PHYTOTHERAPY RESEARCH
Phytother. Res. **19**, 915–917 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002/ptr.1747

SHORT COMMUNICATION

A Kampo Formula Juzen-taiho-to Induces Expression of Metallothioneins in Mice

Naoko Anjiki[1], Ritsuko Hoshino[1], Yasuyuki Ohnishi[2], Kyoji Hioki[2], Yoshifumi Irie[1]*, Atsushi Ishige[1] and Kenji Watanabe[1]

[1]Department of Oriental Medicine, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
[2]Central Institute for Experimental Animals, 1430 Nogawa, Miyamae, Kawasaki 216-0001, Japan

Juzen-taiho-to (JTX), one of the commonly prescribed traditional Japanese herbal medicines (Kampo), is indicated for adjunctive treatment of cancers and autoimmune diseases. To understand the mechanisms underlying the clinical effects of JTX, the effects of orally administered JTX on the expression of metallothioneins (MTs) were examined in the liver, spleen, small and large intestines of mice. In addition, the expression of MTs in specific pathogen free (SPF) mice was examined to understand the participation of intestinal bacteria in the expression of MTs. JTX enhanced expression of MT-I and -II significantly in the liver of SPF mice. Induction of MT-II expression was observed also in the small intestine. Intestinal bacteria appeared to have no effect on MTs expression. Neither expression of MT-III nor its induction was observed in any tissue. These findings strongly suggest that MTs should mediate at least some effects of JTX in mice. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: Juzen-taiho-to; JTX; metallothionein; Kampo; herbal medicine; mouse.

INTRODUCTION

Juzen-taiho-to (JTX) is a prescription of Kampo medicine consisting of ten medicinal herbs in the indicated amounts (g per day); *Astragali Radix* (3.0), *Cinnamomi Cortex* (3.0), *Rehmanniae Radix* (3.0), *Paeoniae Radix* (3.0), *Cnidii Rhizoma* (3.0), *Atractylodis Lanceae Rhizoma* (3.0), *Angelicae Radix* (3.0), *Ginseng Radix* (3.0), *Poria Cocos* (3.0) and *Glycyrrhizae Radix* (1.5). It has been used in Japan for centuries for the treatment of cancers, rheumatoid arthritis, atopic dermatitis, etc. (Saiki, 2000). However, to date, the precise mechanism of action of JTX has not been elucidated.

Metallothioneins (MTs), which have been of our interest, are metal-binding proteins present in various organs/tissues of diverse species, from prokaryotes to mammals (Vallee, 1995). MT-I and -II, the ubiquitous forms of MTs, consist of 61 amino acids and 20 cysteine residues in highly conserved positions, where they bind seven molecules of metals such as Cd, Zn and Cu with high affinities. MTs detoxify heavy metals, buffer essential trace metals such as Cu and Zn, and have an antioxidant property (Vallee, 1995). MT-III that shows limited tissue distribution (i.e. mainly in the central nervous system) has additional functions (Irie and Keung, 2003).

The present study investigated the effects of JTX on the gene expression of MT-I, -II and -III in mice to understand the precise pharmacological actions of JTX. The expression of MTs in germ free (GF) mice was observed to examine the participation of intestinal bacteria on the expression of MTs because Kampo medicines are believed to act after being digested by the intrinsic bacteria.

* Correspondence to: Dr Yoshifumi Irie, Department of Oriental Medicine, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan.
E-mail: iymkdks@yahoo.co.jp

METHODS

Male, 7–9 week-old IQI mice (SPF and GF) were provided from the Central Institute for Experimental Animals (Kawasaki, Japan). Three GF mice were maintained in a bacteria-free condition throughout the experiments. Ten SPF mice were given (a) JTX extract powder (provided by the Tsumura & Co. Ltd, Tokyo, Japan, $n = 5$) dissolved in 0.1 mL of distilled water (= 1.0 g/kg body weight/day) or (b) water alone ($n = 5$) using a steel gastric tube once a day. All mice had free access to standard chow and water. The room light was on between 6:30 and 19:00. After JTX treatment for 14 days, the mice were ether-anesthetized and killed by transcardial bleeding. The liver, spleen, small intestine and large intestine were taken immediately. This protocol was approved by the Guideline for Care and Use of Laboratory Animals at Keio University School of Medicine in accordance with the NIH Guide for Care and Use of Laboratories Animals. Every effort was made to minimize discomfort of the animals.

Total RNA (100 ng), extracted from each organ sample with an isopropanol/ethanol precipitate technique as described previously (Tanji *et al.*, 2003), was submitted to real-time RT-PCR. Reactions including data analysis were performed using the QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Tokyo, Japan) with an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Tokyo, Japan) in 30 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 20 s with

Received 15 March 2004
Accepted 4 July 2005

oligonucleotides for MT-I (5′-ATG GAC CCC AAC TGC TCC TG-3′ and 5′-CAC AGC CCT GGG CAC ATT TG-3′), MT-II (5′-TCC TGC AAG AAA AGC TGC TG-3′ and 5′-CTA TTT ACA CAG ATG TGG GGA-3′), MT-III (5′-ATG GAC CCT GAG ACC TGC CCC TG-3′ and 5′-TCA CTG GCA GCA GCT GCA TTT CTC-3′) and β2-microglobulin (5′-CAG CAA GGA CTG GTC TTT CT-3′ and 5′-CAG CAA GGA CTG GTC TTT CT-3′), respectively. The threshold cycles (Ct) were used to quantify the mRNA levels of the target genes. Relative mRNA was calculated as $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycles for MT- and β2-microglobulin genes. Data were statistically evaluated using the Student's *t*-test.

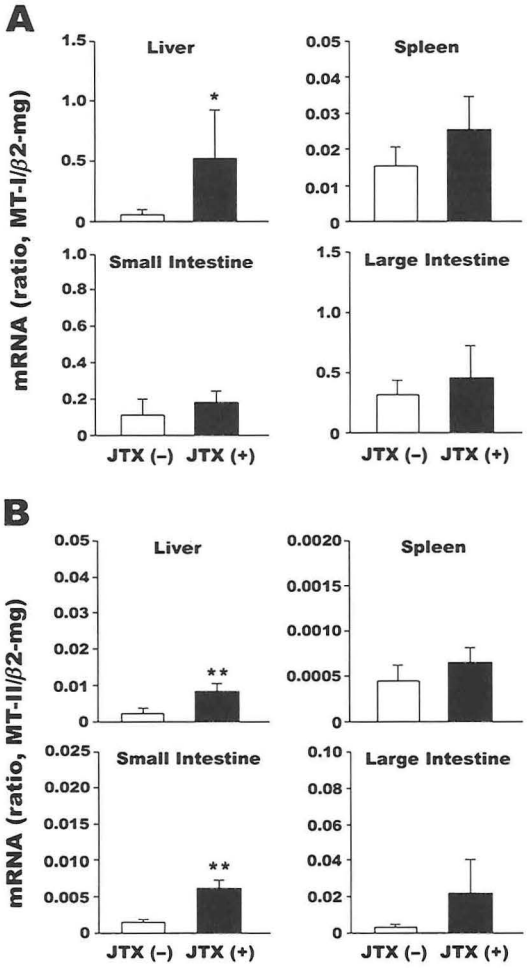

Figure 1. Real-time RT-PCR for expression of MT-I (A) and -II (B) in the liver, spleen, small and large intestine, with (filled bars) or without (open bars) treatment with JTX, respectively ($n = 5$). p values are * < 0.05, ** < 0.001, respectively by the Student's *t*-test.

RESULTS AND DISCUSSION

Expressions of MT-I and -II were detected by RT-PCRs in all tissues examined (liver, spleen, small intestine and large intestine) in GF and SPF mice, while expression of MT-III was not detected in any tissues. No difference was observed between GF and SPF in baseline expression both of MT-I and -II (data not shown), indicating that intestinal bacteria had no effect on the expression of MTs. Thereafter, only SPF mice were used. JTX induced MT-I solely in the liver (Fig. 1A). MT-II showed similar induction by JTX except up-regulation also in the small intestine (Fig. 1B). Induction of MT-III was not observed in these tissues by JTX (data not shown). Together with these results, the fact that MT-I and -II are functionally identical (Vallee, 1995) and that the MT-II expression was negligible (<10% of MT-I, Fig. 1A, B) suggest that the clinical effects of JTX are mediated mostly by MT-I. The reason for the difference in the effect of JTX in different organs remains to be investigated. The absence of expression of MT-III and its induction by JTX in those tissues in the present study is not unexpected because MT-III exhibits rather limited tissue distribution compared with MT-I and -II (Irie and Keung, 2003).

The anti-carcinogenic activity of JTX can be ascribed to the functions of MTs, at least partially. Among transcriptional factors that control carcinogenesis, those with Zn finger motifs need Zn to exert their activities. MTs can modulate the activities of these factors by donating Zn (Ebadi *et al.*, 1995). In addition, MTs protect cells from oxidative stress that damages DNA and cell membranes and causes carcinogenesis (Lazo *et al.*, 1995). The anti-metastatic effects of JTX also appear to be mediated by MTs. For instance, JTX markedly increases the number of T-cells and promotes an anti-tumor effect in mice (Utsuyama *et al.*, 2001), whereas MTs act similarly (Canpolat and Lynes, 2001, etc.). Furthermore, the chemo- and radio-preventive activities of JTX (Ikehara *et al.*, 1992) may be mediated by MTs. Radiation and chemotherapy produce hydroxy-radicals that damage myelocytes, which are scavenged by MTs (Chen *et al.*, 2002).

The induction of other stress proteins by JTX treatment is also of interest, and is being examined in our laboratory.

Acknowledgements

We gratefully thank Dr Gregory A. Plotnikoff for critical reading and correcting of the manuscript.

REFERENCES

Canpolat E, Lynes MA. 2001. *In vivo* manipulation of endogenous metallothionein with a monoclonal antibody enhances a T-dependent humoral immune response. *Toxicol Sci* **62**: 61–70.

Chen Y, Irie Y, Keung WM, Maret W. 2002. S-nitrosothiols react preferentially with zinc thiolate clusters of metallothionein III through transnitrosation. *Biochemistry* **41**: 8360–8367.

Ebadi M, Iversen PL, Hao R *et al.* 1995. Expression and regulation of brain metallothionein. *Neurochem Int* **27**: 1–22.

Ikehara S, Kawamura H, Komatsu Y *et al.* 1992. Effects of medicinal plants on hemopoietic cells. *Adv Exp Med Biol* **319**: 319–330.

Irie Y, Keung WM. 2003. Anti-amyloid beta activity of metallothionein-III is different from its neuronal growth inhibitory activity: structure-activity studies. *Brain Res* **960**: 228–234.

Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KH, Pitt BR. 1995. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *J Biol Chem* **270**: 5506–5510.

Saiki I. 2000. A Kampo medicine 'Juzen-taiho-to'-prevention of malignant progression and metastasis of tumor cells and the mechanism of action. *Biol Pharm Bull* **23**: 677–688.

Tanji K, Irie Y, Uchida Y *et al.* 2003. Expression of metallothionein-III induced by hypoxia attenuates hypoxia-induced cell death *in vivo*. *Brain Res* **976**: 125–129.

Utsuyama M, Seidlar H, Kitagawa M, Hirokawa K. 2001. Immunological restoration and anti-tumor effect by Japanese herbal medicine in aged mice. *Mech Ageing Dev* **122**: 341–352.

Vallee BL. 1995. The function of metallothionein. *Neurochem Int* **27**: 23–33.