NOTE *Surgery*

Immunohistochemical Analysis of Cartilage-Derived Retinoic Acid-Sensitive Protein (CD-RAP)/Melanoma Inhibitory Activity (MIA) in Murine, Canine, Bovine and Equine Cerebrospinal Tissues

Satoshi TOKUNAGA[1)]*, Makoto FUJIKI[1)], Akira YABUKI[1)] and Kazuhiro MISUMI[1)]

[1)]*Department of Veterinary Medicine, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0065, Japan*

(Received 30 October 2011/Accepted 15 November 2011/Published online in J-STAGE 29 November 2011)

ABSTRACT. Cartilage-derived retinoic acid-sensitive protein (CD-RAP)/melanoma inhibitory activity (MIA), which appears abundantly in hypertrophic cartilage at the stage of endochondral ossification, is also detected in cerebrospinal fluid (CSF) following spinal cord injury. In this study, the localization of the CD-RAP/MIA molecule in normal tissues of the spine and brain obtained from mice, rats, dogs, cattle and horses was examined using immunohistochemistry with a specific antibody. The positive signals of CD-RAP/MIA were found at nerve cells in the spinal cords of all species and were especially strong at cerebellar Purkinje cells. The results suggested that CD-RAP/MIA included in normal cerebrospinal tissues could be a biomarker associated with tissue injuries, as the molecules might flow into the CSF.

KEY WORDS: CD-RAP/MIA, cerebellum, spinal cord.

doi: 10.1292/jvms.11-0494; *J. Vet. Med. Sci.* 74(4): 523–526, 2012

Melanoma inhibitory activity (MIA) was identified within growth-inhibiting activities purified from the tissue culture supernatant of the human melanoma cell line HTZ-19 [1]. MIA is translated as a 131-amino acid precursor molecule and processed into a mature 107-amino acid protein after cleavage of a putative secretion signal [1]. It plays a role in tumor progression and spread of malignant melanoma via mediation of active detachment of cells from extracellular matrix (ECM) molecules within their local milieu [11]. In a study on the mRNA from bovine chondrocytes cultured with retinoic acid (RA), PCR products, the expression of which was inhibited by RA treatment, corresponded to MIA [4]. MIA, also referred to as cartilage-derived retinoic acid-sensitive protein (CD-RAP), is detected not only pathologically in malignant melanoma but also physiologically in cartilage tissue [4, 10]. CD-RAP in cartilage appears in association with chondrogenesis of endochondral ossification in articular cartilage and is related to cartilage development and the phenotype of chondrocytes [4]. Interestingly, CD-RAP is also detected in cerebrospinal fluid (CSF) following damage or stress to the neural structures and would be a potential marker of spinal diseases in humans [8].

Aggrecan, which is a well-known proteoglycan in articular cartilage, is significantly increased in CSF from patients with rapidly progressive inflammatory disease of the spinal cord, as compared with that from patients showing slowly progressive disease [9]. Cartilage oligomeric matrix protein (COMP), which is abundantly distributed in cartilage, is a key molecule in the interplay between cells and ECM in cartilage [2, 3, 6, 7, 13]. We previously demonstrated the presence of COMP and its mRNA in the cytoplasm of tumor cells from mammary gland tumors, mast cell tumor and melanoma cells using immunohistochemistry and *in situ* hybridization [14]. The molecules have also been found in the spinal cords of mice, rats and dogs and detected in the CSF of dog intervertebral disc herniation (IVDH) cases with suspected spinal cord injury [12]. Because of the determination of common molecules such as aggrecan, COMP and CD-RAP/MIA, we have hypothesized that some constituent proteins of ECM in the cartilage could correspond to those in the cerebrospinal tissues and be diagnostic biomarkers of cerebrospinal disease. Although the CD-RAP/MIA level increases in the CSF of human patients with spinal injury and disease [8], not only the localization of CD-RAP/MIA in normal cerebrospinal tissues, but also the way it flows into the CSF in injured tissues, has not been yet explained. The aim of this study was to reveal the distribution of CD-RAP/MIA in the normal mouse, rat, dog, cattle and horse spine and brain, using immunohistochemistry with a specific antibody.

Normal spinal cords and brains were obtained from healthy mouse, rat, dog, cattle and horse subjects, which were all euthanized in accordance with the regulatory protocols approved by the animal experiment committee of Kagoshima University. The tissues were fixed in 4% paraformaldehyde and then embedded in paraffin. Serial sections (4 μm thick) were placed on glass slides, and then the slides were deparaffinized with xylene and rehydrated in ethanol. To stimulate the antigen-antibody reaction, the slides were pretreated in citrate buffer (pH 6.0) in a microwave oven for 10 min. Thereafter, the slides were cooled in the citrate buffer at room temperature (RT) for more than

*CORRESPONDENCE TO: TOKUNAGA, S., Department of Veterinary Medicine, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0065, Japan.
e-mail: yqbkp563satoshi@yahoo.co.jp

20 min and washed with phosphate-buffered saline (PBS, pH 7.0) three times for 5 min. After the blocking of endogenous peroxidase activity with 0.3% H_2O_2, the slides were washed with PBS three times for 5 min and again treated with casein buffer (0.01 M PBS containing 0.25% casein) at RT for 1 hr. The slides were then incubated with goat polyclonal antibody against human MIA (sc-17048, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) as the primary antibody (diluted 1:200 in casein buffer) overnight at 4°C. After rinsing with PBS, secondary horseradish peroxidase-labeled rabbit anti-goat IgG antibody (Sigma-Aldrich, Tokyo, Japan), diluted 1:200, was applied and kept for 30 min at RT. After rinsing three times with PBS, the color reaction with the substrate (3,3'-diaminobenzidine) was evaluated microscopically. The slides were also counterstained with Mayer's hematoxylin.

As presented in Figs. 1 and 2, immunohistochemistry with anti-MIA antibody showed positive signals in the spine and cerebellum of all species. No significant signals were detected in the cerebrum (data not shown). The positive signals in the cerebellum were distinctly strong at the Purkinje cells, of which not only the cell body but also the axon was strongly positive in all species (Fig. 1). Furthermore, the signal intensity in the nerve cells of the molecular layer varied among the species (Fig. 1); it was more distinctly positive in dog specimens than in cattle specimens. No positive reaction was seen in the white matter of the cerebellum in any animals (data not shown). In the spinal cords, positive signals were localized at the cell body, axon hillock and dendrites of larger neurons in the gray matter (Fig. 2), but there were no positive reactions at the nerve cells in the spinal white matter (data not shown). The signal intensity differed among the species.

Approximately 20% of the total base sequence of CD-RAP/MIA at the site of the 5'-end varies greatly among the animal species, but the residual 80% of the sequence is preserved with higher homology in the different species (with 100% amino acid identity in the human, mouse, rat, dog, cattle and horse). Polyclonal antibodies recognizing the epitopes near the C-terminus of the whole molecule could cross-react with the molecules in a wide range of animal species. The goat polyclonal antibody we used is raised against a peptide mapping near the C-terminus of MIA of human origin and recommended for immunochemical detection of MIA of the mouse, rat and human. In the same manner as the cerebrospinal tissues of the mouse and rat in this study, positive signals of CD-RAP/MIA were detected in the spine and cerebellum slides but not in the cerebrum slides of the other animal species. Purkinje cells showing distinctly positive signals in the cerebellum play a major role in controlling the information sent to the deep cerebellar nuclei, and degeneration and death of the cells could cause cerebellar dysfunction with a subsequent gait disorder in humans [15]. Although no previous papers have shown an increased CD-RAP/MIA level in CSF depending on the cerebellar diseases, immunohistochemical analysis of CD-RAP/MIA in degenerative cerebellar diseases might be an interesting study in veterinary clinical cases.

In the gray matter of the normal spinal cord, the larger neurons that could be motor or sensory neurons functioning as efferent and afferent nerves, respectively, retained the molecule in the body, axon hillock and dendrites. CD-RAP/MIA concentrations in the CSF were higher as a result of damage to the spinal cord in cases of cervical myelopathy, lumbar canal stenosis and lumbar disc herniation in a human clinical study [8]. We previously reported that COMP localized in the spinal cord could flow into the CSF in association with injury in the area of disc extrusion in dog IVDH [12]. Similar to that speculation, we suggest that CD-RAP/MIA retained in nerve cells could also flow into the CSF following injury to the spinal gray matter, and conversely, the increased CD-RAP/MIA level in the CSF might also be a predictive marker of spinal cord injury.

CD-RAP/MIA is included in the ECM of hypertrophic cartilage and overexpressed during the chondrogenesis phase of endochondral ossification. These phenomena are related to cartilage development and the phenotype of chondrocytes [4]. In a study on the effect of RA in fetal bovine chondrocytes, control chondrocytes presented a typical rounded shape and synthesized type II, IX, XI and III collagens, while RA-treated chondrocytes exhibited a fibroblast-like shape and decrease in the synthesis of total protein [5]. Since mRNA encoding CD-RAP is downregulated by RA *in vitro* in a time- and dose-dependent manner [4, 5], the RA-dependent differences in CD-RAP mRNA levels could provide information relevant to understanding the chondrocyte proliferation and cartilage differentiation. In our study, CD-RAP/MIA could indeed be preserved in the gray matter of the spinal cord and cerebellum in the mouse, rat, dog, cattle and horse, but we did not suggest that function. To explain the role of the CD-RAP/MIA in cerebrospinal tissues, we should analyze the CD-RAP/MIA production in the constituent cells *in vitro* and the change in the distribution in diseased or injured tissues.

This is the first paper demonstrating the presence of CD-RAP/MIA in cerebrospinal tissues. Based on these data, the molecule expressed in the gray matter of both spinal cord and cerebellum could be a credible marker for predicting cerebrospinal tissue diseases.

REFERENCES

1. Bogdahn, U., Apfel, R., Hahn, M., Gerlach, M., Behl, C., Hoppe, J. and Martin, R. 1989. Autocrine tumor cell growth-inhibiting activities from human malignant melanoma. *Cancer Res.* **49**: 5358–5363. [Medline]
2. Chen, F. H., Herndon, M. E., Patel, N., Hecht, J. T., Tuan, R. S. and Lawler, J. 2007. Interaction of cartilage oligomeric matrix protein/thrombospondin 5 with aggrecan. *J. Biol. Chem.* **282**: 24591–24598. [Medline] [CrossRef]
3. Chen, F. H., Thomas, A. O., Hecht, J. T., Goldring, M. B. and Lawler, J. 2005. Cartilage oligomeric matrix protein/thrombospondin 5 supports chondrocyte attachment through interaction with integrins. *J. Biol. Chem.* **280**: 32655–32661. [Medline] [CrossRef]
4. Dietz, U. H. and Sandell, L. J. 1996. Cloning of a retinoic acid-sensitive mRNA expressed in cartilage and during chondro-

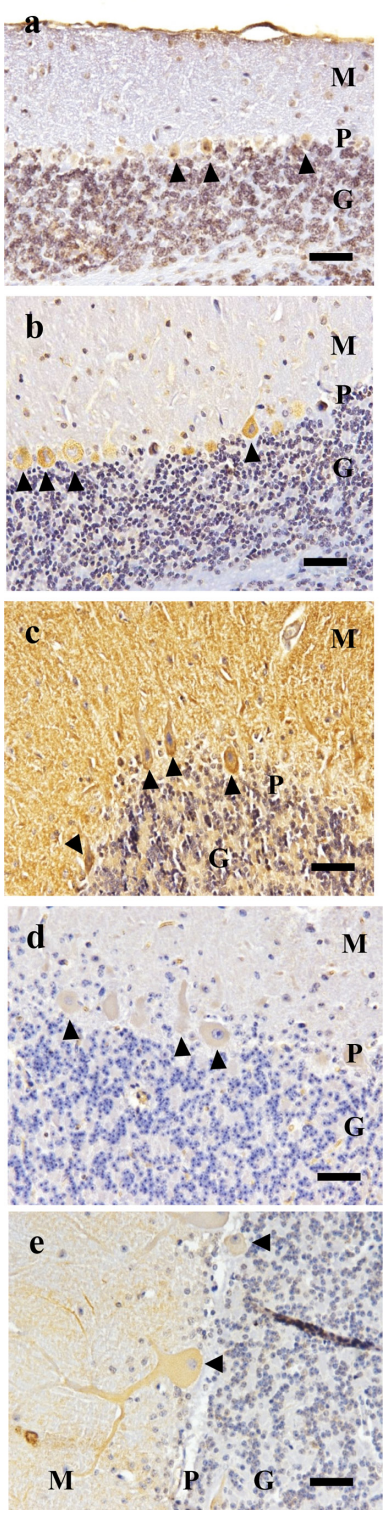

Fig. 1. Immunolocalization of CD-RAP/MIA in the cerebellum of a mouse (a), rat (b), dog (c), cattle (d) and horse (e). Purkinje cells show a distinctly positive signal in all species (arrowheads), but the signal intensity of the nerve cell body and axon in the molecular layer varies among species. M, Molecular layer; P, Purkinje cell; G, Granular layer. Bar, 50 μm.

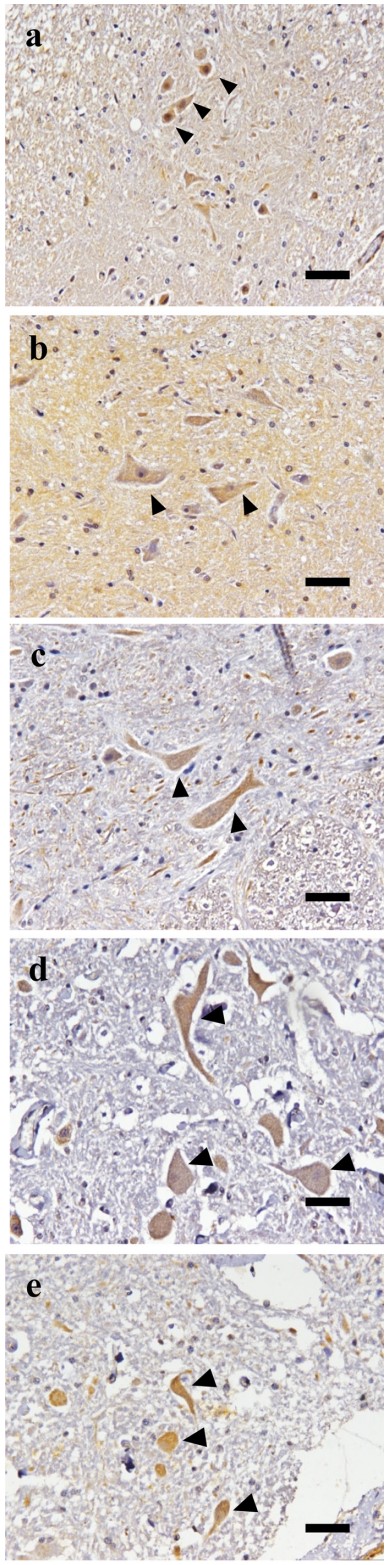

Fig. 2. Immunolocalization of CD-RAP/MIA in spinal gray matter of the mouse (a), rat (b), dog (c), cattle (d) and horse (e). The positive signals are localized at the cell body of larger neurons in the gray matter of all species. Bar, 50 μm.

genesis. *J. Biol. Chem.* **271**: 3311–3316. [Medline] [CrossRef]
5. Freyria, A. M., Ronzière, M. C., Boutillon, M. M. and Herbage, D. 1995. Effect of retinoic acid on protein synthesis by foetal bovine chondrocytes in high-density culture: down-regulation of the glucose-regulated protein, GRP-78, and type II collagen. *Biochem. J.* **305**: 391–396. [Medline]
6. Halász, K., Kassner, A., Mörgelin, M. and Heinegård, D. 2007. COMP acts as a catalyst in collagen fibrillogenesis. *J. Biol. Chem.* **282**: 31166–31173. [Medline] [CrossRef]
7. Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A. and Heinegård, D. 1992. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. *J. Biol. Chem.* **267**: 6132–6136. [Medline]
8. Natsume, N., Kondo, S., Matsuyama, Y., Sumida, K., Inou, H., Kawakami, N., Sandell, L. J. and Iwata, H. 2001. Analysis of cartilage-derived retinoic acid-sensitive protein in cerebrospinal fluid from patients with spinal diseases. *Spine* **26**: 157–160. [Medline] [CrossRef]
9. Nobuhara, Y., Usuku, K., Saito, M., Izumo, S., Arimura, K., Bangham, C. R. and Osame, M. 2006. Genetic variability in the extracellular matrix protein as a determinant of risk for developing HTLV-I-associated neurological disease. *Immunogenetics* **57**: 944–952. [Medline] [CrossRef]
10. Schmidt-Rohlfing, B., Schneider, U., Thomsen, M. and Bosserhoff, A. K. 2002. Correlation of a novel matrix protein with the degree of cartilage degradation. *Rheumatol. Int.* **22**: 165–169. [Medline] [CrossRef]
11. Stoll, R., Renner, C., Zweckstetter, M., Brüggert, M., Ambrosius, D., Palme, S., Engh, R. A., Golob, M., Breibach, I., Buettner, R., Voelter, W., Holak, T. A. and Bosserhoff, A. K. 2001. The extracellular human melanoma inhibitory activity (MIA) protein adopts an SH3 domain-like fold. *EMBO J.* **20**: 340–349. [Medline] [CrossRef]
12. Tokunaga, S., Yamanokuchi, K., Yabuki, A., Fujiki, M. and Misumi, K. 2010. Cartilage oligomeric matrix protein in canine spinal cord appears in the cerebrospinal fluid associated with intervertebral disc herniation. *Spine* **35**: 4–9. [Medline] [CrossRef]
13. Xu, K., Zhang, Y., Ilalov, K., Carlson, C. S., Feng, J. Q., Di Cesare, P. E. and Liu, C. J. 2007. Cartilage oligomeric matrix protein associates with granulin-epithelin precursor (GEP) and potentiates GEP-stimulated chondrocyte proliferation. *J. Biol. Chem.* **282**: 11347–11355. [Medline] [CrossRef]
14. Yamanokuchi, K., Yabuki, A., Yoshimoto, Y., Arai, K., Fujiki, M. and Misumi, K. 2009. Gene and protein expression of cartilage oligomeric matrix protein associated with oncogenesis in canine tumors. *J. Vet. Med. Sci.* **71**: 499–503. [Medline] [CrossRef]
15. Zhang, C., Zhu, Q. and Hua, T. 2010. Aging of cerebellar Purkinje cells. *Cell Tissue Res.* **341**: 341–347. [Medline] [CrossRef]