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Research Paper

Modulation of thioacetamide-induced hepatic inflammations, angiogenesis and fibrosis by andrographolide in mice

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ABSTRACT

Ethnopharmacological relevance: Liver fibrosis is a complex disease in which several pathological processes, such as inflammation and angiogenesis, are closely integrated.
Materials and methods: We hypothesised that treatment with the pharmacological agent, andrographolide (AP), which has multiple mechanisms of action, will provide a greater understanding of the role of AP during the multiple pathological processes that occur in advanced liver disease.
Results: Liver fibrogenesis was induced in mice using thioacetamide (TAA), which was administrated for 6 weeks. Andrographolide (5, 20 or 100 mg/kg) was then given once daily following TAA injection. Liver collagen was examined using hydroxyproline and α-SMA, while the inflammatory response was quantified by Western blot and RT-PCR assays. Liver angiogenesis, neutrophil infiltration and hypoxia were assessed using CD11b+, vWF and HIF-1α immunostaining. Mice with liver injuries that were treated with andrographolide showed improved inflammatory response and diminished angiogenesis and hepatic fibrosis. Andrographolide treatment inhibited liver neutrophil infiltration, while a decreased in TNF-α and COX-2 signalling indicated macrophage activation. Andrographolide decreased overall liver hypoxia, as shown by the downregulation of hypoxia-inducible cascade genes, such as VEGF. Andrographolide treatment resulted in a significant decrease in hepatic fibrogenesis, α-SMA abundance, and TGF-βR1 expression.
Conclusions: The present results suggest that multi-targeted therapies directed against angiogenesis, inflammation, and fibrosis should be considered for the treatment of advanced liver injury. They further suggest that andrographolide treatment may be a novel therapeutic agent for the treatment of liver disease.

1. Introduction

Patients with chronic liver disease undergo an intense process of tissue remodelling that is characterised by chronic inflammation, neoangiogenesis, and fibrogenesis (Tugues et al., 2007; Friedmam, 2008). The pathological process during the development of fibrosis consists of a complex network of interacting cells, fibrogenic mediators and extracellular matrix molecules. Angiogenesis, which has been shown to play an important role in the development of liver fibrosis (El-Assal et al., 1998; Yoshiji et al., 2003), involves a tightly regulated network of cellular and molecular mechanisms that result in the formation of functional vessels. Angiogenesis is important during repair and is also a hallmark of the inflammatory response where both phenomena are closely integrated. Mediators of inflammation have direct angiogenic activities, and angiogenesis, in turn, contributes to the amplification of the inflammatory response due to the expression of adhesion molecules and chemokines in the neovessels, which further promote inflammation status. Several mechanisms participate

Abbreviations: α-SMA, α-smooth muscle actin; vWF, von Willebrand factor; HIF-1α, hypoxia-inducible factor-1α; TNF-α, tumour necrosis factor-α; COX-2, cyclooxygenase-2; VEGF, vascular endothelial growth factor; TGF-βR1, transforming growth factor-β receptor 1

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in the regulation of vascular endothelial growth factor (VEGF), a well-established angiogenic factor, including the recruitment of inflammatory cells, which then amplify angiogenesis via secretion of cytokines, such as TNF-α, TGF-β, IL-1, and IL-6, that enhance angiogenesis either directly or via the upregulation of VEGF (Pertovaara et al., 1994; Cohen et al., 1996). Most chronic liver diseases are characterised by fibrosis and inflammation. Fibrotic tissue leads to decreased blood flow and oxygen delivery, thus becoming hypoxic. Proinflammatory mediators, as well as other hypoxic stimuli, can elicit an angiogenic response through the induction of hypoxia-inducible factor-1α-dependent transcriptional activity, including VEGF production (Masferrer et al., 2000; Richard et al., 2000). The therapeutic potential of select cyclooxygenase-2 (COX-2) inhibitors has been shown in tumour-related angiogenesis (Subbaramaiah and Dannenberg 2003). However, these inhibitors may be effective in nontumoural hepatic angiogenesis as COX-2 activation also occurs in chronic liver inflammation (Williams et al., 1999). In addition, VEGF was recently found to have potent proinflammatory properties during hepatic fibrosis. In the liver, activated hepatic stellate cells (HSCs) express VEGF and VEGF receptors after treatment with carbon tetrachloride (CCl_4) and may respond to hypoxia by expressing VEGF in vitro (Ankoma-Sey et al., 2000). The effect of VEGF is mediated, in part, by its ability to facilitate intragraft mechanisms of leucocyte recruitment and to promote endothelial activation responses, including adhesion molecule and chemokine production (Reinders et al., 2003). This hypothesis has recently generated considerable interest because if confirmed, angiogenesis could be used as a potential prognostic marker of disease progression and as a novel therapeutic target for the liver disorders.

Andrographis paniculata (Burm.f.) Nees (Acanthaceae), a plant indigenous to Southeast Asian countries, has been used as an accepted herbal medicine in China for many years. The bioactive andrographolide from the leaves of *Andrographis paniculata* extracts and their components exhibit a wide spectrum of biological activities, including antitumour, antiviral and anti-inflammatory properties (Calabrese et al., 2000; Gabrielian et al., 2002; Abu-Ghefreh et al., 2009; Parichatikanond et al., 2010; Lee et al., 2011; Stansbury et al., 2013). Andrographolide (AP), a bicyclic diterpenoid lactone, is the major constituent of *Andrographis paniculata*. It efficiently regulates immune responses and works as an anti-inflammatory agent by reducing the generation of ROS in human neutrophils (Calabrese et al., 2000; Shen et al., 2002) and macrophage (Lee et al., 2011). A previous study demonstrated a role for AP in regulating the expression of α-smooth muscle actin (α-SMA), which is a key marker of activated hepatic stellate cells (HSCs) (Lee et al., 2010). However, the anti-inflammatory properties of AP during HSCs activation and subsequent angiogenesis have not been previously investigated.

Andrographolide may be beneficial both due to its specific anti-inflammatory effects in vitro (Lee et al., 2011) and to its antifibrotic activity in vivo (Lee et al., 2010). Therefore, the overall objective of this study was to determine if andrographolide causes resistance to lobular fibrosis caused by thioacetamide (TAA). The hypothesis was that the inflammatory response is involved in the pathogenesis of angiogenesis during hepatic remodelling in liver fibrosis. Our results demonstrate that andrographolide ameliorates local inflammation and liver damage. Taken together with the observations that chronic inflammation and angiogenesis are associated in liver disorders, andrographolide may be used as a therapeutically agent to modulate inflammation in fibrosis.

2. Methods

2.1. Chemicals and drugs

Andrographolide was from Chromadex Inc. (Irvine, CA); TAA, α-SMA and β-actin antibodies were from Sigma-Aldrich (St. Louis, MO); TNF-α, IL-1β and IL-6 kits were from R&D Systems (Minneapolis, MN); PGE_2 kit and ECL were obtained from Amersham (Piscataway, NJ); anti-neutrophil antibody was from Abcam (Cambridge, MA); CD11b antibody was from Thermo Scientific (Rockford, IL), F4/80 was from Biolegend; anti-HIF-1α antibody was from Santa Cruz; vWF antibody was from Millipore (Billerica, MA); DAPI antibody was obtained from Invitrogen (Carlsbad, CA); and anti-VEGFR1 antibody was from Epitomics (Burlingame, CA).

2.2. Animal models and drug treatment

Hepatic fibrosis was induced in mice by intraperitoneal injection of thioacetamide (TAA, 100 mg/kg body weight) twice weekly for 6 weeks in total. Andrographolide (AP) was dissolved in dimethyl sulfoxide (DMSO), which was then serially diluted in normal saline prior to use. BALB/c mice ($n=38$) were obtained from the National Laboratory Animal Center (Taiwan) and were divided into seven groups. Animals groups were treated as follows: Group 1 was control (untreated) mice ($n=5$); Group 2 receive AP intraperitoneal injections at a dose of 100 mg/kg ($n=5$); Group 3 received TAA injections twice for 1 week only ($n=5$); Group 4 received TAA injections twice a week for 6 weeks; ($n=5$); Group 5 received TAA injections for 6 weeks plus AP injections at 5 mg/kg ($n=6$); Group 6 received TAA injections for 6 weeks plus AP injections at 20 mg/kg ($n=6$); and Group 7 received TAA injections for 6 weeks plus AP injections at 100 mg/kg ($n=6$). Indeed, we found that AP (100 mg/kg) decreased cholestasis-induced ALT and TNF-alpha levels when given after bile duct ligation induction by more than 70% (Lee et al., 2010). Therefore, we chose the dose of AP (100 mg/kg) treatment in order to evaluate the efficacy at 6 weeks follow-up TAA injection. All injections were given intraperitoneally. AP injections were administered once a day for 4 weeks after an initial 2-weeks treatment with TAA. All animal experiments were approved by the Institutional Animal Care Committee at the Chang Gung University and were conducted humanely. Liver injury in BALB/c mice resulted in severe fibrosis, whereas C57BL/6 mice developed comparatively minimal fibrosis (Shi et al., 1997). On the other hand, chronic carbon tetrachloride (CCl_4) treatment is frequently used in rats as an experimental model of hepatic fibrosis (Iredale, 2007; Amin and Mahmoud-Ghoneim, 2009, 2011; Yang et al., 2014). CCl_4 is the most common agent to cause liver damage and induced liver fibrosis; however, application to BALB/c mice was associated with an intolerable high mortality rate of 50%. In order to lower burden for experimental animals, we administered thioacetamide (TAA) to establish our liver fibrosis model. In addition, TAA is frequently applied in mice and serves as a second, independent approach to confirm data obtained from CCl_4-treated animals (Liedtke et al., 2013).

2.3. Biochemical measurement

Animals were sacrificed using CO_2 inhalation. Blood was collected by intracardiac puncture and stored overnight (4 °C). Serum was then collected and used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Livers were harvested and weighed, then fixed and embedded for histopathological and immunohistochemical analyses. Some liver samples were snap-frozen in liquid nitrogen and stored at −80 °C until required.

Liver homogenate for lipid peroxidation was prepared with 2 mL of 50 mM potassium phosphate buffer, pH 7.4, and TBARS were determined (Lee et al., 2007). Results were expressed in nmol/mg of protein, and protein concentrations were determined using the method described by Lowry et al. (1951).