Short Communication

Transgenic Mice Over-Expressing the C-99 Fragment of β PP with an α -Secretase Site Mutation Develop a Myopathy Similar to Human Inclusion Body Myositis

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Inclusion body myositis (IBM) is the most common muscle disease in the elderly. Amyloid- β protein ($A\beta$) has been shown to accumulate abnormally in the vacuolated fibers and to localize to amyloid-like fibrils in muscles from IBM patients. We studied the skeletal muscles from a line of transgenic mice over-expressing the carboxyl-terminal 99 amino acids (C99) of the β -amyloid precursor protein (β PP) with a substitution of lysine-612 to valine (K612V), intended to abolish α -secretase recognition and to preserve the $A\beta$ domain of C99. The majority (87%) of the 24-month-old transgenic mice showed myopathic changes, and approximately one-third of them had degenerating fibers with sarcoplasmic vacuoles and thioflavin-S-positive deposits. Ultrastructurally, the inclusions were aggregates of short thin amyloid-like fibrils, 6 to 8 nm in diameter. These features are similar to those of human IBM. Immunocytochemistry using an antibody against $A\beta$ showed membranous staining in most muscle fibers of transgenic mice, as well as granular or vacuolar cytoplasmic staining in the atrophic fibers. Western blots showed a high level of accumulation of carboxyl-terminal fragments of β PP in the muscles of the transgenic mice with the most severe IBM-like lesions. The expression of IBM-like lesions was age dependent. These transgenic mice provide a model for the study of IBM and for the peripheral expression of a key element in the pathogenesis of Alzheimer disease. (*Am J Pathol* 1998, 153:1679-1686)

The similarities in the pathogenetic mechanisms underlying two age-associated disorders, Alzheimer disease (AD) and inclusion body myositis (IBM), are receiving increasing attention. IBM is the most common muscle disease in patients over the age of 55 years.^{1,2} It is a slowly progressive, debilitating disorder that is unresponsive to steroid therapy. Cryostat sections of muscles with IBM stained with hematoxylin and eosin (H&E) show angulated atrophic fibers, mononuclear cell inflammation, and characteristic rimmed vacuoles in the degenerating muscle fibers. The vacuolated muscle fibers are Congo-red positive,³ consistent with amyloid deposition. By electron microscopy (EM) the vacuolated fibers contain filamentous cytoplasmic and/or nuclear inclusions.^{1,2} Cytoplasmic filaments of 15 to 19 nm appear to have a tubular structure and a peculiar helical arrangement and have been termed either twisted tubulofilaments or paired helical filaments.⁴ There are also aggregates of short thin fibrils 6 to 8 nm in diameter that are decorated with antibodies against amyloid- β protein ($A\beta$).⁵ The filamentous inclusions are surrounded by membranous whorls and cellular debris. The inclusions, or some material associated with them, also immunoreact with antibodies for other epitopes of β -amyloid precursor protein (β PP),^{6,7} hyperphosphorylated tau,^{4,8} α_1 -antichymotrypsin,⁹ apolipoprotein E,^{10,11} ubiquitin,^{12,13} cellular prion protein,¹⁴ and presenilin-1.¹⁵ All of them are proteins that are likely to play key roles in the degeneration of neurons in AD and other neurodegenerative disorders. Therefore, AD and IBM appear to share some common pathogenetic mechanisms, or at least similar mechanisms of amyloidogenesis.

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There is strong evidence that intracellular events mediated by β PP metabolites may cause neuronal death. Several laboratories have reported that, by overexpression, either full-length^{16,17} or the amyloidogenic carboxyl-terminal fragments (CTFs) of β PP^{18,19} are neurotoxic *in vitro* and cause AD-like lesions *in vivo*. Our laboratory has studied the effects of a fusion protein termed S β C composed of the carboxyl-99 residues of β PP fused to the 17-amino acid signal peptide of β PP. S β C overexpression in neuron-like cells resulted in altered proteolysis and was associated with neurotoxicity.^{20–22} The toxic effects were related to CTFs of approximately 14 and 15 kd that contain intact A β domains and are potentially amyloidogenic. These CTFs are normal metabolic products of β PP in the human brain.²³ Several β PP mutations linked to familial AD cause intraneuronal accumulation of potentially amyloidogenic CTFs.²⁴ So far, however, no intraneuronal amyloid has been found in these model systems.

Here we report a line of transgenic mice showing an abnormal accumulation of CTFs in the skeletal muscles, with associated lesions similar to IBM. This is the first animal model for this untreatable disease.

Materials and Methods

Transgenic Mice

The K612V substitution in pCA-S β CK612V was made by modifying the pCA-S β C construct²¹ using the Alter Sites Mutagenesis System (Promega, Madison, WI). The pCA-S β CK612V vector encodes a 17-amino-acid signal peptide fused to C99 of β PP containing a valine in place of the lysine at the site of α -secretase cleavage (K612V of β PP695). Expression is under the control of the cytomegalovirus enhancer/chicken β -actin promoter. The sequence of the entire mutagenized cDNA was confirmed by automated DNA sequencing (Department of Pharmacology, University of Washington). A 3.1-kb *SalI/PstI* fragment containing the enhancer, promoter, and mutated cDNA sequences was isolated and injected into the fertilized eggs produced by mating between F1s of C57BL/6 and C3H mice. Founders were backcrossed to C57BL/6 mice (three to four backcrossings at the time of this analysis).

Western Blot Analysis

Quadriceps muscle from one side was homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Quadriceps muscle from the other side was saved for histochemistry and immunocytochemistry. Fifty micrograms of each sample were run on a 16.5% SDS-PAGE gel and transferred to Immobilon-P (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat milk and sequentially incubated with B994, a polyclonal antibody to the carboxyl-terminal 39 amino acids of β PP (generously provided by Thomas R. Hinds) and a polyclonal antibody to desmin (Sigma Chemical Co., St. Louis, MO). The signal was detected using biotinylated

secondary antibodies, Vectastain peroxidase (Vector Laboratories, Burlingame, CA), and the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Histochemistry and Immunocytochemistry

Quadriceps muscles from control and transgenic mice were divided transversely into two halves. One half was immediately fixed in formalin, and some small pieces from it were fixed in 2.5% glutaraldehyde for electron microscopic examination. The other half was immediately frozen in isopentane cooled in liquid nitrogen. Adjacent cryostat sections were stained with H&E and modified Gomori's trichrome stains as well as NADH dehydrogenase reactions. Adjacent formalin-fixed, paraffin-embedded tissue sections were stained with H&E, Gomori's trichrome, thioflavin-S, myosin, and A β immunocytochemistry. Standard peroxidase-anti-peroxidase²⁵ or avidin-biotin complex (ABC)²⁶ techniques with hematoxylin counterstain were used for immunocytochemistry. Tissue pretreated with formic acid²⁷ was used for the immunocytochemistry applying A β 1-28 antibody (Athena Neurosciences, South San Francisco, CA).

Electron Microscopy

One-millimeter pieces of quadriceps were fixed in 2.5% glutaraldehyde in 0.1 mmol/L sodium cacodylate buffer. Each tissue sample was post-fixed in buffered osmium tetroxide and embedded in PolyBed (Polysciences, Warrington, PA). Plastic sections (1 μ m) were prepared from each tissue block, and these sections were stained with Azure II methylene blue. Thin sections were picked up on copper grids and stained with uranyl acetate and lead citrate. Each thin section was examined with a Philips 410 electron microscope at 80 kV.

Results

S β CK612V Transgenic Mice Showed No Brain Lesions

Most molecules of β PP undergo proteolytic release from the cell membrane by the action of an α -secretase that cleaves in the middle of the A β domain²⁸ and abolishes the amyloidogenicity of CTFs. To make the product of S β C construct more amyloidogenic, we replaced lysine at amino acid 612 of β PP-695²⁹ with valine (K612V mutation) and produced transgenic mice harboring the S β CK612V construct. As the major action of the α -secretase is directed at either amino or carboxyl to K612, it was expected that the K612V substitution would abolish much of the α -secretase recognition and preserve an intact A β domain. This effect has been demonstrated in a COS-1 cell transfection system.³⁰ Our S β CK612V construct, when transfected into P19 cells, directed the over-expression of 10- to 12-kd proteins specifically recognized by B994, a polyclonal antibody to the carboxyl-terminal 39 amino acids of β PP (M. Hearn, in preparation). We

studied line 5590, which has two unlinked arrays of S β CK612V transgenes, as revealed by Southern blot analysis using *Bgl*II-digested genomic DNA probed with DNA from the S β C construct. One array contained approximately three copies and the other one copy of the transgene (data not shown). Transgenic mice and nontransgenic sibs were sacrificed for histological and immunochemical analysis at ages 9 to 24 months. By Western blots, the relative abundance of the transgene protein products among various organs were found to be similar to those of S β C mice without the K612V mutation³¹; the brain showed comparatively less expression than the heart, intestine, kidney, and skeletal muscle (data not shown). As in the case of S β C mice,³¹ no brain lesions were found in S β CK612V mice of all ages. Modified Bielschowsky silver and A β immunocytochemical stains failed to demonstrate any senile plaque formation in the transgenic brains.

Muscles of 24-Month-Old Transgenic Mice Showed a Myopathy Similar to Human IBM

Although the quadriceps muscle of the S β CK612V mice showed at least a fivefold greater expression of the transgene protein product (C99K612V) as compared with the brain (data not shown), they did not show any lesions or any evidence of amyloid formation at ages 9 to 13 months. However, at 24 months of age, the transgenic mice exhibited myopathic changes of varying severity in the quadriceps muscle. In the mild cases, variation of muscle fiber size, increased central nuclei, and split fibers were found. In more advanced cases, the quadriceps demonstrated scattered angulated atrophic fibers, occasional necrotic fibers with macrophage infiltration, occasional regenerating fibers with basophilic cytoplasm, and markedly increased central nuclei (many fibers showed a caravan pattern of central nuclei). The modified Gomori's trichrome stain showed a patchy increase in subsarcolemmal red granular staining, indicating an increased number of mitochondria in these fibers. The trichrome stain did not show any increase in interstitial fibrous tissue. These are nonspecific myopathic changes and were seen in 26 of the 30 transgenic mice examined. Ten nontransgenic sibs examined showed none of these changes, except for one with a slight increase of subsarcolemmal mitochondria and two with a few muscle fibers with central nuclei. Although the presence of angulated atrophic fibers may indicate neurogenic atrophy, it is not likely due to the absence of fiber type grouping in NADH dehydrogenase reactions and myosin immunocytochemically stained sections (data not shown). In 9 of 30 transgenic mice examined, more striking myopathic changes were noted. As shown in Figure 1, in addition to the severe myopathic changes indicated above, there were patchy lymphocytic infiltrates around endomysial blood vessels and connective tissue (Figure 1A) seen on H&E-stained paraffin sections as well as cryostat sections. On H&E-stained cryostat sections, but not on paraffin sections, there were sarcoplasmic vacuoles, which were oval or irregular in small degenerating

fibers with small basophilic granules clinging to their edges (Figure 1C). The basophilic granules were also irregularly scattered within these vacuole-containing fibers. These granules stained red with a modified Gomori's trichrome stain (Figure 1D). Muscle fibers with vacuoles were much less frequent relative to those with other myopathic features and were not found in the nontransgenic sibs.

As we anticipated that the transgene protein product would have a high propensity to form amyloid, we screened the mouse quadriceps with thioflavin-S stains. There were thioflavin-S-positive clusters of inclusions in small fibers in the vacuole-containing muscles, indicating amyloid deposits (Figure 1B), but fibers with these thioflavin-S-positive deposits were rather rare. Immunocytochemistry using an antibody against A β 1-28 showed no staining in the control nontransgenic muscles (Figure 1E) whereas the muscles from the transgenic mice showed pronounced sarcolemmal staining in most fibers (Figure 1F). Anti-A β 1-28 also highlighted the cytoplasm of almost all angulated atrophic fibers (Figure 1F), many of which showed A β -positive, vacuole-like inclusions (Figure 1G) or coarse granules (Figure 1H) in approximately one-third of S β CK612V mice, the features of which included angulated atrophic fibers, lymphocytic infiltration, sarcoplasmic vacuoles, and thioflavin-S- and A β -positive sarcoplasmic deposits. This combination of pathological changes is similar to that seen in human IBM.

Sarcoplasmic Inclusions of Amyloid-Like Fibrils Were Seen in Degenerating Muscle Fibers by Electron Microscopy

We carried out electron microscopic examination to find additional evidence of IBM-like pathology. On semi-thin sections stained with Azure II methylene blue, myopathic changes corresponding to those found on H&E sections were noted in transgenic mice. In particular, round to oval, rather pale sarcoplasmic inclusion bodies were detected in six of the nine transgenic mice with IBM-like lesions (Figure 2A). Corresponding to the sarcoplasmic vacuoles seen on the cryostat sections, these inclusion bodies were relatively less frequent (involving less than 1% of the muscle fibers on sections) compared with other pathological features and tended to occur in a small cluster, often subsarcolemmal (Figure 2D), in one degenerating fiber. These inclusion bodies had a smooth contour with dark granules of various sizes within or around the inclusions. The muscle fibers harboring these inclusion bodies invariably were small, with disorganized myofibrils and dense debris. Focal zones of degeneration within one muscle fiber were frequent (Figure 2, A and C). There were small clusters of dark granules in the degenerating fibers (Figure 2B), which are the characteristic feature of human IBM on resin sections.² Specimens from two such transgenic mice and one nontransgenic sib were studied by EM. The above inclusions consisted of

Figure 1. Quadriceps muscle from 24-month-old S β CK612V transgenic mice showing dense foci of perivascular and interstitial mononuclear cell infiltration (A, H&E stain) and thioflavin-S-positive intracellular deposits in a small muscle fiber, indicated by an arrow (B, thioflavin-S stain). On cryostat sections, there are degenerating fibers with sarcoplasmic vacuoles (C, H&E stain; D, modified Gomori's trichrome stain). A β immunocytochemistry (E to H) shows no staining in the muscles from nontransgenic sibs (E) and wide-spread sarcolemmal membrane staining in muscles from transgenic mice (F) as well as intracytoplasmic staining in an angulated atrophic fiber (arrow). Note that there are many central nuclei in F but not in E. G and H show two examples of A β -positive degenerating fibers showing vacuolar (G, arrows) and granular (H) cytoplasmic staining. Magnification, $\times 400$ (A, B, and H), $\times 1000$ (C, D, and G), and $\times 200$ (E and F).

Figure 2. Quadriceps muscle from a 24-month-old $S\beta CK612V$ transgenic mouse. **A to C:** Azure II methylene-blue-stained, plastic-embedded semi-thin sections. **A:** An atrophic fiber at the center with loss of sarcoplasmic architecture, dark granules, and vacuoles (arrow). A neighboring fiber shows dark granules scattered among the myofibrils and a central zone of degeneration (open arrow). **B:** A degenerating muscle fiber with many isolated or small clusters of dark granules (arrows) of various sizes. **C:** A longitudinal section of a muscle fiber shows regions of degeneration admixed with dark granules. Magnification, $\times 1000$. **D to F:** Electron micrographs. **D:** A degenerating fiber with Z-band streaming and two subsarcolemmal inclusions indicated by arrows, in contrast to a normal fiber in the left lower corner. **E:** The vacuoles in **A** are inclusion bodies, one of which is shown here, with membranous whorls and irregular blotches of osmophilic granules. **F:** Inclusions are composed of tufts of short, thin (6 to 8 nm in diameter) fibrils, admixed with small osmophilic granules. The fibrils are beaded with a poorly defined periodicity. Magnification, $\times 5100$ (**D**, 1 cm = 204 nm), $\times 8100$ (**E**, 1 cm = 126.6 nm), and $\times 51,000$ (**F**, 1 cm = 196.1 nm).

aggregates of short thin fibrils, 6 to 8 nm in diameter (Figure 2, E and F), not unlike the amyloid-like fibrils that were decorated with antibodies against $A\beta$ in human IBM.⁵ The fibrils were haphazardly arranged. The individual fibrils appear straight and beaded with a poorly defined periodicity. There were also granular materials admixed with the fibrils in the inclusions. The filamentous

inclusions were surrounded by osmophilic membranous whorls and cellular debris (Figure 2, D and E). The inclusions resided solely in the sarcoplasm. No such inclusions were found in the control muscles. Thus, EM confirmed the presence of inclusions composed of amyloid-like fibrils in the degenerating muscle fibers from the transgenic mice with severe muscle pathology.

Figure 3. Fifty micrograms of protein extracted from quadriceps muscle were run on a 16.5% SDS-PAGE gel. The lanes were labeled with the mouse identification number and C (control, nontransgenic) or T (transgenic). Western blots were probed sequentially with antibodies to βPP (B994) and desmin.

Transgenic Mice with IBM-Like Pathology Exhibited High Levels of Transgene Protein Product in Their Quadriceps Muscles

Western blots were performed to estimate the relative abundance of the transgene protein products in the quadriceps muscles of both transgenic and nontransgenic mice. The CTFs of βPP, ranging in size from 10 to 12 kd, were visualized by B994, an antibody to the carboxyl-terminal 39 amino acids of βPP (Figure 3). CTFs were not present in the muscles from the nontransgenic sibs at any ages. Muscles obtained from the transgenic mice at ages ranging from 9 to 13 months showed a baseline expression level (approximately five times that of the brain) of CTFs (data not shown), although there were no pathological changes found in these muscles. In contrast, the muscles obtained from the 24-month-old mice showed a large variation in the abundance of CTFs above the baseline. As the occurrence of the inclusions in the muscle sections was relatively rare, it was impossible to make a correlation between the number of inclusions and the abundance of CTFs. A subjective three-tier histological grading of the severity of myopathic changes with a consideration of all myopathic features was performed in a blind fashion by the neuropathologist (L-W. Jin). The levels of CTFs were normalized with the levels of desmin detected on the same blot. The histological grades and the levels of CTFs did not correlate well (data not shown). However, it is noteworthy that among the nine transgenic mice with inclusions, five showed very high

levels of CTFs (at least five times the average) and more severe myopathic changes as well.

Discussion

In the 24-month-old SβCK612V transgenic mice, we found histopathological changes that are strikingly similar to those of human IBM. In addition to nonspecific myopathic changes, in the degenerating muscle fibers there were sarcoplasmic vacuoles and amyloid-like filamentous inclusions. It was noted that sarcoplasmic vacuoles may be observed in other conditions,³² such as neurogenic disorders, limb-girdle muscular dystrophy, or oculopharyngeal muscular dystrophy. However, the combination of vacuoles with Aβ-positive deposits, thioflavin-S-positive deposits, angulated atrophic fibers, and chronic inflammatory infiltration is rather diagnostic of IBM, if in human subjects. At the EM level, we did not detect the 15- to 21-nm cytoplasmic twisted tubulofilaments, which are the major abnormal filaments in IBM. Also, there were no intranuclear inclusions, which are sometimes present in human IBM. What we detected were short thin fibrils 6 to 8 nm in diameter, not unlike the amyloid-like fibrils that were decorated with antibodies against Aβ in human IBM.⁵ This is consistent with reports that twisted tubulofilaments are immunoreactive with ubiquitin, hyperphosphorylated tau, apolipoprotein E, and prion protein, but not Aβ. It appears that at least some of these other molecules may be required for the development of a full picture of IBM. The presence of angulated atrophic fibers is interesting, as it is also a feature of human IBM and may be considered a sign of denervation rather than myopathy. In human IBM, clinical and electrophysiological observations have suggested a coexistent neurogenic component, although it is still rather controversial.^{33–35} The lack of muscle fiber type grouping in our transgenic mice does not support a denervation-induced atrophy. Future electrophysiological study on our mice may help resolve this issue.

Among the aged (24 months old) transgenic mice, the phenotypes varied. At the protein level, the expression of CTFs in the muscles also varied widely among the transgenic mice. At the histological level, the changes varied from no lesions in 4 of 30 transgenic mice to IBM-like lesions in 9 of 30 transgenic mice. The level of CTFs and the severity of myopathy did not correlate well. One possible explanation for the lack of correlation is that the quadriceps for the Western blot analysis and the quadriceps for the microscopic examination were from different sides. In human IBM, the muscle lesions may be rather focal. There would be sampling errors if there was asymmetric involvement of IBM-like pathology in the transgenic mice, which may have also occurred in a rather focal fashion. For example, in one case we found moderately severe myopathy in the semi-thin sections but no lesions in the proportionally much larger H&E-stained sections. This focal nature of the lesions has posed difficulties for both adequate sampling for pathological evaluation and Western blot analysis of transgene expression. Other uncontrolled variables may have contributed

to this variability. The most interesting possibility would be differential contributions of background alleles, as the transgenic mice were only backcrossed (to C57BL/6 mice) three to four times.

A heterogeneous group of β PP molecules is generated by alternative splicing and post-translational modification as β PP molecules transit the secretory pathway. Although the majority of β PP molecules remain associated with internal membranes, especially the Golgi, some full-length β PP is transported to the cell surface.³⁶ The extracellular domains of approximately 30% of the β PP molecules at the cell surface are secreted.³⁷ The remainder of the molecules are internalized and either recycled or processed in the endosomal/lysosomal pathway. Internalization of β PP is mediated through regions of the cytoplasmic domain.^{38,39} The S β CK612V mice express the CTFs of β PP, which contains A β as well as the cytoplasmic domain. Immunocytochemistry demonstrated the A β -positive sarcolemmal staining in almost all fibers from transgenic mice. This is consistent with the data of Sisodia,³⁰ who showed that β PP-770 molecules with the same K to V substitution are associated with the plasma membrane instead of being secreted. Interestingly, the positive cytoplasmic staining was present only in the degenerating fibers, indicating abnormal metabolism of CTFs and accumulation of A β -containing metabolites in the cytoplasm. In view of the low abundance of inclusions or amyloid deposition relative to the high levels of A β epitopes in the degenerating fibers, most A β -containing fragments in the cytoplasm were not in the amyloid form. It cannot be ascertained at this point whether the high level of A β -containing fragments has caused individual fiber degeneration or the fiber degeneration has caused an accumulation of A β -containing fragments. The accumulation of osmiophilic membranous whorls and cellular debris in or around the filamentous inclusions on EM may argue for toxicity induced by the amyloid-like filaments. However, in most fibers, myopathic changes (central nuclei, fiber splitting, and increased number of mitochondria) occurred in the absence of inclusions or cytoplasmic A β positivity.

The extent to which the K612V mutation enhances the myopathy remains to be determined by quantitative comparison of the muscle pathology of mutant and wild-type constructs having similar degree of expression. The laboratory of K. Fukuchi (see related paper in this issue) has shown similar myopathy in the absence of the K612V mutation. Their transgenic mice,³¹ however, had a genetic background different from ours and, therefore, direct comparisons cannot be readily established. Our discovery of these lesions was made completely independently.

Similar to human IBM, age appears to be a risk factor for the development of lesions in the transgenic mice. Transgenic mice at ages of 9 to 13 months, although expressing high levels of CTFs in the muscles, had not developed any muscle lesions. It is possible that the high levels of CTFs in the sarcoplasmic membranes in the transgenic mice render them more susceptible to the oxidative stress that accumulates with age. Environmental factors, such as cumulative minor traumas, may contribute to the CTF accumulations with age.

The study of muscle degeneration and amyloidosis in IBM may yield insights into the mechanism of neuronal degeneration and amyloidosis in AD. The idea that amyloid can form intracellularly is gaining acceptance^{40,41} and may have a broader pathological significance. For example, intracellular amyloid has been observed in scrapie-infected hamster brains⁴² and in a case of telencephalic variant of Gerstmann-Sträussler syndrome.⁴³ In this regard, our transgenic mice should facilitate the study of the pathogenesis of IBM and the mechanism of amyloid formation in general. Its greatest significance, however, may be its potential utility as a surrogate for investigation of the cerebral β -amyloid cascade hypothesis as a primary mechanism in the neuronal lesions of AD and its pharmacological modulation.⁴⁴ The biochemical and morphological evolution of the peripheral muscle lesions in our transgenic mice should be amenable to longitudinal studies via biopsy.

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