Japanese Journal of Veterinary Research 58(2): 143–148, 2010

INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph.D.) in Veterinary Medicine on June 30, 2010 to 5 recipients.
The titles of theses and other information are as follows:

Characterization of the Plasma Membrane Targeting and the Endoplasmic Reticulum-associated Degradation of Bovine Anion Exchanger 1

Hirokazu Adachi

Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

The present study demonstrated that the intracellular distribution of the R664X mutant of anion exchanger 1 (AE1) in transfected HEK293 cells is totally different from that of ΔF508 mutant of cystic fibrosis transmembrane conductance regulator (ΔF508-CFTR), when proteasomes are inhibited, although both proteins are found predominantly in the endoplasmic reticulum (ER) of the transfected cells under steady state condition. The ΔF508-CFTR forms aggresomes at the pericentriolar locus by the function of microtubules, whereas R664X AE1 does not. This finding was strengthened by the observation that R664X AE1 was localized in aggresomes in association with co-transfected ΔF508-CFTR due to a characteristic association of R664X AE1 with ΔF508-CFTR. These results indicate that R664X AE1 can form aggresomes once dislocated into the cytosol, suggesting, in turn, that ER-associated degradation (ERAD) of AE1 would occur on the ER membrane without apparent ubiquitylation and retrotranslocation of the polypeptides into the cytoplasmic space. The present study also showed that the conserved amino acid sequence, the EL(K/Q)(L/C)LD(A/G)DD sequence, within the C-terminal tail is structurally important. The data demonstrate that the conserved amino acid residues ELXXLD (X is any amino acid residue) in this region have essential structural consequences in stable expression of AE1 at the plasma membrane regardless of the ability in binding to carbonic anhydrase II of this region. AE1 mutants, in which the conserved sequence was disrupted by substitution mutations, showed retardation in the ER followed by proteasomal degradation. The molecular structure within the cytoplasmic region of the polytopic proteins would thus be included in recognition by ERAD molecules in addition to the putative function for exit from the ER. Taken together, these findings suggest that the N-terminal and C-terminal cytoplasmic regions of the AE1 protein may possess some signals for intracellular trafficking and/or degradation via the ERAD pathway.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43250
Some parts of this thesis have been published in *Jpn. J. Vet. Res.* **57**: 135–146 (2009) and *Jpn. J. Vet. Res.* **58**: 101–110 (2010).

Modification of Spectrin in Red Cell Membranes by the Lipid Peroxidation Product 4-Hydroxy-2-nonenal Associated with the Changes in Red Cell Membrane Properties

Nobuto Arashiki

Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Spectrin strengthens the red cell membrane through its direct association with membrane lipids and through protein-protein interactions. Spectrin loss reduces the membrane stability and results in various types of hereditary spherocytosis. However, less is known about acquired spectrin damage. The present study showed that α- and β-spectrin in human red cells are the primary targets of the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) by immunoblotting and mass spectrometry analyses. The level of HNE adducts in spectrin (particularly α-spectrin) and several other membrane proteins was increased following the HNE treatment of red cell membrane ghosts prepared in the absence of MgATP. In contrast, ghost preparation in the presence of MgATP reduced HNE adduct formation, with preferential β-spectrin modification and increased cross-linking of the HNE-modified spectrins. Exposure of intact red cells to HNE resulted in selective HNE-spectrin adduct formation with a similar preponderance of HNE-β-spectrin modifications and marked reduction in the membrane mechanical properties. These findings indicate that HNE adduction occurs preferentially in spectrin at the interface between the skeletal proteins and lipid bilayer in red cells and suggest that HNE-spectrin adduct aggregation results in the extrusion of damaged spectrin and membrane lipids under physiological and disease conditions.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43145
This thesis has been published in part in *Biochem. Biophys. Res. Commun.*, **391**: 1543–1547 (2010).

Canine Reticulocyte Exosomes: Parallel and Selective Extrusion of Na,K-ATPase and Stomatin during Reticulocyte Maturation in Dogs with HK and LK Red Cell Phenotypes

Tomohiko Komatsu

Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Reticulocyte maturation in dogs is associated with marked changes in contents of various membrane proteins. The present study showed that this membrane remodeling process in dogs

involves the selective extrusion of Na,K-ATPase and a lipid raft-associated protein stomatin via the exosome pathway, as well as several other proteins incorporated into reticulocyte exosomes such as Hsc70 and transferrin receptor (TfR). In exosomes, the Na,K-ATPase, TfR, and about half of the stomatin were found in detergent-soluble fraction that was distinct from the lipid raft-derived fraction. Moreover, Na,K-ATPase and a portion of stomatin were distributed differentially to Hsc70, TfR, and ganglioside GM1 in vesicles separated by sucrose density gradient centrifugation. This study also showed that the levels of stomatin decrease in parallel with those of Na,K-ATPase during reticulocyte maturation. The stomatin content of HK reticulocytes is higher than that of LK reticulocytes, and remains in the erythrocytes at levels compatible with that in human erythrocytes. However, it is almost absent from LK erythrocytes with the *lk*/*lk* genotype; similar to the deficiency seen in human red cells with overhydrated stomatocytosis. LK erythrocytes from *hk*/*lk* genotype dogs show reduced, but not negligible, levels of stomatin. These findings demonstrate that a heterogeneous group of exosomes participates in membrane remodeling during reticulocyte maturation in dogs and suggest that Na,K-ATPase and stomatin can associate together in non-lipid raft segments of the membrane in reticulocytes and subsequently in some population of exosomes, leading to their parallel reduction during reticulocyte maturation. The findings of the present study also indicate that the erythrocyte stomatin level is a suitable genotypic marker for the HK/LK red cell phenotype, and suggests that again a functional association between stomatin and Na,K-ATPase. The absence of morphological abnormalities in the erythrocytes of stomatin-deficient LK dogs also confirms that stomatin deficiency and stomatocytic shape change are independent from each other. The mechanisms remain to be solved include those for selective incorporation of Na,K-ATPase and stomatin and for HK/LK phenotypic diversity and its causative gene regulation in dogs.

The full text of this thesis (PDF) appears at http://eprints.lib.hokudai.ac.jp/dspace/handle/2115/43146
Some parts of this thesis have been published in *Jpn. J. Vet. Res.*, **58**: 17–27 (2010) and *J. Vet. Med. Sci.*, **72**, in press.

Studies on cellular factors affecting the formation of abnormal isoform of prion protein

Satoshi Nakamitsu

Laboratory of Prion diseases, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Cells infected with prions have been used for analyses of cell biological mechanisms of prion propagation and formation of the abnormal isoform prion protein (PrP^{Sc}). Studies using prion-infected cells have provided considerable insights into prion biology, including knowledge of the mechanism of PrP biosynthesis and the cell biological mechanism of prion propagation. However, the precise mechanism of prion propagation in cells remains to be elucidated. To facilitate further understanding of the mechanism of prion propagation, I attempted to

identify host factors and cellular environments associated with prion propagation using prion-persistently infected Neuro2a (N2a) mouse neuroblastoma subclones.

In Chapter I, I have described the results obtained from the investigation of the mechanism of the fluctuation of the level of protease resistant prion protein (PrP-res) in prion persistently-infected N2a cells. The level of PrP-res in N2a cells infected with prions decreased to 50% of that of the initial level over the first 48 hr and recovered 72–96 hr after seeding. The level of cellular prion protein (PrP^{C}) also appeared to fluctuate but did not influence the fluctuation of the PrP-res level. Prion-infected N2a cells, co-cultured with a higher number of prion-unsusceptible cells had twice as much PrP-res than those cultured without unsusceptible cells, suggesting that cell density influences the fluctuation of PrP-res levels. Direct cell-to-cell contact between cells rather than soluble factors was involved in the cell density-dependent increase in the PrP-res level. Cholesterol content and localization, which are known to influence PrP-res formation, also changed depending on cell density. Taken together, these results suggest that alterations in cellular microenvironments controlled by cell density influence PrP-res formation.

In Chapter II, I have described the results obtained from knockdown of the expression of the *Clc-5* gene to study the involvement of acidification of the endosomal vesicles and endosomal recycling machinery in PrP^{Sc} formation. The knockdown of *Clc-5* gene expression by siRNA against the *Clc-5* gene reduced the PrP-res level in prion-infected N2a-5 cells to 40% of that of cells transfected with negative control siRNA, but did not affect the expression of PrP^{C}. Unexpectedly, the knockdown of Clc-5 expression in N2a-5 cells resulted in enhanced acidification of the endosomal-lysosmal vesicles. Bafilomycin A1 treatment could overcome the enhanced vesicular acidification but it did not inhibit the reduction in the PrP-res level induced by the Clc-5 siRNA, suggesting that enhanced acidification is not a major factor in the reduction of PrP-res levels by Clc-5 knockdown. N2a-5 cells transfected with Clc-5 siRNA showed slower trafficking of transferrin than cells transfected with negative control siRNA. Furthermore, the knockdown of Clc-5 expression affected intracellular cholesterol distribution and altered the localization of rab11, a marker molecule for recycling endosome. These results suggest that the endosomal recycling pathway was impaired by the reduction of Clc-5 expression. Since the enhanced acidification of endosomal-lysosomal vesicles did not appear to be the major cause of the reduction in the PrP-res level, impairment of the endosomal recycling pathway by the knockdown of Clc-5 expression may account for the reduction in the PrP-res level.

In my thesis, I reported that (i) cellular microenvironments controlled by cell density and/or direct cell-to-cell contact and (ii) the endosomal recycling pathway are involved in PrP^{Sc} formation in cells. Although my findings are far from providing a complete understanding of the mechanism of prion propagation in cells, I believe that they are useful for promoting further efforts to elucidate the mechanism of prion propagation.

The original paper of this thesis appeared in *J. Gen. Virol.*, **91**: 563–569 (2010).

Differentiation of bone marrow mesenchymal stem cells through culture with chondrocytes to produce adequate cartilage constructs in horses

Vivien Lettry

Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Being joint injuries and joint diseases the most common cause of lameness in horses that perform athletic events, limiting efficiency and even demanding early retirement, this study was devoted to the investigation of the current novelties for the diagnosis and treatment of joint cartilage degradation.

In the first part, the performance of a system that indentifies cartilage turnover and degeneration through measurement of keratan sulfate (KS) epitope concentrations present in equine sera was evaluated. The aim was to analyze measurements obtained using anti KS antibody 5D4 (KS5D4) compared to High Sensitive Keratan Sulfate (HSKS) ELISAs, and to estimate changes of cartilage metabolism in young athletic horses under regular training schedules. Results showed that HSKS can sensitively detect early signs of cartilage metabolic change. Therefore, allowing prompt intervention to protect articular cartilage before irreversible deterioration has occurred.

An additional way to estimate articular cartilage turnover would be through the application of biomarkers targeting collagen type II synthesis and cleavage sites. In part II, changes in synovial fluid concentrations of procollagen II C propeptide (CPII) and collagen II cleavage site (C2C) were investigated. Such peptides can function as markers of join cartilage synthesis and degeneration, respectively. They were measured in horses with osteochondrosis dissecans (OCD) or intraarticular fracture, and the relationship between arthroscopic findings of the affected joints and these biomarkers levels were examined. Within each disease group of horses there was no correlation between biomarkers levels and arthroscopic findings. Therefore, although CPII and C2C have diagnostic potential, further knowledge is required to provide accurate analysis.

There are still many contradictory reports on the efficacy of an early and precise diagnosis of articular cartilage damage using biomarkers as shown in parts I and II. A suitable therapy has not yet been established so far, which recovers cartilage damage and overcome local limitations of tissue healing. Currently, mesenchymal stem cells (MSCs) have been considered a potentially suitable alternative for tissue regeneration, including articular cartilage. The subsequent part of the study focused attention at MSCs harvesting, characterization and differentiation in horses.

Following bone marrow aspiration from equine sternebra, protocols already established for differentiation of the selected cells to chondrogenic, osteogenic and adipogenic lineages were performed. Harvested cells were then characterized as equine MSCs.

When considering bone marrow derived MSCs to repair cartilage defects, these cells should be properly stimulated so that they could differentiate morphologically and hold cellular synthetic features closer to maturely differentiated chondrocytes, this way avoiding spontaneous differentiation of MSCs into divergent lineages. In order to increase MSCs

value for cartilage repair, improvement of chondrogenic differentiation of such cells was investigated using coculture of MSCs with mature equine articular chondrocytes. The use of coculture system produced enhanced differentiation of equine MSCs, improving expression of cartilage-specific genes and resulting in a more homogeneous production of extra cellular matrix within the newly formed cocultured cartilage. Such differentiation approach could play an important role in articular cartilage repair and regeneration in the near future.

The original papers of this thesis appeared in *Jpn. J. Vet. Res.*, **57**: 197–206 (2010), *Jpn. J. Vet. Res.*, **57**: 207–212 (2010) and *Jpn. J. Vet. Res.*, **58**: 5–15 (2010)