Immunohistochemical Localization of Metallothionein in the Olfactory Pathway of Dogs

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ABSTRACT. Dogs raised in the open air were used in this study. Metallothionein (MT) immunoreactivity was observed in the nucleus and/or cytoplasm of sustentacular cells of the olfactory epithelium in the nasal mucosa, whereas there was few MT-positive cells in the respiratory epithelium. MT immunoreactivity was also observed in astrocytes in all layers of the olfactory bulb cortex; glial cells surrounding the glomeruli in the olfactory bulb showed prominent immunoreactivity for MT. Adult dogs exhibited stronger immunoreactivity for MT than young. Northern blot analysis demonstrated substantial levels of MT mRNA in the olfactory mucosa and olfactory bulb. Physiological roles of MT localized in the olfactory pathway of dogs were discussed. — KEY WORDS: canine, immunohistochemistry, metallothionein, Northern blotting, olfactory pathway.


Metallothionein (MT), a 6 kDa protein with 61 amino acid residues including 20 cysteines, is bound to a total of 7 equivalents of certain bivalent metal ions (Cu2+, Zn2+) with high affinity [2]. MT is found in a wide variety of eucaryotes, known to be induced by exposure to these metal ions. Therefore, MT is considered to play a role in their absorption, tissue uptake, transport, storage and detoxification [23, 32]. Other physiological roles of MT, such as scavenging of free radicals and regulation of cell differentiation, have been proposed, since its synthesis can be induced by many physiological and nutritional factors including starvation and imposition of various types of physical or inflammatory stresses [11, 22]. Roles of MT in normal physiology have not been fully understood [9].

The olfactory system is unique in that the olfactory epithelium is directly exposed to the external environment and that the primary olfactory neurons (project to the main olfactory bulb) are directly connected to the brain at the olfactory bulb. It has been demonstrated that the olfactory system can provide a direct entry into the central nervous system for a variety of agents including virus [15, 30], solvents [7] and metals [8, 25]. This enabled some researchers to suggest that the exogenous environmental etiologic factor(s) entering the brain through the olfactory pathway may be responsible for neurodegenerative disorders such as Alzheimer’s disease [24] and amyotrophic lateral sclerosis and Parkinsonism-dementia complex of Guam [26].

There is an increasing attention that millions of tons of trace metals are produced each year from the mines resulting in the greatly increased circulation of toxic metals through the soils, water and air [21]. Dahl and Hadley [5] emphasized the importance of activities of xenobiotic metabolizing enzymes in the olfactory organ of mammals for protection of the neuroepithelium against pollutants. These enzymes were also demonstrated in the olfactory epithelium of fish [16]. The existence of MT in the olfactory system is plausible since environmental pollutions including toxic metals are likely to be inhaled, transported along the olfactory pathway and beyond the olfactory bulb [8]. To our knowledge, there is no report describing localization of MT in the olfactory pathway including the olfactory mucosa. In the present study, we examined the distribution of MT immunoreactivity and the expression of MT mRNA in the olfactory pathway of those dogs which had been raised in the open air.

MATERIALS AND METHODS

Animals: Twenty dogs, 12 females and 8 males, were used in this study. Twelve were old, ranging from 11 to 19 years of age (mean 14.3), and eight were young, one week to 6 years of age. All the dogs were raised in the open air. The dogs were killed by euthanasia with sodium pentobarbital or died of a variety of disorders including heart worm disease, renal failure, and tumor of visceral or genital organs.

Tissue preparations: For immunohistochemistry, the olfactory bulb and nasal conchae containing the olfactory mucosa were taken immediately after death. The samples were then fixed in 10% neutral buffered formalin for three days. Five-mm-thick coronal slices were obtained at various levels of the nasal conchae and olfactory bulb. The tissue slices were then routinely processed and embedded in paraffin. Serial sections (6 µm) were processed for immunohistochemistry.

For Northern blotting, fresh samples from the olfactory mucosa, olfactory bulb and liver were taken immediately at autopsy, frozen with liquid nitrogen, and stored in a freezer at −80°C.

Immunohistochemistry: The primary antibody used in this
study was MT monoclonal antibody against horse MT-1 and MT-2 (DAKO, Glostrup, Denmark). The antibody is specifically reactive with a conserved epitope common to several mammalian species of MT [10]. Monoclonal antibody to glial fibrillary acidic protein (GFAP) (DAKO, Glostrup, Denmark) was also used to identify astrocytes.

After blocking endogenous peroxidase activity with 3% H₂O₂ in PBS, sections were treated with 5% normal goat serum, incubated with primary antibodies overnight at 4°C and then sequentially incubated with biotinylated goat anti-mouse IgG (DAKO, Glostrup, Denmark) (1:500 in PBS) for the monoclonal antibodies or with biotinylated goat anti-rabbit IgG (DAKO, Glostrup, Denmark) (1:500 in PBS) for the polyclonal antibody for 2 hr at room temperature, and with peroxidase-conjugated streptavidin (1:500 in PBS) for 1 hr. Primary antibodies were diluted with PBS containing 5% bovine serum albumin and 0.3% Triton-X 100 (1:100 for MT; 1:100 for GFAP). After the incubations with antibodies and streptavidin, the sections were washed 3 times for 10 min each in PBS and then developed with 0.02% 3,3’-diaminobenzidine tetrahydrochloride and H₂O₂ (DAB-H₂O₂). The sections were counterstained with methyl green or hematoxylin. Positive control sections from dog kidney were incubated with and without primary antibody. The sections were incubated with either nonimmune mouse serum instead of the primary antibody or with PBS. The specificity of the MT monoclonal antibody was tested by pre-absorption techniques in which the antibody was preabsorbed with an excess amount (100 μM) of the initial antigen (horse MT) (SIGMA, St. Louis, U.S.A.).

Probe preparation: Dog MT complementary (c)DNA containing a 102-bp fragment was used as a hybridization probe. The cDNA was obtained by reverse transcription of a mRNA from adult dog liver, followed by polymerase chain reaction (PCR). Mixed primers representing all possible coding combinations, 20-mer 5’TG(C/T)AA(A/G)TG(C/T)AA(A/G)GA(A/G)TG(C/T)AA (corresponding to the amino acid sequence CKCCEK) and 20-mer 5’CC(C/T)TT(A/G/T)AT(A/G)CACC(C/T)TG (corresponding to CKCIGQ) were constructed from the known amino acid sequence of the dog MT [14]. The primers were ordered to Sawadei Technology Co., Ltd. (Tokyo, Japan). The PCR-generated fragment was subcloned into pBluescriptII KS+. Sequencing of the obtained cDNA was performed by the dideoxynucleotide method of Sanger et al. [27]; the base sequence was 82% homologous to that of mouse MT-1 cDNA described previously [29].

RNA extraction and Northern blotting: Total RNA was extracted from the olfactory mucosa, olfactory bulb and liver by the method of Chomczynski and Sacchi [3]. For Northern blotting, 20 μg of total RNA was fractionated on 1% agarose gel and transferred to a Hybond N+ nylon membrane (Amersham, Amersham, UK). Membranes were prehybridized and then hybridized with the [³²P] dCTP-labeled probes according to the manufacturer’s instruction. After hybridization, the membranes were washed, and signals were detected by autoradiography.

RESULTS

Immunohistochemistry: Positive control sections from dog kidney showed intense immunoreactivity for MT. The absorption of the MT antibody with horse liver MT abolished all staining (Figs. 1A and 1B). There was no staining observed when the MT antibody was omitted from the procedure. MT immunoreactivity was demonstrated in the olfactory epithelium in a patchy fashion (Fig. 2A); the respiratory epithelium attaching to the nasal conchae showed few immunopositive cells. The intensity of immunostaining and the number of immunopositive cells differed from case

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Fig. 1. Serial sections through the olfactory epithelium (A, B) from a 5-year-old dog. Note that the absorption of the MT antibody with an excess amount of the initial antigen has abolished all staining (B). MT-immunostaining, × 320. Bars=30 μm.
immunoreactivity for MT. The presence of MT immunoreactivity was demonstrated in a population of glial cells in the all layers of the olfactory bulb cortex (Fig. 4A). These glial cells showed nuclear and/or cytoplasmic staining for MT (Figs. 4B and 4C). Glial cells surrounding the glomeruli exhibited prominent immunostaining for MT (Fig. 4C). Occasional glial cells with a weak staining for MT were observed in the marginal area between the cortex and the medulla of the olfactory bulb. Serial sections demonstrated that MT-positive glial cells corresponded to astrocytes, which are characterized by GFAP-positive processes (Figs. 5A and 5B).

**Northern blot analysis:** Northern blotting showed a high level of MT mRNA in the liver and lesser levels in the olfactory mucosa and olfactory bulb (Fig. 6).

**DISCUSSION**

The amount of toxic metals circulating in the environment through the air is increasing [21]. It has been observed that experimentally administered metals through the olfactory mucosa enter the olfactory bulb by way of the axonal transport [8, 25]. This observation prompted us to study MT localization in the olfactory pathway of dogs, since MT is known to be induced by exposure to metal ions [2]. With the use of mouse monoclonal MT antibody, we demonstrated MT immunoreactivity in the olfactory epithelium and olfactory bulb of dog. In addition, Northern blot analysis detected MT mRNA in these tissues.

There are two kinds of mucosae attaching to the nasal conchae: respiratory and olfactory. The epithelium of the respiratory mucosa consists of ciliated epithelial cells mixed
with goblet cells, and that of the olfactory mucosa consists of olfactory (sensory) cells, sustentacular cells and basal cells [6]. In the present study, MT immunoreactivity was observed exclusively in the olfactory epithelium; olfactory primary neurons exhibited no staining for MT. This finding is in agreement with the previous reports describing immunohistochemical localization of MT in the epithelial components of a variety of organs including the liver [17, 23], kidney [17, 23], thymus [28], skin [13, 31] and genital organs [19, 20] and in the epithelial tumor tissues [1, 12, 18]. A recent report by Monod et al. [16] also demonstrated the presence of xenobiotic metabolizing enzymes, not in the sensory cells but in the sustentacular cells, in the olfactory epithelium of rainbow trout (Oncorhynchus mykiss).

In the olfactory bulb of dogs, MT localization was largely confined to the cortex. In addition, prominent immunostaining for MT was observed in astrocytes surrounding the glomeruli, where synaptic endings of the primary olfactory neurons were located. These findings may also imply physiological roles of MT in storage or detoxification of metals transported from the olfactory mucosa. There is a report which shows evidence of selective accumulation of orally exposed Cd within the olfactory bulbs [4]. This evidence may be partly explained by the presence of the MT-rich astroglial network in the olfactory bulb; the network demonstrated in the present study would be of great advantage in binding metals to MT when exposed to them.

The dogs used in the present study had been exposed to the open air throughout the life after birth. Adult dogs
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Fig. 5. Serial sections through the granular layer of the olfactory bulb (A, B) from an adult dog. Note that glial cells (arrows) and glial processes surrounding the blood vessel (arrow head) are immunoreactive for MT (A) and for GFAP (B). A, MT-immunostaining, x 600. B, GFAP-immunostaining, x 600. Bars=30 μm.

1 2 3

28S -

18S -

MT -

Fig. 6. Northern blot analysis of MT mRNA in the olfactory bulb (lane 1), olfactory mucosa (lane 2) and liver (lane 3) from a 16-year-old dog. Liver total RNA was analyzed for a comparative purpose.

generally showed stronger immunoreactivity for MT than younger. In addition, the intensity for MT staining differed from case to case. The differences in the MT expression between adult and young dogs, and among cases may reflect the different histories of individual dogs on the opportunity or the duration for the exposure to the air containing certain amounts of pollutants. In this context, MT may be localized in the olfactory pathway for the protection against pollutants including toxic metals. MT may, however, also play a role in providing a reservoir with essential trace elements required in the metabolic activities [23, 32] especially related to olfaction. Future analysis of metals bound to MT

REFERENCES


