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**Expressions of the multidrug resistance-related proteins in the rat olfactory epithelium:
A possible role in the phase III xenobiotic metabolizing function.**

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Abstract

The xenobiotic metabolizing system is considered to play important roles in the olfaction by the chemical homeostasis. Several phase I and phase II xenobiotic metabolizing enzymes are expressed in the olfactory epithelium in vertebrates. Multidrug resistance-related proteins (MRPs) are the phase III xenobiotic metabolizing pumps that eliminate some conjugated ligands from cells. However, the MRP-expressions in the olfactory epithelium have not been confirmed in the mammals. We investigated gene and protein expressions of MRP type 1 (MRP1) and type 2 (MRP2) isoforms in the adult rat olfactory epithelium in order to clarify the existence of phase III xenobiotic metabolizing pumps in the olfactory organs. Expressions of MRP1 mRNA were detected in the nasal cavity by reverse transcriptase polymerase chain reaction (RT-PCR). The nucleoside sequence of the RT-PCR products were completely identical to that found in other organs of rat. On the contrary, the analysis did not detect expressions of MRP2 mRNA in the nasal cavity. By in situ hybridization using a digoxigenin-labeled MRP1 cRNA probe, signals for MRP1 mRNA were observed preferentially in the perinuclear regions of supporting cells. However, the respiratory epithelial cells did not show the signals for MRP1 mRNA. By immunohistochemistry using a specific antibody to MRP1, MRP1-immunoreactivities were seen mainly on the supporting cells. These findings suggest that MRP1 is involved in olfaction as a part of the “olfactory signal termination” by the chemical homeostasis in the “perireceptor events” of the olfactory epithelium.

Keywords: Olfactory epithelium, Multidrug resistance-related protein, Xenobiotic metabolism, Immunohistochemistry, In situ hybridization, Rat

Xenobiotic metabolism is generally referred to as phases I, II and III, where phase I includes oxidation of lipophilic xenobiotics, phase II deals with the conjugation of phase I products, and phase III eliminates some conjugated xenobiotics with glucuronide, sulfate or glutathione from cells in various tissues [8]. Since several phase I and phase II xenobiotic metabolizing enzymes were already detected in the olfactory epithelium of the mammals, the xenobiotic metabolizing system is considered to play important roles in the chemical homeostasis of the olfactory epithelium [2, 4, 15]. In particular, the existences of olfactory specific isoforms were reported in cytochrome P450 as phase I enzyme [17] and UDP-glucuronosyl transferase as phase II enzyme [14] in mammalian olfactory epithelia. The possibility of the conjugated xenobiotics being extruded from the cells of olfactory epithelia has not yet been investigated. In other mammalian cells and tissues, such as the liver, kidney, trachea and tumour cells, multidrug resistance P-glycoproteins (MDRs) and multidrug resistance-related proteins (MRPs) are known to extrude xenobiotics as phase III xenobiotic metabolizing pumps, and these molecules show polarity localization in each cell [1, 7, 21]. MRPs were more strongly than MDRs involved in the excretion of phase II conjugated xenobiotics [10, 19]. However, expressions of their phase III xenobiotic metabolizing pumps in the olfactory epithelia have not been confirmed in the mammals. In this study, we investigated gene and protein expressions of MRP type 1 (MRP1) and type 2 (MRP2) isoforms in the adult rat olfactory epithelia by employing molecular biological and histochemical techniques.

Eight-week-old male Wistar rats (n=20) weighing 250±30 g (Seac Yoshitomi, Fukuoka, Japan) were provided for the present study. The care and use of animals followed 'The Guiding Principles for the Care and Use of Animals', approved by our university in accordance with the principles of the Declaration of Helsinki. Rats were deeply anesthetized with an intraperitoneal injection of 5 mg of pentobarbital sodium per 100 g body weight and

perfused intracardially with physiological saline. After perfusion, the nasal cavities, including the olfactory and respiratory epithelia, and livers were carefully dissected and removed. Total RNA was prepared from the rat nasal cavities and livers (n=8, respectively) using the ISOGEN (Nippongene, Tokyo, Japan). Poly (A)⁺ RNA was isolated by oligo (dT)-Latex beads (Oligotex-dT30<Super>; Takara, Otsu, Japan). Five hundred nanograms of poly (A)⁺ RNA from the nasal cavities and livers were reverse transcribed using an random primer pd (N)₉ (Takara) and Super ScriptTMII RNaseH⁻ Reverse Transcriptase (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. Oligonucleotides for MRP1 and MRP2 cDNA amplification were as follows: MRP1 forward primer (rat MRP1: 2270-2291), 5'-ACCTTATTCAGTCCACCATCCG-3', MRP1 reverse primer (rat MRP1: 2809-2830), 5'-TAAATGTGGCTGAATAGGCATG-3', MRP2 forward primer (rat MRP2: 2295-2312), 5'-GGGATAAATCTCAGTGGT-3', MRP2 reverse primer (rat MRP2: 3145-3162), 5'-ATATGCTCCACAGAGTTG-3' designed from a coding region of known MRP sequences (GenBank accession nos. RN0277881 and D86086, respectively). The PCR conditions were: the first cycle, denaturation at 94°C for 3 min, annealing at 55°C for 5 min, and extension at 72°C for 5 min; 35 cycles of incubation, 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and finally extension at 72°C for 5 min for 3' A overhangs using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplicons were T-A ligated to pCRII-TOPO plasmid vector (TOPO TA Cloning Kit; Invitrogen, San Diego, CA) and sequenced. After the plasmids were linearized, digoxigenin (DIG)-labeled antisense and sense cRNA probes were prepared with T7 and SP6 RNA polymerase by using a DIG RNA Labeling Kit based on the protocol provided by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Each tissue from nasal cavities (n=12) was fixed with 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2; PB) for 72 h at 4°C, decalcified (only nasal cavities) with 0.2

mol/L EDTA in 0.1 mol/L PB for 2 wk at 4°C, and embedded in paraffin. Serial sections of approximately 5 µm in thickness were prepared using a microtome and mounted on glass slides. Deparaffinized sections (n=10) were digested with 10 µg/mL proteinase K for 20 min at 37°C, and then treated with 0.2 mol/L HCl for 10 min and 2.5 g/L acetic anhydride in 0.1 mol/L triethanolamine-HCl (pH8.0) for 10 min. DIG-labeled MRP1 or MRP2 cRNA probes (200 ng/100 µL) were applied to each glass slide. The sections were incubated for 18 h at 50°C in a moist chamber. Post hybridization washing and immunohistochemical detection for hybridized signals were performed by using the method of Kudo et al. [11]. In control experiments, the sense RNA probes were hybridized to tissue sections as described for the antisense RNA probes. Alternatively, the sections were treated with 20 µg/mL RNase A for 30 min at 37°C prior to in situ hybridization. Sections adjacent to those used for the above hybridization were counterstained with Delafield's hematoxylin and eosin staining. The double immunofluorescence labeling was performed in order to compare the localization between MRP1 and OMP, as an olfactory receptor neuron cell-marker molecule [12], in the same sections. Deparaffinized sections (n=8) were irradiated with 800W microwaves (Micromed T/T microwave equipment; Milestone, Sorisole, Italy) for 20 min. Antigen-retrieved sections treated with 10% normal donkey serum in phosphate-buffered saline (PBS) for 30 min, and primary immunoreacted with goat anti-MRP1 antibody (N-19; Santa Cruz Biotechnology, Santa Cruz, CA) [20] at a dilution of 1:150 and then with rabbit anti-salmon OMP antibody [12] at a dilution of 1:200 each in PBS for 18 h at 4°C. After PBS washing, sections were reacted with mixture of Alexa Fluor 546 donkey anti-goat IgG conjugate and Alexa Fluor 488 donkey anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR) each at a dilution of 1:200 in PBS for 2 h at room temperature. All incubations were carried out in a humid and dark chamber. Sections were coverslipped with 90% nonfluorescent glycerol containing 7.5 µg/mL of 4', 6-diamino-2-phenylindole

dihydrochloride (DAPI) in PBS, and digitally captured on a Carl Zeiss Axioskop 2 plus fluorescence microscope equipped with a Carl Zeiss AxioCam digital camera. The specificities of the antibodies for MRP1 [20] and OMP [12] have already been demonstrated in the rat tissues. The specificities of the above immunohistochemical reactions were confirmed by a preabsorption test with antigenic peptide for them.

Amplified both MRP1 and MRP2 cDNA fragments of the expected sizes (561 and 868 bp, respectively) were detectable in the liver using RT-PCR with each specific primer sets (Fig 1). Only MRP1 cDNA was detected in the amplification of MRPs cDNA in the nasal cavity. Nucleotide sequences of these RT-PCR products were completely identical to the corresponding region of both MRP1 and MRP2 sequences (GenBank accession nos. RN0277881 and D86086, respectively) previously examined in the rat astrocyte [6] and liver [9], respectively.

Signals of MRP1 mRNA using a labeled cRNA antisense probe were seen mainly in the supranuclear portions of the supporting cells near upper regions and a part of olfactory receptor neuron near middle regions in the olfactory epithelium of the turbinate (Figs. 2A and 2C). The respiratory epithelium, which could be distinguished in the goblet cells, did not show the signals for MRP1 mRNA. Hybridization with a labeled sense control probe identified no detectable hybridized signals in adjacent sections to Fig 2A of the nasal cavity (Fig. 2B). Double immunofluorescence labeling of MRP1 (Fig. 3A) and OMP (Fig. 3B) in olfactory epithelium of the turbinate revealed that immunoreactivities for MRP1 were independently localized the cytosol of supporting cells. However, distinctly colocalization of immunoreactivities for MRP1 and OMP was not observed in the olfactory receptor neurons (Fig. 3D). Figure 3C shows the nuclei by DAPI staining. The above immunoreactivities were not detected by the preabsorbed antibodies with each antigenic peptide. No clear differences in the localization and the signal intensity for MRP1 were observed between the

nasal septum and turbinates in both immunohistochemistry and in situ hybridization examinations.

RT-PCR analysis in the present study revealed the expression of MRP1 mRNA in the rat nasal cavities, but did not detect expression of MRP2 mRNA. Previous study revealed that MRP1 expression indicated widely distribution of various organs including the brain [5]. This report indicated that MRP2 expression could not detect in the brain. Present study by in situ hybridization revealed that the expression of MRP1 mRNA was detected in the olfactory epithelial regions, and not in the respiratory epithelial regions in the rat nasal cavity. These results suggest that MRP1 is involved in the olfaction in the nasal cavity, and has more important roles about xenobiotic metabolism than MRP2 in the neural tissues. Gene and protein expressions of MRP1 were preferentially localized in the supporting cells of the olfactory epithelia by both histochemical techniques. However, mammalian phase II enzymes, including glutathione S-transferase (GST), were expressed in the supporting cells of olfactory epithelia [e.g., 3, 18]. Our previous study demonstrated, zinc-deficient rats that exhibited olfactory dysfunction, a marked reduced expression of GST class mu at both mRNA and protein levels in supporting cells of olfactory epithelia [13]. These results suggested that MRP1 in the supporting cells eliminated the phase II conjugated substances, although the localization of MRP1 did not show the polarity in the supporting cells. This elimination may relate to the chemical homeostasis as one of the “perireceptor events” in the olfactory epithelium. In fact, phases I and II xenobiotic metabolic molecules in the olfactory epithelium have been proposed to underlie the termination of odorant signals, as well as the protection of the olfactory receptor neurons against airborne toxic compounds [e.g., 4, 14]. In vertebrate olfactory organs, the expressions of phase III molecules including MRPs are confirmed in the olfactory receptor neurons of only *Xenopus* [16]. The present study didn't indicate the protein expression in the olfactory receptor neurons, although signals for MRP1

mRNA were located in a part of the olfactory receptor neurons. MRP1 protein may be restrictively localized on the cell membrane of dendritic portion that adjoins the supporting cells. Further work is required to determine the ultrastructural localization of MRP1 in rat ORNs by post-embedding immunoelectron microscopy (immuno-gold method).

The present study is the first to describe the expressions of MRP1 mRNA and protein in the mammalian olfactory epithelium. These findings imply that MRP1 is involved in olfaction as a part of the “olfactory signal termination” by the chemical homeostasis in the “perireceptor events” of the olfactory epithelium.

Acknowledgements

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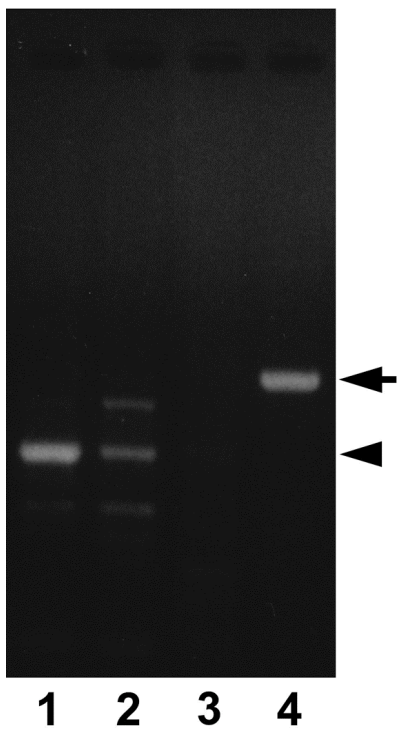
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Figure legends

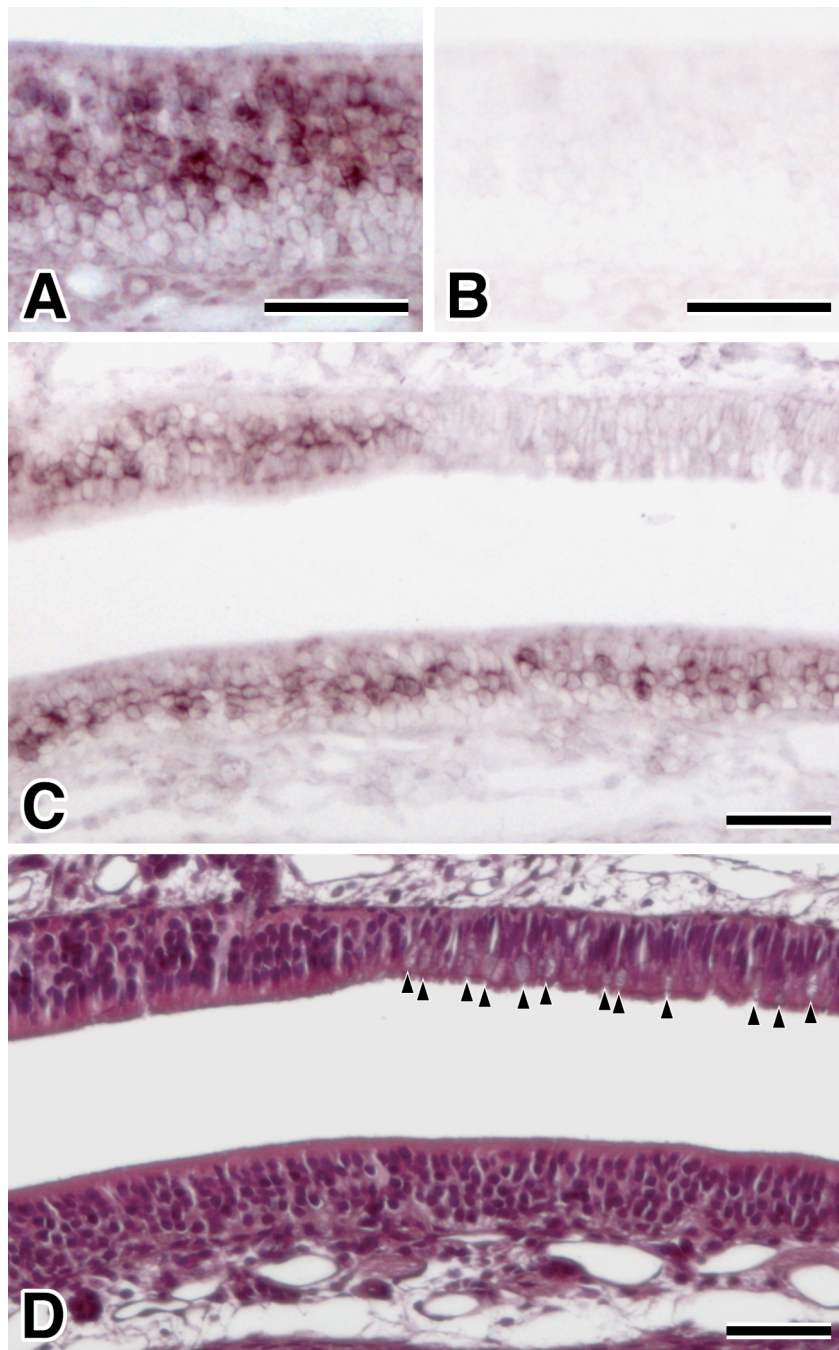
Fig. 1. Agarose gel electrophoresis showing MRP mRNAs expression in rat nasal cavity (lanes 1 and 3) and liver (lanes 2 and 4). The cDNAs were amplified with MRP1 coding primer sets (lanes 1 and 2) and MRP2 coding primer sets (lanes 3 and 4). RT-PCR amplified MRP1 cDNA of 561 bp (arrowhead) and MRP2 cDNA of 868 bp (arrow) predicted size. This amplification did not detect expressions of MRP2 mRNA in the nasal cavity. These products were cloned, and strands sequenced and confirmed as MRP1 and MRP2 fragments, respectively.

Fig. 2. Expression of MRP1 mRNA in the olfactory epithelium of rat turbinate by digoxigenin-labeled in situ hybridization (A). A section adjacent to that used for Fig. 2A represents level of signals using a labeled sense control probe for MRP1 mRNA (B). Expression of MRP1 mRNA in the olfactory and respiratory epithelium of rat turbinate by labeled in situ hybridization (C). Adjacent section to that used for Fig. 2C was counterstained with Delafield's hematoxylin and eosin staining (D). Signals of MRP1 mRNA were observed preferentially in the perinuclear regions of the supporting cells and a part of olfactory receptor neurons. The respiratory epithelial cells did not show the signals for MRP1 mRNA. Arrowheads indicate the goblet cells. Bars: 50 μ m.

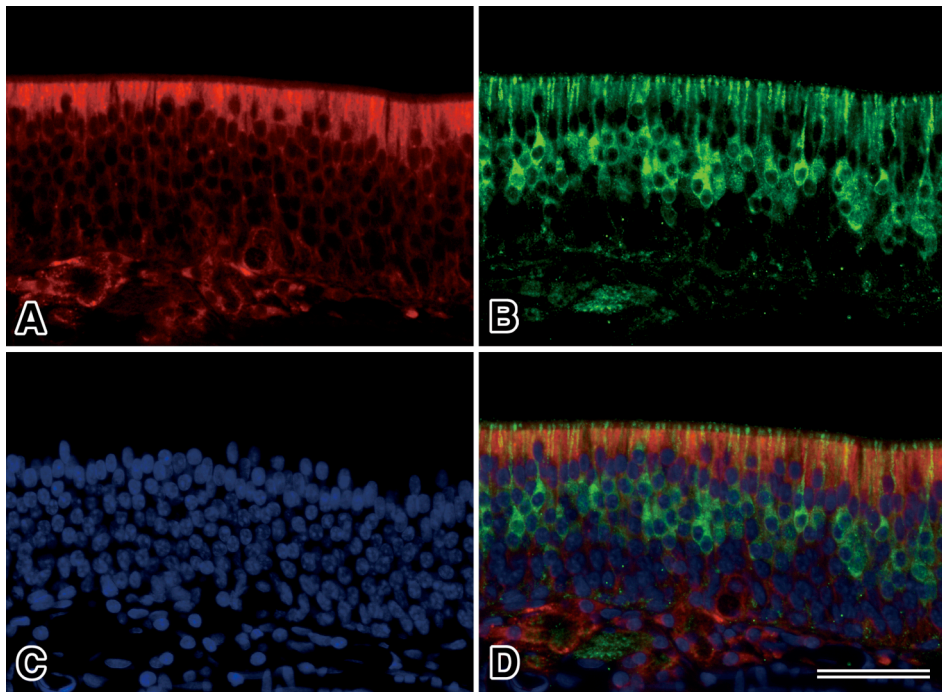
Fig. 3. Double immunofluorescence labeling of MRP1 (red signal; A) and OMP (green signal; B) in olfactory epithelium of the rat turbinate. Blue signals indicate nuclei by DAPI staining (C). Combined double-labeled image of MRP1 and OMP shown in Fig. 3D. Bar: 50 μ m.



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Fig. 1



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Fig. 2



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Fig. 3