the Angus breed, the clinical signs are observed at birth or up to 3 months of age. A congenital noninherited cerebellar abiotrophy with progressive clinical signs at birth and degeneration of Purkinje cells has been described in Holstein cattle in southern Brazil. The inherited hypermetria described in Shorthorn cattle in this report can be differentiated from cerebellar abiotrophy and other similar diseases by the absence of histologic and ultrastructural lesions in Purkinje cells. Also, in cerebellar abiotrophy clinical signs are usually progressive, whereas in the Shorthorn cattle disease described here clinical signs were always nonprogressive.

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References


Ubiquitin immunocytochemistry of spinal cord in an inherited porcine motor neuron disease

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Ubiquitin is a 76-amino acid polypeptide that is highly conserved in all eukaryotic cells. It is a member of the heat-shock (cell stress) family of proteins, and it shows high levels of expression when cells are subject to specific stresses such as heat, hypoxia, irradiation, and viral infection. A major function of ubiquitin involves covalent attachment to target proteins (“ubiquitination”), thereby designating ubiquitin-conjugated proteins for ATP-dependent nonlysosomal proteolysis. Some ubiquitinated proteins may undergo catabolism via the lysosomal system. Immunoocytochemical studies using antibodies to ubiquitin have documented ubiquitinated inclusions in selected human neurodegenerations, as well as in some chronic hepatic and muscular disorders. Ubiquitin-positive intraneuronal inclusions are now recognized in Alzheimer’s disease, Down’s syndrome, diffuse Lewy body disease, amyotrophic lateral sclerosis (ALS), infantile motor neuron disease, and normal aged brains, among others. It is unclear whether ubiquitin is an integral part of these inclusions or if instead it is tagging proteinaceous inclusions to attempt catabolism. Detection of the intracytoplasmic skein-like or granular ubiquitin-positive inclusions, some of which are closely associated with Bunina bodies, in spinal cord motor neurons from human ALS patients has been advocated as the most reliable way to confirm histologically a clinical diagnosis of ALS.

There are few studies of ubiquitin expression in the neural axis of animals with chronic neurodegenerations. Hereditary porcine neuronal system degeneration (HPNSD) is a genetically determined lower motor neuron disease of pigs that was originally identified in England. Typical clinical signs involve paraparesis of the pelvic limbs, with quadriaparesis in some severely affected pigs. Alpha motor neurons in the spinal cord become atrophic, and many develop intracytoplasmic vacuoles before they disappear (Fig. 1). This is a rare, dominantly inherited trait that has been recognized only in pigs in England and in a colony of pigs kept initially at the Central Veterinary Laboratory in Weybridge, England (1983-1991) and then at the Wyoming State Veterinary Laboratory (WSVL) (1992-the present). The subtility of histologic lesions in the spinal cord may hamper diagnosis. The purpose of this study was to determine, using an immuno-histochemical method and 5 antibodies to conjugated ubiquitin, whether abnormal ubiquitin patterns occurred in the spinal cords of 4 piglets with typical clinical signs of HPNSD. Documentation of such inclusions could be useful diagnos-
Coronal sections of spinal cord were obtained from 4 young pigs with typical clinical signs of HPNSD that varied from mild (pig no. 1) to moderate (pig no. 4) to severe (pigs no. 2 and no. 3) (Table 1). Clinical signs developed between 33 and 59 days of postnatal life, and pigs were either killed (nos. 1, 2, 3) or died (no. 4) between 83 and 163 days of age. Two pigs (nos. 1, 2) were born in 1986 at the Central Veterinary Laboratory in Weybridge, England, and formalin-fixed spinal cord at C7 was stored for 6 years before attempted immunostaining. Two affected pigs (nos. 3, 4) were litter-mates born at the WSVL in Laramie, Wyoming, in 1992. Segments of cervical, thoracic, and lumbar spinal cord from pigs no. 3 and no. 4 were fixed for 1 day in neutral phosphate-buffered formalin. Spinal cord segments from the 4 HPNSD-affected pigs were dehydrated in alcohol and embedded in paraffin wax. For negative control tissue, cervical, thoracic and lumbar spinal cord segments were collected into formalin at necropsy from an unrelated, clinically normal, freshly killed pig (no. 5) age 84 days from the University of Wyoming swine herd. Following fixation for 1 day, spinal cord from this control pig was processed identically to tissue from pigs no. 3 and no. 4.

Five sets of antibodies to conjugated ubiquitin were used: murine monoclonal antibodies (MAbs) 4-3H8 and 4-2D8 and lapine-origin polyclonal antibodies (PAbs) UH 19, Z458. Both MAbs are specific for ubiquitin conjugated by glutaraldehyde to laprine serum albumen, detect epitopes between ubiquitin amino acid residues 34 and 54, and were used in immunohistochemical studies of Alzheimer’s disease and of β-mannosidosis encephalopathy of Salers calves. PAb UH19 was raised to ubiquitin conjugated by glutaraldehyde to keyhole limpet hemocyanin, detects both conjugated and unconjugated ubiquitin, has specificity for ubiquitin that was confirmed in earlier absorption studies, and was used in immunohistochemical studies of several chronic human neurodegenerations. PAbs 221M and Z458 were raised to ubiquitin derived from bovine erythrocytes and conjugated to gammaglobulin; specificity for ubiquitin

Table 1. Staining of cytoplasmic inclusions in spinal cord from normal and HPNSD-affected pigs with antiubiquitin antibodies.
was confirmed by the commercial suppliers by immunoblotting and immunoelectrophoresis (both PAbs), as well as by indirect enzyme-linked immunosorbent assay (Z458 only). PAb Z458 was raised in-house by the supplier, whereas PAb 221M was obtained from an outside source the supplier was unwilling to disclose. Preliminary studies using control-positive tissue (cerebral cortex from a human patient with Alzheimer’s disease) and spinal cord from a clinically normal pig indicated that use of a published “universal” immunochemical protocol was successful only when sections were pretreated for antigen retrieval using microwave heating. This method had the disadvantage that most or all porcine spinal motor neurons in the ventral horn were lost from tissue sections following the harsh microwave protease digestion step. Immunocytochemical staining was therefore done using an avidin-biotin (ABC) kit according to the manufacturer’s directions. The 5 antiubiquitin antibody solutions were diluted in phosphate-buffered saline (PBS) as follows: 1:40 (4-2D8 1:10 (4-3H8), 1:200 (UH19), pre-diluted (221M), and 1:160 (Z458). No enzymatic or microwave oven antigen retrieval was required. Deparaffinized tissue sections were incubated with specific sera for 60 minutes at room temperature. Following secondary incubations, sections were incubated with DAB (3,3’ diaminobenzidine tetramchloride/nickel chloride) and counterstained with Mayer’s hematoxylin solution. For negative controls, PBS or pooled sera from 3 healthy rabbits (diluted 1:200) was substituted for specific antiubiquitin PAbs, and PBS or an irrelevant MAb (MAb to bluetongue virus) was substituted for antiubiquitin MAbs. Duplicate sections from all blocks were stained with hematoxylin and eosin (HE).

In control Alzheimer’s disease tissue, typical ubiquitin immunoreactive patterns were detected in cerebrocortical neurofibrillary tangles and senile plaques (Fig. 2) and as dot-like deposits in subcortical white matter. Immunoreactive patterns were identical in distribution and intensity in Alzheimer’s disease cerebral cortex for all 5 antiubiquitin antibodies at the dilutions specific above. Staining of these structures was absent when normal laprine serum, PBS, or an irrelevant MAb was substituted for antiubiquitin antibodies. In normal porcine spinal cord, there was weak to moderate ubiquitin immunoreactivity in many nuclei of macroglial and neuronal cell populations. Weak positive ubiquitin immunoreactivity was present in some cell processes adjacent to the central canal’s ependymal lining and in the cytoplasm of some oligodendroglia in white matter. Intracytoplasmic ubiquitin immunoreactivity was detected in ventral horn motor neurons of cervical, thoracic, and lumbar spinal cord from the clinically normal pig used as a control.

There was no evidence of abnormal ubiquitin immunoreactive patterns in the 4 HPNSD-affected pigs using 3 of 5 antiubiquitin antibodies (Table 1). A slight increase in chromagen deposition in markedly atrophic neurons and in axonal spheroids was interpreted as increased background rather than specific staining. Two of 5 antibodies (221M and Z458; Table 1) resulted in identical patterns of moderate positive staining in perikarya of all vacuolated and some nonvacuolated alpha motor neurons in 3 of 4 HPNSD-affected pigs. Other neuronal populations, in dorsal horn and in dorsal root ganglia as well as nonneuronal cells, were unstained. Ubiquitin-immunoreactive inclusions were absent in the spinal cord of the clinically normal pig (no. 5) and of HPNSD piglet no. 1, which had the mildest clinical signs (Table 1). In vacuolated atrophic neurons, positive staining comprised round inclusions (Fig. 3). Aggregated inclusions generally filled perikarya of affected vacuolated neurons. In nonvacuolated neurons, positively stained inclusions were small (1-3 µm) and either ring-like or solid (Fig. 4). When numerous, inclusions encircled the nucleus but did not completely fill the perikaryon. No structures that corresponded to these inclusions in nonvacuolated neurons were evident in HE-stained sections of spinal cord. Ubiquitin-immunoreactive perikaryal inclusions were sparse in cervical spinal cord at C7 of pig no. 2 (the only level available for examination). In pig no. 4, inclusions were absent in cervical spinal cord and sparse in thoracic and lumbar spinal cord. In pig no. 3, inclusions were absent in cervical spinal cord, sparse in thoracic spinal cord, and relatively common in lumbar spinal cord. Specific staining of inclusions was absent when pooled normal laprine serum was substituted for ubiquitin-specific antibody solutions.

These immunocytochemical findings are evidence that in a proportion of lower motor neurons from HPNSD pigs there is increased expression of intracytoplasmic ubiquitin. The subcellular nature of these inclusions is uncertain, because in conventional HE preparations equivalent structures are
not visible. Only 2 of 5 antiubiquitin antibodies used for this study detected inclusions. Because all 5 antibodies resulted in identical staining patterns in Alzheimer’s disease cerebral cortex used as a ubiquitin-positive control, it may be significant that both successful antibodies were raised to conjugated ubiquitin using similar (or identical) protocols in rabbits. It is unlikely that staining of intraneuronal inclusions is due to the presence of contaminating nonubiquitin-specific antibodies. Intracytoplasmic staining was clearly associated with atrophic neurons in particular and was absent in spinal cord from a clinically normal pig. Pooled serum from clinically normal rabbits, when substituted for ubiquitin-specific serum, did not result in staining of inclusions. The presence of ubiquitinated inclusions in this disease may reflect increased turnover of short-lived proteins by stressed, degenerating alpha motor neurons. Ubiquitin immunocytochemistry may be of some value in mapping the distribution of neuronal degeneration in brain stem and spinal cord of pigs with HPNSD.

Ubiquitin-immunoreactive intracytoplasmic inclusions in these pigs (round with unstained cores) differed from the principal types of inclusions found in human ALS. In ALS, ubiquitinated inclusions in alpha motor neurons are loose filamentous and skein-like or solid ball-like inclusions with frayed margins. Ultrastructurally, these correspond to bundles of 10-15 µm diameter filaments (skein type) and 15-25 µm diameter filaments (ball type). There is evidence that ubiquitin expression in ALS is most pronounced in the earlier clinical stages of the disease. In the motor neuron degeneration (Mnd) mouse, there is abnormal ubiquitin expression in both the presymptomatic and symptomatic stages of the disease. Intra-neuronal ubiquitin-protein conjugates have also been detected in lysosome-like bodies in brains of scrapie-inoculated mice, in nonmyelinated dystrophic neurites in Down’s syndrome brains, and in myelinated dystrophic neurites in aged human and canine brains. Round inclusions with a more intensely stained ubiquitin-immunoreactive periphery do occur in ALS but are rare. Diffuse cytoplasmic staining for ubiquitin has also been described in infantile motor neuron disease and in the Mnd mouse, but this pattern of immunostaining was absent in these HPNSD pigs.

It would be interesting to extend these findings to pigs at different stages of clinical progression of HPNSD and to establish whether similar inclusions occur in other forms of lower motor neuron disease in animals, such as spinal muscular atrophy (SMA) in Brown Swiss calves and hereditary canine spinal muscular atrophy of Brittany spaniels, particularly in the context of the proposed intermediate filament-ubiquitin family of neurodegenerations.

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