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SHORT COMMUNICATION

Expression of Neuronal Growth Inhibitory Factor (Metallothionein-III) in the Salivary Gland

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Summary

Metallothioneins (MTs) are metal-binding proteins that have been regarded as intrinsic factors for protecting cells and tissues from metal toxicity and oxidants. Among the three major classes of MTs, MT-III is different from other MTs because it has neuronal inhibitory activity and is only expressed in the central nervous system. Recent studies, however, have confirmed that MT-III is also expressed in organs other than the brain. These findings not only indicate that MT-III has a much wider tissue distribution than was originally thought, but also suggest that it might have other unknown activities. In the present study, we examined the human salivary and thyroid glands and demonstrated that the MT-III gene is also expressed in the salivary but not in the thyroid gland. While salivary ducts showed intense immunoreactivity with anti-MT-III, weak immunoreactivity was observed in acinar cells. This, together with the findings that some neuromodulators (i.e. nerve growth factor, etc.) exist in the salivary gland and that MT-III may participate in the transport in renal tubules, suggest that MT-III may have other functions than cytoprotection in the salivary gland.

Key words

Metallothionein-III • MT-III • GIF • Salivary gland • NGF

Metallothioneins (MTs) are low molecular weight (~6 kD) metal-binding proteins that are expressed in many tissues. They are known to protect cells and organs from the toxicity of metals and oxidants (Vallee 1995). Members of the MT family are designated MT-I, -II, -III, and -IV. MT-I and -II consist of 61 amino acids and contain 20 cysteine residues in highly conserved positions, where they bind metals such as Cd, Zn, and Cu with high affinities. Expression of MT-I and -II can be induced by kainic acid, glucocorticoids, interferon, lipopolysaccharides, and metals such as Cd, Zn, and Cu (Vallee 1995).

MT-III, the brain-specific MT, differs from other MTs in several respects (Uchida *et al*. 1991): it consists of 68 amino acids with a unique amino acid sequence insert; it is mainly found in the brain, especially in astrocytes (Yamada *et al*. 1996); and its expression is not induced by typical MT inducers described above (Vallee

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1995). MT-III was originally isolated as a neuronal growth inhibitory factor (Uchida *et al*. 1991) that inhibits sprouting of neuronal processes and survival of neurons in culture. Several studies have demonstrated that the level of MT-III is down-regulated in the brains of patients with Alzheimer's disease, suggesting that it may be involved in the pathogenesis of the disease (Uchida *et al.* 1991, Yu *et al.* 2001). Recent studies have shown that MT-III protects cerebral cortical neurons in culture against the neurotoxic effects of various types of agents including glutamate (Montoliu *et al.* 2000), amyloid β peptides (Irie and Keung 2001), nitrosative stress (Chen *et al*. 2002), hydrogen peroxide (Chen *et al.* 2002), high-concentration oxygen (Uchida *et al.* 2002), and oxygen-free radicals (Ren *et al.* 2001). In a previous report, we have shown that the expression of MT-III is induced by hypoxia and that MT-III has a prominent protective effect on cells under hypoxia *in vitro* (Tanji *et al.* 2003). Under the same experimental conditions, MT-I and -II exhibited little, if any, cytoprotective activities (Irie and Keung 2001, Chen *et al*. 2002, Tanji *et al.* 2003). Thus, on the basis of these findings, one may regard MT-III as an intrinsic neuroprotective factor.

Since MT-III was first isolated and identified from the brain (Uchida *et al.* 1991), numerous studies have shown that this protein is also present in the kidney (Garret *et al.* 1999a), prostate (Garret *et al.* 1999b), urinary bladder (Sens *et al.* 2000), and breast (Sens *et al.* 2001). These findings suggest that in addition to its neuroprotective function in the brain, MT-III may be expressed and exert protective or other functions in tissues outside the central nervous system. These findings prompted us to investigate the expression of MT-III in tissues that have not been examined in previous studies. It is known that MT-III is absent in many organs such as liver, pancreas, intestine, heart, testis, ovary (Palmiter *et al*. 1992), spleen, muscle (Tsuji *et al*. 1992), etc. Among major organs, lung, stomach, thyroid and salivary gland, etc. have not been tested. In this study, the salivary gland was chosen to be examined because the nerve growth factor (NGF) that apparently has an opposite effect (neuronal growth) to that of MT-III (neuronal growth inhibitory) was discovered for the first time in this gland. Thyroid was tested for comparison.

Human total RNA from the salivary gland (No. 64110-1) and thyroid (No. 64104-1) were purchased from Clontech (Palo Alto, CA). Total RNA from normal human astrocytes NHA6700 was extracted using an RNeasy kit (QIAGEN, Hilden, Germany). cDNA fragments of MT-III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (Tanji *et al.* 2003). The PCR products were separated by electrophoresis in a 2 % agarose gel and visualized under UV in the same manner as described previously (Tanji *et al.* 2003).

Autopsy samples of human salivary glands (submandibular and sublingual) were obtained from three subjects (50-year-old female, 71-year-old male, and 72-year-old male) who had died of stroke. Brain tissues were also obtained from three other patients who had died with cerebral infarcts. For histological examinations, tissues were fixed with formalin, embedded in paraffin, sectioned and then stained with hematoxylin and eosin. Four-μm thick sections were immunostained using the avidin-biotin-peroxidase complex method with diaminobenzidine as the chromogen. The immunolabeled sections were counterstained with either methyl green or hematoxylin. The primary antibody used (diluted 1:5 000) was an anti-human MT-III polyclonal antibody (Richarz and Bratter 2002) that was provided by courtesy of Prof. Milan Vašák (Department of Biochemistry, University of Zurich, Switzerland). The specificity of this antibody had been confirmed by a conventional technique of dot-blot assay using recombinant human MT-I, -II, and -III protein (Irie and Keung 2001) conjugated on nitrocellulose membrane (data not shown).

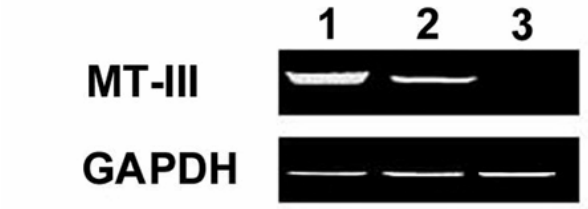

Fig. 1. Detection of MT-III expression in human salivary gland by RT-PCR. Upper panel represents the results of RT-PCR from cultured normal human astrocytes (lane 1), human salivary gland (lane 2), and human thyroid (lane 3). Lower panel represents the expression of GAPDH in the same tissues.

We examined the expression of MT-III gene in human salivary and thyroid glands. In a previous study using RT-PCR, we have shown strong expression of MT-III gene in cultured normal human astrocytes NHA6700 (Fig. 1, lane 1). In this study, we showed that MT-III was also expressed in the salivary, but not in the thyroid gland (Fig. 1, lanes 2 and 3, respectively).

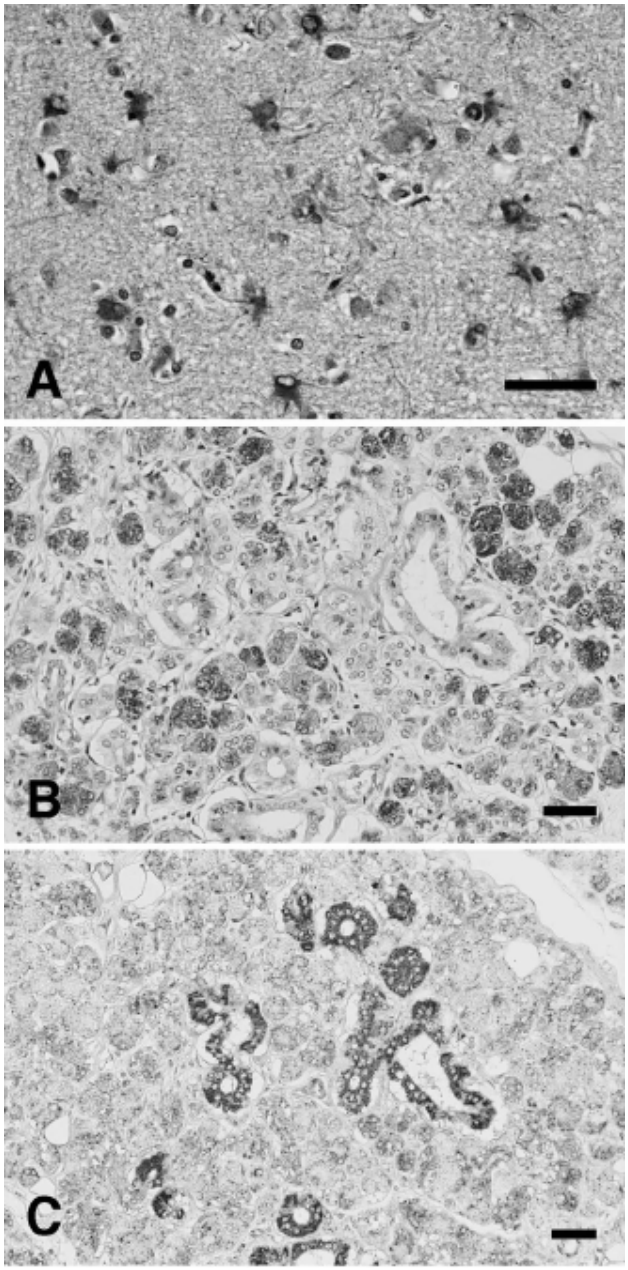

Fig. 2. MT-III immunoreactivity in the human brain and salivary gland. (**A**) Sections of the cerebral infarcts showing MT-III immunoreactivity in reactive astrocytes. (**B** and **C**) Serial sections of the submandibular gland stained with hematoxylin-eosin (B) and anti-MT-III (C) showing intense MT-III immunoreactivity in the salivary ducts. Acinar cells of the serous alveoli are also weakly immunostained. Bar 50 μm.

To confirm the presence of MT-III protein in the salivary gland, we examined the human submandibular and sublingual glands from three autopsied patients using immunohistochemical methods. The brain tissues from patients with cerebral infarction were also included for comparison. MT-III immunoreactivity of reactive astrocytes adjacent to the cerebral infarcts was intense, however, that of neuronal cells was not clear (Fig 2A). This observation is inconsistent with those in the previous studies that MT-III localizes mainly in astrocytes but weakly in neurons (Uchida *et al.* 1991, Hozumi *et al.* 1996), where the specificity of this anti-MT-III antibody was confirmed. In the submandibular gland, the duct cells were intensely immunostained with anti-MT-III antibody (Fig 2B, C). The acinar cells were also weakly immunolabeled with anti-MT-III. The sublingual gland showed similar results (data not shown).

Previously, the expression of MT-I and -II has been reported in various tissues (Vallee 1995), including the salivary gland (Sunardhi-Widyaputra *et al.* 1995). The present study demonstrated for the first time that MT-III is expressed in the salivary gland. This, together with the findings that MT-III is also expressed in the kidney (Garret *et al.* 1999a), prostate (Garret *et al.* 1999b), urinary bladder (Sens *et al.* 2000), and breast (Sens *et al.* 2001), appears to suggest that MT-III may have an even wider tissue distribution and implicate more diverse biological functions for this protein beyond that of neuroprotection.

The present immunohistochemical study showed that MT-III is concentrated in the salivary ducts. Recently, Kim *et al.* (2002) reported that MT-III immunoreactivity is present in the proximal tubules of the human kidney and that the function of a human renal cell line retains properties of the proximal tubules. These findings suggest that MT-III is involved in the transport function in the kidney. Hecht *et al.* (2002) reported that MT-I plays important roles in the differentiation of acinar cells in the salivary gland. It is possible to consider that the kidney, salivary gland, and the other endo- or exocrine organs may share MT-III for its common activity in transport. The precise function of MT-III in these organs should be investigated in future studies.

Many neuro- and immunomodulating factors such as the nerve growth factor (NGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), renin and kallikreins are found in the salivary gland that are secreted into the blood stream and may affect the function of the gastrointestinal tract (Sabbadini and Berczi 1995). MT-III may be secreted into the saliva and act in a similar way. The present study cannot make it clear if MT-III is secreted into the saliva. However, it may be possible because it has been suggested that MT-III is secreted by astrocytes *in vitro* (Uchida *et al.* 2002). NGF is of our interest because both NGF and MT-III act on growth of neuronal cells. NGF promotes the growth of neuronal cells while MT-III has concentration-

dependent biphasic effect on neuronal cells: growth promoting at low but inhibitory at high concentrations (Irie and Keung 2001). Co-localization of these two counteracting factors in the same organ implies that NGF and MT-III may act coordinately in growth/ differentiation and in other function(s) of the salivary gland. The interaction of MT-III and the other molecules, including NGF, should also be investigated. In addition, expression of other MTs should be examined in a future study to better understand the roles that MTs play in the salivary gland.

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