Short Communication

Application of Prohibitin Immunohistochemistry to Rat Livers Treated with Methapyrilene Hydrochloride

Kaoru Toyosawa1, Takatoshi Koujitani1, Izumi Matsumoto1, Shinichi Mikami1, Mami Kouchi1, Yoshiko Michimae1, Tadashi Inoue1, and Takaki Seki1

1Safety Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Enoki 33–94, Suita, Osaka 564–0053, Japan

Abstract: An immunohistochemical staining method for prohibitin, a 30-kDa protein located in the inner membrane of mitochondria, is used for detection of mitochondria on formalin-fixed paraffin-embedded sections. Methapyrilene hydrochloride (MP), which is known to increase mitochondria in periportal hepatocytes, was administered once by oral gavage to male rats at a dose of 100 mg/kg. Hypertrophy with eosinophilic granules was noted in the periportal hepatocytes treated with MP. Immunohistochemical staining with prohibitin antibody demonstrated a positive reaction to eosinophilic granules. Electron microscopy showed an increased number of mitochondria in the periportal hepatocytes treated with MP compared to the control. Although electron microscopy is a useful tool to detect ultrastructural changes, an immunohistochemical examination with anti-prohibitin antibody is a simple and easy method for detecting cytoplasmic mitochondria quantitatively. (J Toxicol Pathol 2008; 21: 119–122)

Key words: prohibitin, mitochondria, immunohistochemistry, eosinophilic, methapyrilene hydrochloride (MP)

In hematoxylin-eosin (HE) stained sections, eosinophilic changes in hepatocytes are common findings in toxicological studies1–5. Such changes are typically caused by 1) increases of eosinophilic components within the cell organelles, including mitochondria, smooth endoplasmic reticulum, or peroxisomes; 2) decreases of basophilic components within the cell organelles, such as rough endoplasmic reticulum or ribosomes; or 3) atrophy of the hepatocyte by a decrease of glycogen, resulting in high density of the cell organelles. In the case of hypertrophy of the hepatocyte, cause 1) is suspected; that is, mitochondria, smooth endoplasmic reticulum, or peroxisomes often increase5. Toxicological pathologists are required to demonstrate which of the components increase, and for this purpose electron microscopy has been routinely used. In the present study, we demonstrated that immunohistochemical staining with anti-prohibitin antibody6,7, a 30-kDa protein that is located in the inner membrane of mitochondria, is useful and convenient for the detection of mitochondria on formalin-fixed, paraffin-embedded sections of liver tissue.

The experimental procedures and use of animals followed accredited in-house animal welfare principles. Male CD(SD) rats purchased from CLEA Japan (Kanagawa, Japan) were housed individually in polycarbonate cages (215W × 320D × 181H mm), in an animal room with a controlled temperature of 23 ± 2°C, a relative humidity of 55 ± 10%, ventilation of 15 times/hour, and a 12 consecutive hour/day light cycle. Rodent chow and tap water were available ad libitum. The rats at 7 weeks of age were randomly assigned to two groups, a test group and a control group, consisting of 4 rats each.

It has been demonstrated by electron microscopy that methapyrilene hydrochloride (MP) increases mitochondria in the periportal hepatocytes after single oral administration in rats8. MP (Lot No. 37F0929, Sigma-Aldrich Co., St. Louis, USA) was administered once by oral gavage to the test group at a dose of 100 mg/kg, while vehicle (0.5 w/v% methylcellulose aqueous solution) was given to the control group. Twenty-four hours after the single administration, all animals were euthanized under sodium pentobarbital anesthesia and necropsied.

The left lateral lobe of the liver was preserved in phosphate-buffered 10% formaldehyde solution, then processed to paraffin-embedded sections and stained with HE for microscopic examination. Prohibitin in these sections was also stained immunohistochemically, using anti-rat prohibitin rabbit polyclonal antibody (1:100 dilution, Fitzgerald Industries International, Inc., MA, USA). The tissue sections were boiled in citrate buffered solution for 10 minutes in a pressure cooker (above 120°C, 2 atmospheres pressure), incubated with a 3% (v/v) solution of hydrogen peroxide for quenching endogenous peroxidase, and

Received: 28 November 2007, Accepted: 21 February 2008
Mailing address: Kaoru Toyosawa, Safety Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Enoki 33–94, Suita, Osaka 564–0053, Japan
TEL: 81-6-6337-5923 FAX: 81-6-6337-7053
E-mail: kaoru-toyosawa@ds-pharma.co.jp
immersed in blocking reagent (Block Ace™, DS Pharma Biomedical Co., Ltd., Osaka, Japan) to prevent non-specific reaction. The antibody was applied to the tissue sections for 60 minutes at room temperature and was subsequently detected using Histofine® Simple Stain MAX-PO (MULTI) (Nichirei Co., Ltd., Tokyo, Japan). For electron microscopic examination, pieces of the left lateral lobe of the livers were preserved in 2.5% glutaraldehyde solution for 3 hours,

Fig. 1. Microscopic findings in liver tissue of a rat treated with MP. HE stain. ×40. Eosinophilic changes in periportal hepatocytes.

Fig. 2. Microscopic findings in liver tissue of a rat treated with MP. HE stain. ×200. Hypertrophy with eosinophilic granules and single cell necrosis (arrow head) of the periportal hepatocytes, mononuclear cell infiltration around Glisson’s sheath, and anisonucleosis were observed in the liver tissues of a rat treated with MP.

Fig. 3. Serial sections with HE stain (Figs. 3-1 and 3-3) and immunohistochemical stain for prohibitin (Figs. 3-2 and 3-4): sections from a control rat, Figs. 3-1 and 3-2; sections from a rat treated with MP, Figs. 3-3 and 3-4. Eosinophilic granules with HE staining showed a positive reaction to anti-prohibitin protein antibody. ×300
postfixed in 1% osmium tetroxide solution for 1 hour, then processed to epoxy resin-embedded ultrathin sections and stained with uranyl acetate and hafnium chloride.

In all treated rats, eosinophilic change (Fig. 1) or hypertrophy with eosinophilic granules (Fig. 2) of the perportal hepatocytes was noted. Single cell necrosis of the perportal hepatocytes, mononuclear cell infiltration around Glisson’s sheath, and anisonucleosis were also observed in the livers treated with MP (Fig. 2). Eosinophilic granules demonstrated a positive reaction with anti-prohibitin protein antibody (Figs. 3-3 and 3-4). Perportal hepatocytes from a control rat had clear cytoplasm with glycogen and were little stained with anti-prohibitin protein antibody (Figs 3-1 and 3-2). Electron microscopy showed an increased number of mitochondria in the perportal hepatocytes treated with MP compared to the control (Figs. 4-1 and 4-2).

Single oral administration with MP induced hypertrophy with eosinophilic granules that was consistent with increased mitochondria numbers in the perportal hepatocytes. In the present study, immunohistochemical staining with prohibitin antibody directly demonstrated that the eosinophilic granules were indeed mitochondria. Electron microscopy is one method used to demonstrate increases of mitochondria number; however, immunohistochemical examination for prohibitin is simple, and easily demonstrates quantitative changes of mitochondria.

Prohibitin has antiproliferative activity and available data\textsuperscript{7,9,10} suggest a role in such diverse processes as normal cell cycle regulation, replicative senescence, cellular immortalization and tumor suppression. There is evidence that prohibitin has functions in addition to its roles as chaperone for imported proteins in mitochondria and inhibitor of cell proliferation\textsuperscript{11}. Understanding the biology of prohibitin is still at a very early stage.

Acknowledgement: We thank Ms. Kaori Kunito, Ms. Izuru Mise, and Mr. Yasufumi Ibuchi for technical expertise.

References

1. Malarkey DE, Devereux TR, Dinse GE, Mann PC, and Maronpot RR. Hepatocarcinogenicity of chlordane in B6C3F\textsubscript{1} and B6D2F\textsubscript{1} male mice: evidence for regression in B6C3F\textsubscript{1} mice and carcinogenesis independent of ras proto-oncogene activation. Carcinogenesis. \textbf{16}: 2617–2625. 1995.


8. Reznik-Schuller HM and Lijinsky W. Morphology of early changes in liver carcinogenesis induced by methapyrilene.

