, Lot. GR1010881-1), and Goat anti-rabbit IgG H&L (Alexa Fluor 488, Lot. GR158636-1) were all purchased from Abcam (Hong Kong, China). Authentic standards atractylenolide I, atractylenolide II, and atractylenolide III (Lot. 73069-13-3, 83-48-7, and 73030-71-4, respectively) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).