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Research Paper

Mistletoe alkaloid fractions alleviates carbon tetrachloride-induced liver fibrosis through inhibition of hepatic stellate cell activation via TGF-β/Smad interference

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ABSTRACT

Ethnopharmacological relevance: Mistletoe (*Viscum coloratum (Kom.) Nakai*) has long been categorized as a traditional herbal medicine in Asia. In addition to its application in cancer therapy, mistletoe has also been used in the treatment of chronic hepatic disorders in China. In the present study, we investigated the antifibrotic effect and mechanisms of action of mistletoe extracts in a rat model of carbon tetrachloride (CCl_4)-induced hepatotoxicity.

Materials and methods: An experimental model of hepatic fibrosis was established by intraperitoneal injection of rats with CCl_4 for 8 weeks. Rats were subsequently treated with a mistletoe alkaloid fraction preparation via oral administration (120 mg/kg daily for 8 weeks) or with distilled water as a control. Histopathological changes were observed by hematoxylin and eosin staining and Masson's trichrome staining. The expression of markers relevant to hepatic stellate cell (HSC) activation in the liver was assessed by real-time reverse transcription-polymerase chain reaction, immunohistochemistry and western blotting. The anti-fibrosis activity and mechanisms of action of mistletoe **alkaloid fractions** were further investigated in the HSC-T6 HSC line, following treatment with mistletoe **alkaloid fractions** (12 mg/ml) for 48 h.

Results: Hepatic fibrosis decreased markedly in CCl_4-treated animals following treatment with mistletoe **alkaloid fractions**, compared to controls. The mRNA levels of transforming growth factor-β1 (TGF-β1), procollagen I and tissue inhibitors of metalloproteinases (TIMPs) were significantly downregulated, by about 40%, 40% and 45%, respectively, in liver tissues from rats treated with mistletoe **alkaloid fractions**. Furthermore, significant downregulation of TGF-β1, TGF-β1 receptor, phosphorylated Smad 2 and alpha smooth muscle actin (α-SMA) proteins, by about 45%, 30% and 40%, respectively, was also observed in liver tissues from mistletoe **alkaloid fractions**-treated rats. In contrast, Smad 7 levels were significantly increased by about 30% in mistletoe **alkaloid fractions**-treated rats. Treatment of HSC-T6 cells with mistletoe **alkaloid fractions** significantly induced Smad 7 expression and inhibited the expression of α-SMA, TGFβ1, TGF-β1 receptor, Smad 2 and TIMP-1, in vitro.

Conclusion: We demonstrate that mistletoe **alkaloid fractions** decrease extracellular matrix accumulation by inhibiting HSC activation. Mechanistically, this may occur via inhibition of TGF-β1/Smad 2 and Smad 7 signal transduction, thereby blocking the synthesis of procollagen I and TIMP-1. These findings suggest that mistletoe **alkaloid fractions** may be a potential therapeutic agent for the treatment of hepatic fibrosis.

1. Introduction

Hepatic fibrosis is a common consequence of chronic liver injury of multiple etiology. Prolonged unresolved hepatic fibrosis can lead to cirrhosis, liver failure, and even hepatocellular carcinoma (Friedman, 2008a). Therefore, the process of hepatic fibrosis is a critical step which decides the clinical outcome of chronic liver diseases. Control or resolution of fibrosis is an important issue to avoid the development of end-stage diseases. Although continuous updates on the mechanisms underlying hepatic fibrosis are available, the development of anti-fibrotic therapy is still a challenging task (Friedman, 2010). Currently, there is no approved treatment designed for hepatic fibrosis in humans. Therefore, an urgent need exists for seeking and developing antifibrotic strategies that can prevent, halt, or reverse hepatic fibrosis.

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Hepatic fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) due to an imbalance between the production and resorption of ECM. Formation of fibrous septa and distorted liver architecture eventually impairs normal liver function (Wynn, 2007). Increasing evidence has shown that progressive liver fibrosis is mediated by multiple factors, including cytokines, growth factors, metabolic toxins, and stress molecules, via multiple mechanisms and pathways. Hepatic stellate cells (HSCs) are the primary effector cells, and orchestrate the deposition of ECM in normal and fibrotic liver (Iredale, 2001; Friedman, 2008b). The activation, proliferation, transdifferentiation, and collagen secretion of HSCs have been shown to contribute to liver fibrosis and cirrhosis (Bataller and Brenner, 2001; Rippe and Brenner, 2004). Transforming growth factor-β1 (TGF-β1) has been recognized as a key mediator in the pathogenesis of liver fibrosis by activating its downstream Smad signaling pathway (Derynck and Zhang, 2003; Leask and Abraham, 2004; Inagaki and Okazaki, 2007). Smads are a unique family of signal transduction molecules that can transmit signals directly from cell surface receptors to the nucleus. The TGF-β/Smad signaling pathway plays a prominent role in the activation of HSCs and the regulation of the production, degradation, and accumulation of ECM proteins. Thus, the TGF-β signal transduction pathway has become a new effective target for the prevention and treatment of hepatic fibrosis (Derynck and Zhang, 2003).

European mistletoe (*Viscum album L.*) has long been categorized as a traditional herbal medicine in Europe (Frans, 1985) and Asia (Yoon et al., 1995). Mistletoe extract has been used as complementary therapy for several types of cancer for several decades. The therapeutic benefit of mistletoe extract, when used along with surgery, chemotherapy, or radiotherapy, contributes to the overall improvement in the quality of life in cancer patients (Hajto et al., 1990; Kim et al., 2004; Augustin et al., 2005; Bussing et al., 2005; Klopp et al., 2005; Kienle and Kiene, 2010; Kienle et al., 2011). In addition, mistletoe extract is also implicated as a conventional phytotherapeutic in the treatment of several conditions associated with cardiovascular diseases (Wagner et al., 1986), dementia (Schumacher et al., 1994), diabetes (Gray and Flatt, 1999; Orhan et al., 2005), immuno-inflammatory disorders (Hajto et al., 1990; Elluru et al., 2006) and chronic hepatitis (Tusenius et al., 2005).

Chinese mistletoe (*Viscum coloratum (Kom.) Nakai*) has high similarity with European mistletoe in its biological characteristics and therapeutic benefit (Editorial Board of Zhonghua Bencao, 1999; Han et al., 2002). Chinese mistletoe was used as a herbal remedy in the ancient Chinese pharmacopoeia and has been used in traditional Chinese medicine against a variety of diseases, such as arthritis, rheumatism, and hypertension. Hu Qi San is a traditional Chinese medicinal formula that has been traditionally used for patients suffering from various hepatic disorders, such as chronic hepatitis, fibrosis and hepatoma (Gao et al., 1998, 2001; Yang et al., 2009). One of the main components responsible for this effect was identified as mistletoe (Zhang et al., 2005, 2010; Wen et al., 2008). Chinese mistletoe and mistletoe extract have also been used in the treatment of hepatocellular carcinoma as a complementary therapy (Wen et al., 2008; Yang et al., 2009). Chinese mistletoe is a hemiparasitic plant growing on various deciduous trees. It is also composed mainly of mistletoe lectins (Olsnes et al., 1982; Li et al., 1995; Gong et al., 2001, 2007), viscotoxins (Lee et al., 1992; Park et al., 1998; Kong et al., 2004), alkaloids (Khwaja et al., 1986) and several other low molecular proteins, flavonoids, oligo- and poly-saccharides, thionin and others (Franz et al., 1981; Jordan and Wagner., 1986; Kong et al., 1988, 1992; Khwaja and Manjikian, 1990; Li et al., 2002; Urech et al., 2006). Chinese mistletoe lectins showed 91% of identity to European mistletoe lectins at primary structure level and in its biological activity (Kong et al., 2004; Ma et al., 2008). While the mistletoe lectins have been studied intensively, less is known about the mistletoe **alkaloids**.

Despite therapeutic use of mistletoe for the past several decades, the mechanisms of action of mistletoe are not completely understood. Mistletoe extract has been shown to have antioxidant (Orhan et al., 2005; Yao et al., 2007), anti-angiogenic (Yoon et al., 1995; Park et al., 2001; Van Huyen et al., 2002), and immunomodulatory effects (Tusenius et al., 2005; Elluru et al., 2006; Van Huyen et al., 2006). Our previous studies have demonstrated that mistletoe alkali has a protective effect against carbon tetrachloride (CCl_4) toxicity by inhibiting the oxidative damage and stimulating glutathione S-transferas activities (Shi et al., 2006). Several mutually non-exclusive mechanisms have also been proposed, which include induction of apoptosis and cytotoxicity in tumor cells and inhibition of angiogenesis (Park et al., 2001; Van Huyen et al., 2002; Klopp et al., 2005; Xu et al., 2013). Mistletoe lectin induces apoptosis by inducing ROS production and a loss of $\Delta\Psi_m$, in which c-Jun NH_2-terminal kinase (JNK) phosphorylation plays a critical role in these events. We also reported the inhibitory effect of mistletoe alkali and Hu Qi San on rat prehepatocarcinoma induced by diethylnitrosamine and 2-acetyl aminofluorene (Shi et al., 2006; Li et al., 2007). Our study showed that mistletoe alkali and Hu Qi San might induce apoptosis by reducing the inhibitory effects of X-linked inhibitor of apoptosis protein (XIAP) on caspase-3 in tumor cells. The protective effects against hepatic carcinogenesis were mediated by multiple mechanisms, including the reduction of the expression level of c-jun, c-fos, and c-myc oncogenes, promotion of $HtrA_2$/Omi expression and release from mitochondria, activation of caspase-3, regulation of metabolic enzymes, as well as improvement mitochondrial function (Li et al., 2007; Zeng et al., 2013). Recently a study has reported that CM-1, a mistletoe lectin-I isolated from Chinese mistletoe, down-regulated some miRNAs by degrading their precursors, which contributes to its prominent anti-cancer activity (Li et al., 2011). However, to our knowledge, the mechanism of anti-fibrosis properties of mistletoe has not yet been clearly elucidated.

In the present study, we investigated the antifibrotic effect and mechanisms of mistletoe preparations on a rat model of CCl_4-induced hepatotoxicity. Our data show that mistletoe **alkaloid fractions** alleviate CCl_4-induced liver fibrosis through inhibition of HSC activation via TGF-β/Smad pathway interference. These results provide experimental data for mistletoe treatment of liver fibrosis. It indicates the great potential of mistletoe **alkaloid fractions** as a possible therapeutic agent for treating hepatic fibrosis.

2. Materials and methods

2.1. Mistletoe *alkaloid fractions* preparation

Viscum coloratum (kom) Nakai was supplied from Beijing Weiren Chinese Herbal Slices Factory. The stems and leaves of mistletoe were collected from the Herbei province, China in December 2011. Each botanical sample was identified by Professor Li Jing-sheng by comparison to fully registered specimen (voucher specimen number: 275) stored in the herbarium of the Beijing Weiren Chinese Herbal Slices Factory. Mistletoe **alkaloid fractions** were prepared according methods of Beijing Traditional Chinese Medicine Hospital (Beijing, China) as previously described (Park et al., 1998; Zeng et al., 2013) with our own modifications. In brief, dried stems and leaves of mistletoe were pulverized to particles, 1 kg of which was then immersed into 1.5% HCl solution for 48 h. The ratio of particles to HCl was 20:1. After filteration, the aqueous solution was distilled thrice for 2 h at 50 °C under reduced pressure to remove the solvent. Then the residue (extractum)