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Chinese herb Radix Polygoni Multiflori as a therapeutic drug for liver cirrhosis in mice

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Abstract

Liver regeneration not only plays a functional role in directing the restoration of liver mass after resection or injury, but also may have participated in effective therapy of liver cirrhosis. Additionally, hepatocyte growth factor (HGF) appears to be a factor of great importance in liver regeneration and attenuated progression of experimental liver cirrhosis. The aim of this study is to use Radix Polygoni Multiflori (POMU) extract, a Chinese herb traditionally used for liver-protective therapy, as a reagent for the evaluation of its potential medicinal use in liver cirrhosis. We used *in vitro* coculture system to show that POMU could promote the expression of HGF by hepatic nonparenchymal cells, consequently the proliferation of primary liver cells and phagocytic activity of Kupffer cells using fluorescein-labeled *Escherichia coli* as the target, and inhibit the proliferation of stellate cells. Using dimethylnitrosamine-induced liver cirrhosis animal, POMU even at 20 mg/(kg day) dosage, was illustrated to reverse the pathogenic progression of the disease, decrease the hydroxyproline content and increases the expression of HGF messenger RNA in liver tissue. The survival rate was significantly increased in the POMU-treated animal. In conclusion, our study showed the promise of POMU in the medicinal use for the treatment of liver cirrhosis.

Keywords: Liver regeneration; Hepatocyte growth factor; Nonparenchymal cells; Phagocytosis; *In vitro* coculture; Herb

1. Introduction

Liver regeneration can propel *in vivo* regenerative growth of liver in adult animals, resulting in the restoration of hepatic mass and re-establishment of the specific functions of the liver after various types of liver injury (Fausto et al., 2006). This type of liver regeneration likely involves a cascade production of various cytokines and growth factors (Michalopoulos and DeFrances, 1997). Hepatocyte growth factor (HGF) is thought to assume an important role in liver development and regeneration (Boros and Miller, 1995; Schmidt et al., 1995). The expression of HGF messenger RNA was rapidly up-regulated in various types of liver injuries in experimental animals (Zarnegar et al., 1991). Studies have shown that infusion or transgenic expression of HGF can result in the resolution of fibrosis and the improvement of survival rate in cirrhotic rats (Matsuda et al., 1997; Ueki et al., 1999). However, the mechanism leading to the impairment of liver regeneration in patients with liver cirrhosis is at the moment largely unknown. Liver cirrhosis is a pathological process of chronic hepatic disease, and is induced by many factors, including chronic hepatitis virus infections, alcohol drinking and drug abuse. Liver cirrhosis is the irreversible end result of chronic fibrous scarring in conjunction with diminished ability of liver regeneration in patients (Yamanaka et al., 1993). Hepatic stellate cell (HSC) has been implicated in participating in the pathogenesis of liver fibrosis (Eng and Friedman, 2000). Under pathological state, HSC is activated by transforming growth factor (Friedman, 1999) and consequently produces a large quantity of extracellular

Abbreviations: HGF, hepatocyte growth factor; HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; POMU, Radix Polygoni Multiflori; DMN, dimethylnitrosamine; NPC, nonparenchymal cell; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescence isothiocyanate; Hep, hepatocyte; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hep, hepatocyte; ECM, extracellular matrix

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matrix proteins (ECM) contributing to liver fibrogenesis. Liver injury is associated with both increased expression of platelet-derived growth factor (PDGF) and PDGF receptor by HSC (Pinzani et al., 1995, 1996). HSC can be activated *in vitro* with PDGF to exhibit myofibroblast-like phenotype that gains high proliferative potential and high production capacity for ECM (Pinzani et al., 1989). Therefore, suppression of HSC activation is thought to be one of the therapeutic targets against liver fibrosis.

Kupffer cells (KC) constitute 80–90% of the tissue macrophages present in the body and play an important role in the pathogenesis of inflammatory liver diseases leading to fibrosis (Winwood and Arthur, 1993). By liberating different mediators, Kupffer cells not only play an important role in the body's defense system but may also contribute to liver damage. However, recent study demonstrates that macrophages play pivotal but divergent roles, favoring ECM accumulation during ongoing injury but enhancing matrix degradation during recovery (Duffield et al., 2005). Numerous efforts have been directed at the development of effective liver-specific antifibrotic therapies. Radix Polygoni Multiflori (the root tubers of *Polygonum multiflorum* Thunb) (POMU) is a traditional Chinese medicine for the treatment of liver diseases, anemia, and other diseases commonly associated with aging. Thus, we set out to evaluate the effects of POMU on liver regeneration both *in vitro* and in animal model. We concluded that POMU promoted the secretion of HGF from hepatic nonparenchymal cells containing Kupffer cells and HSCs, and the phagocytic capacity of Kupffer cells, and inhibited PDGF-induced proliferation of HSCs *in vitro*. POMU was also shown to impede the hepatic deposit of collagen and significantly improve survival rate in mice with dimethylnitrosamine (DMN)-induced liver cirrhosis.

2. Materials and methods

2.1. Animals

Pathogen-free, C57BL/6J mice, male 10 to 12 weeks of age, were obtained from National Laboratory Animal Center (Taipei, Taiwan). Animals were housed at a constant temperature and fed with laboratory chow (PMI, Brentwood, MO, USA) and water *ad libitum*. The protocol of the experiments was approved by the Animal Research Committee of National Yang-Ming University (Guide for Animal Experiments, National Yang-Ming University).

2.2. Preparation of POMU and its isolates

Emodin and physcion standard samples were purchased from Sigma (St. Louis, MO, USA) and POMU was purchased from a local wholesale distributor (Taipei, Taiwan). Two kilograms of dried POMU was extracted with 5 L of methanol three times. The extracts were combined and evaporated to dryness under reduced pressure, which yielded 430 g of dry power and stored at −70 °C before use. The HPLC analyses were performed with a Nucleosil C-18 column (250 × 4.6 mm I.D.) at a room temperature. For analysis of the POMU, the mobile phase was methanol and water in gradient mode as follows: 20:80–100:0 in 60 min. The effluent was monitored at 280 nm and flow rate was set at 1.0 mL/min constantly. Identification of HPLC peak fractions was carried out by comparing their retention times and UV spectra with those of standard samples. POMU mainly contained emodin (1 mg/g) and physcion (1.5 mg/g) as a standard preparation throughout our experiments.

2.3. Manipulations of mice hepatocytes and nonparenchymal cells and coculture

Enriched hepatocytes and nonparenchymal cells were obtained using previously published protocols (Seglen, 1976; Kreamer et al., 1986; Sun et al., 2005). Basically, liver cells were dispersed by collagenase (Worthington, Lakewood, NJ, USA) perfusion and then fractionated via Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation. Hepatocytes (5×10^3 cells per well) were plated onto 1% gelatin coated 96-well plates and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 30 μg/mL L-proline, 10^{-7} M dexamethasone and 5 μg/mL insulin (Sigma), after 2 h incubation, NPC (1.5×10^4 or 5×10^4 cells per well) was added and coculture for an additional 3 h, after which the old culture media were replaced by fresh media as mentioned above except with the addition of 10 ng/mL epidermal growth factor and the omission of FBS. The cultures were continued for 48 h in the absence or presence of POMU (1–1000 μg/mL), which was pulsed with [^{3}H]thymidine (1 μCi/mL) (NEN, Boston, MA, USA) at 24 h after the addition of POMU. The incorporations were assayed in a scintillation counter after 24 h of labeling. When needed, anti-c-Met (50 μg/mL) and matching IgG (50 μg/mL) control were added to the culture 30 min before the addition of POMU.

2.4. HSC isolation and proliferation assay

HSC was isolated from C57BL/6J mice and cultured in DMEM supplemented with 10% FBS as previously described (Yu et al., 2004). For proliferation assay, HSC (1×10^4 cells per well), was incubated in serum-free DMEM with POMU (1–1000 μg/mL) and 20 ng/mL PDGF-BB (Biosource, Camarillo, USA) for 24 h, and pulsed with 1 μCi/mL [^{3}H]thymidine for 6 h as outlined by Pinzani et al. (1989).

2.5. Kupffer cells isolation and phagocytosis assay

Kupffer cells were purified from NPC by Percoll density centrifugation (Smedsrod et al., 1985). Kupffer cells (1×10^5 cells per well) were incubated in RPMI-1640 with POMU (1–1000 μg/mL) for 2 h, and then in the presence of heat-killed fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, Ore, USA) labeled *Escherichia coli* (20:1; bacteria:cell ratio) for additional 2 h. The resulting cells were washed, and FluoroQuench dye (Canoga Park, CA, USA) was added to quench the signal from externally bound FITC. Phagocytic activity was estimated by a fluorescence plate reader