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Snake coiled fibres in rat soleus muscle in chloroquine induced myopathy share immunohistochemical characteristics with amyloid depositions in Alzheimer's disease brain tissue

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Summary. Pathological and immunopathological studies were carried out on snake coiled fibres (SCF) which occurred in affected soleus muscle in chloroquine treated rats. The SCF began to appear in denervated soleus muscle by 8 days after chloroquine injection. By day 14, typical SCF were observed with an unusual swirling pattern of the myofibrils, presenting a bizarre appearance. By day 21 or later, the SCF became less remarkable, and were fragmented and broken apart to form large vacuoles. Immunopathological studies demonstrated that the amyloid β (Aβ) and N and C-terminal regions of amyloid precursor protein (APP), and the amyloid associated proteins tested, apolipoprotein E (apoE), SP-40,40, α_1-antichymotrypsin (α_1-ACT), and ubiquitin, which are known to be components of amyloid depositions found in Alzheimer's disease (AD) affected brains, were present in the SCF. ApoE, SP-40,40, α_1-ACT, and ubiquitin are induced following certain cell challenges (e.g. heat shock, various drugs and injury). The significance of APP, Aβ, and amyloid associated proteins are discussed in respect to snake coiled fibre formations in chloroquine rat myopathy and in the amyloidogenesis of AD.

Keywords: Alzheimer's disease, amyloid β protein, amyloid precursor protein, amyloid associated proteins, chloroquine myopathy, rimmed vacuole, snake coiled fibre

Snake coiled fibres (or whorled fibres) (SCF) are characterized by the unusual swirling patterns of the myofibrils in transverse section, and are observed in a variety of diseases: denervation atrophy, limb girdle dystrophy, chronic neuropathies, Becker dystrophy, spinal muscular atrophy, myositis ossificans, myosclerosis, and other miscellaneous disorders (Dubowitz 1985;

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Mastaglia & Detchant 1992). Snake coils are thought to be formed in a focal degenerative process which eventually leads to cell death and may, at least in part, play a role in causing muscle atrophy (Dubowitz 1985; Mastaglia & Detchant 1992). However, snake coiled fibre pathogenesis and its significance remain unclear.

Chloroquine, a potent lysosomotropic agent, induces myopathy in experimental animals, which is similar to human myopathy with rimmed vacuoles (RVs) (Whisnant *et al.* 1963). Recently, we and others have demonstrated, for the first time, immunohistochemical evidence that amyloid precursor protein (APP), amyloid β (Aβ) and cathepsin D, a lysosomal enzyme, accumulate in vacuolated rat soleus muscle in this chloroquine induced myopathy (Murakami *et al.* 1995; Tsuzuki *et al.* 1994b). Moreover, all amyloid associated proteins tested so far, (apoE, SP-40,40, α_1-ACT, and ubiquitin) appear to co-localize with Aβ in vacuolated muscle fibres in chloroquine induced myopathy (Tsuzuki *et al.* 1995).

In this study, we observed SCF in the soleus muscle of chloroquine treated rats and demonstrated immunohistochemically, for the first time, that SCF contain the N and C-terminal and Aβ domains of APP, and the so-called amyloid associated proteins, apoE, SP-40,40, α_1-ACT, ubiquitin, and cathepsin D. The possibility was discussed that SCF in chloroquine induced myopathy may provide a peripheral animal model for understanding the role of amyloid associated proteins as well as Aβ in amyloid deposits in Alzheimer's disease (AD) brain.

Materials and methods

Animals

The experimental rats were treated as described elsewhere (Tsuzuki *et al.* 1994b; 1995). Briefly, the right hind leg of adult male Wister rats was denervated by ligating the sciatic nerve. One day after ligation, chloroquine, (50 mg/kg body weight) or an equal volume of saline was injected intraperitoneally once a day. Pathological and immunohistochemical studies were performed with innervated (left) and denervated (right) soleus muscles on days 0, 3, 6, 8, 10, 12, 14, 16, 18, 21 and 28 days after the initial injection.

Histochemical study

For the histochemical study, the soleus muscles were frozen in isopentane cooled in liquid nitrogen. Transverse cryostat sections, 8 μm in thickness, were stained with haematoxyline-eosin (HE) and modified Gomori-trichrome.

Antibodies

Monoclonal antibodies (mcAb) used in this experiment were previously well characterized (Tsuzuki *et al.* 1994a). Briefly, the APP mcAbs used are as follows: mcAb 109/6 was raised against the N-terminal region of APP (NT-1), mcAbs 90/12 against Aβ, and mcAb 127/2 against the C-terminal region of APP (CT-2) (Tsuzuki *et al.* 1994a). Anti apoE mcAbs were also used (unpublished). A polyclonal anti SP-40,40 was raised in rabbit as described (Tsuzuki *et al.* 1995). Rabbit anti sera to apoE, α_1-ACT, ubiquitin, and cathepsin D were purchased from Chemicon, Calbiochem, DAKOPATTS, and Cosmo Bio Chem., respectively.

Immunohistochemical studies

Immunostainings were performed as described elsewhere (Tsuzuki *et al.* 1994b; 1995). Transverse paraffin sections of denervated soleus muscles were stained using the standard streptavidin-biotin peroxidase technique (Vectastain ABC kit; PK-4000, Vector Lab. Burlingame, CA). Deparaffinized sections were treated with 99% formic acid for 30 seconds. Endogenous peroxidase was inhibited by 0.3% hydrogen peroxide in methanol for 30 minutes. After washing with 10 mM phosphate buffered saline (PBS), pH 7.4, the sections were incubated overnight in PBS with 10% bovine serum albumin or PBS with 10% normal serum of the species in which the secondary antibodies were raised to eliminate non-specific binding. The sections were incubated in primary antibodies diluted 1:100–1:1000 with PBS for 1 hour. The sections were then sequentially incubated in biotinylated secondary antibody for 1 hour, then streptavidin–biotin–horseradish peroxidase for 1 hour. The sections were reacted with 3,3′-diaminobenzidine/H_2O_2 and counterstained with haematoxylin.

Specificities of antibodies were determined by (1) applying PBS instead of the primary antibodies, (2) replacing the primary antibodies with non-immune sera, and (3) absorbing the primary antibodies with their antigens. Briefly, 100 μg of synthetic peptides for mcAb NT-1, Aβ, CT-2, purified proteinaceous antigens, apoE (Chemicon), SP-40,40, α_1-ACT (Calbiochem), and ubiquitin (Sigma) were incubated overnight at 4°C with 1 ml of diluted antibodies ($\times$100). After centrifugation at 15000 ***g***, the supernatant was diluted finally at $\times$500.

Results

Histochemical findings in soleus muscle

Most denervated soleus muscles of experimental chloroquine treated rats appeared to be normal by day 3. On day 6, both atrophic and hypertrophied muscle fibres appeared. Vacuoles with or without rims, were also observed. Some had red rims and occasionally contained red granular material. This same red material was also seen distributed throughout the lumen. By day 14, vacuoles increased in number and contained many red granules. There were marked variations in atrophic fibre size, and these fibres appeared in clusters (Figure 1A). With Gomori-trichrome stain, typical SCF were clearly visualized. The unusual whorled patterns of SCF in transverse section appeared evident. Vacuoles of varying shape and size, including ring or crescent shaped vacuoles were seen in SCF. Some had red granular rims along the vacuoles (Figure 1B).

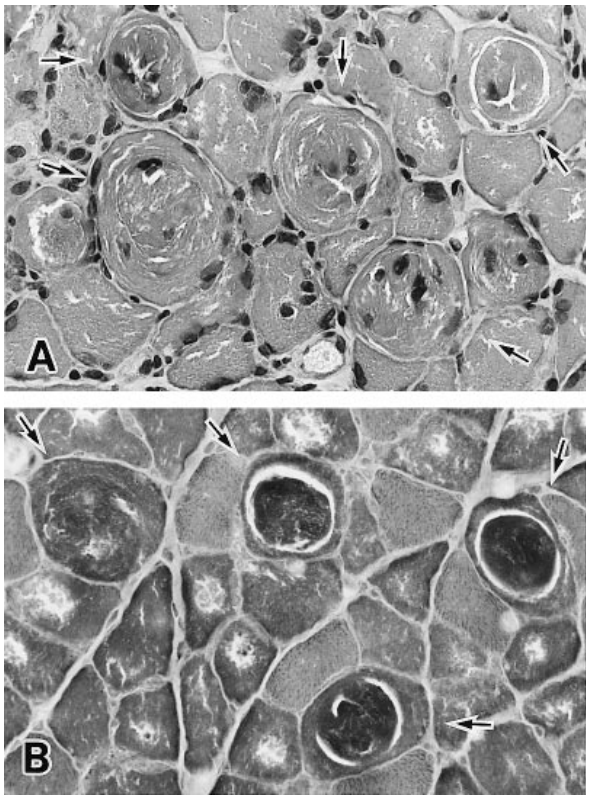

Figure 1. Photomicrographs of transverse cryostat sections of chloroquine treated rat soleus muscle after 14 days of treatment stained with A, HE and B, modified Gomori-trichrome. Arrows indicate the snake coiled fibres. ×290.

Morphological development of SCF was observed. By day 8, SCF appeared hypertrophied and stained a dark blue with HE. Normal pattern of muscle fibre became disoriented and swirling (Figure 2A). By day 14, SCF were sometimes seen in clusters. The orderly pattern seen in transverse section was completely disorganized and disoriented with a bizarre appearance. Internal migration of the nuclei was apparent. Some cells within the muscle fibres were ring shaped. Thin spindle shaped mononucleated cells were often observed lying along the whorled fibres. Vacuoles of varying size and shape, including crescent vacuoles, were found in SCF (Figure 2B). By day 18, some SCF were fragmented and broken apart to form large irregular or round vacuoles with or without apparent phagocytotic cells, which often contained unidentified red granular materials. Only the connective tissue appeared to be proliferating (Figure 2C). By day 21, muscle destruction and connective tissue proliferation became more prominent, and SCF were seen less frequently. Instead, clusters of large vacuoles were found adjacent to the remaining SCF (Figure 2D).

Immunohistochemical studies

Figure 3 shows affected transverse muscle serial sections on day 14 that are HE stained and immunostained using mcAbs against Aβ and N-terminal and C-terminal APP regions. These antibodies clearly reacted with the SCF which were observed using HE staining. Numerous granular structures irregular in shape and size other than vacuoles reacted with the antibodies with varying intensities. Vacuoles were either roundish or thin and crescent shaped. The roundish vacuoles reacted with the antibodies both at the rims and in the lumen, but the thin crescent shaped vacuoles usually reacted at the rims. The immunolocalization of all the APP domains appeared to be the same.

Figure 4 shows immunostaining of the same serial section series of affected muscle used in Figure 3 but using anti apoE, SP-40,40, Aβ, α_1-ACT, ubiquitin, and cathepsin D antibodies. These antibodies clearly stained SCF, and vacuoles of varying sizes and shapes. The positive immunoreactions were located in basically the same structures but subtle differences in immunostaining were observed when these so-called amyloid associated proteins' mcAbs were applied. All these immunoreactions were abolished when the primary antibody was omitted or replaced by non-immune sera or primary antibodies were absorbed with their antigens (data not shown).

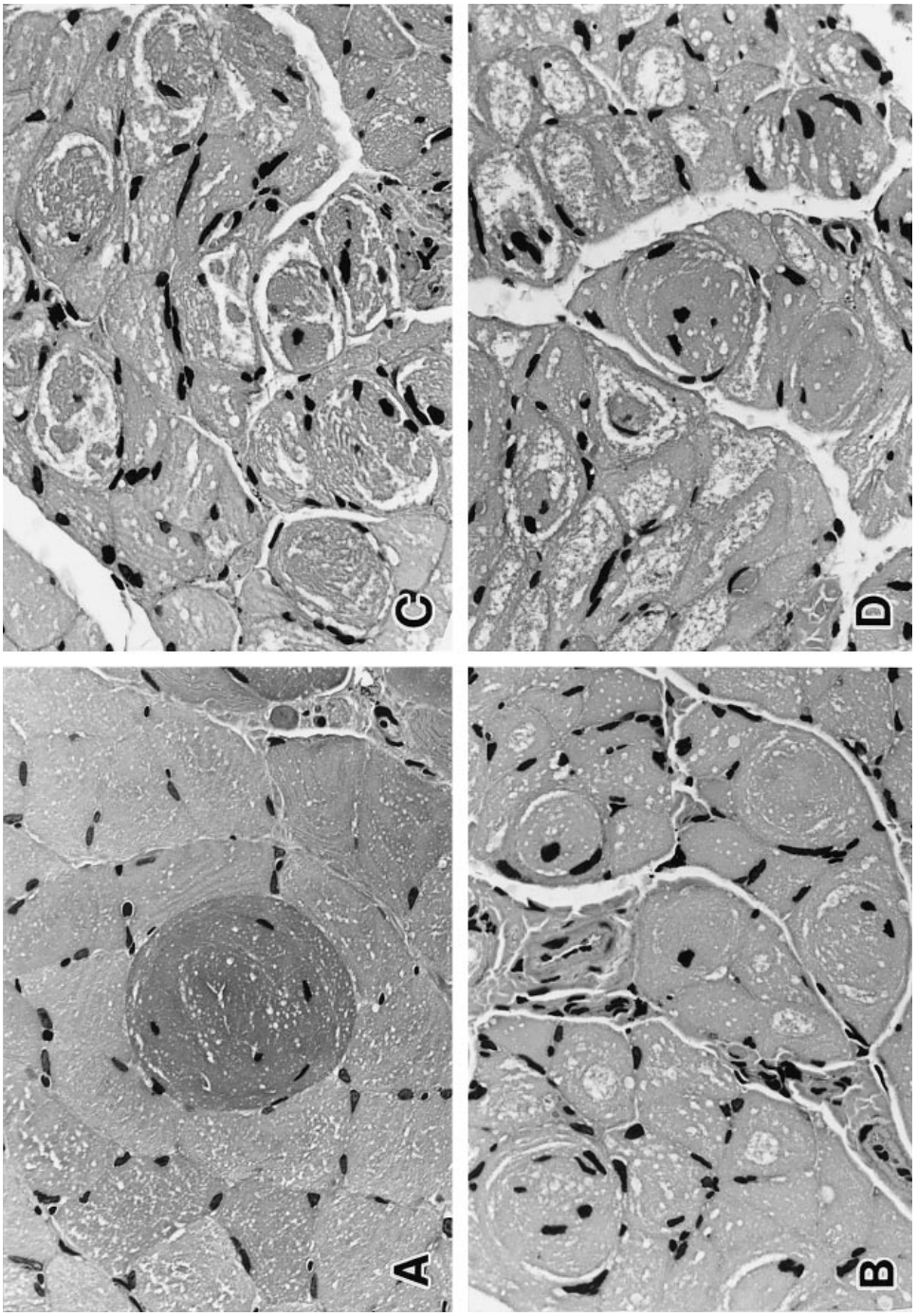

Figure 2. Photomicrographs of transverse paraffin sections of rat soleus muscles after A, 8; B, 14; C, 18 and D, 21 days of chloroquine treatment using HE staining. ×470.

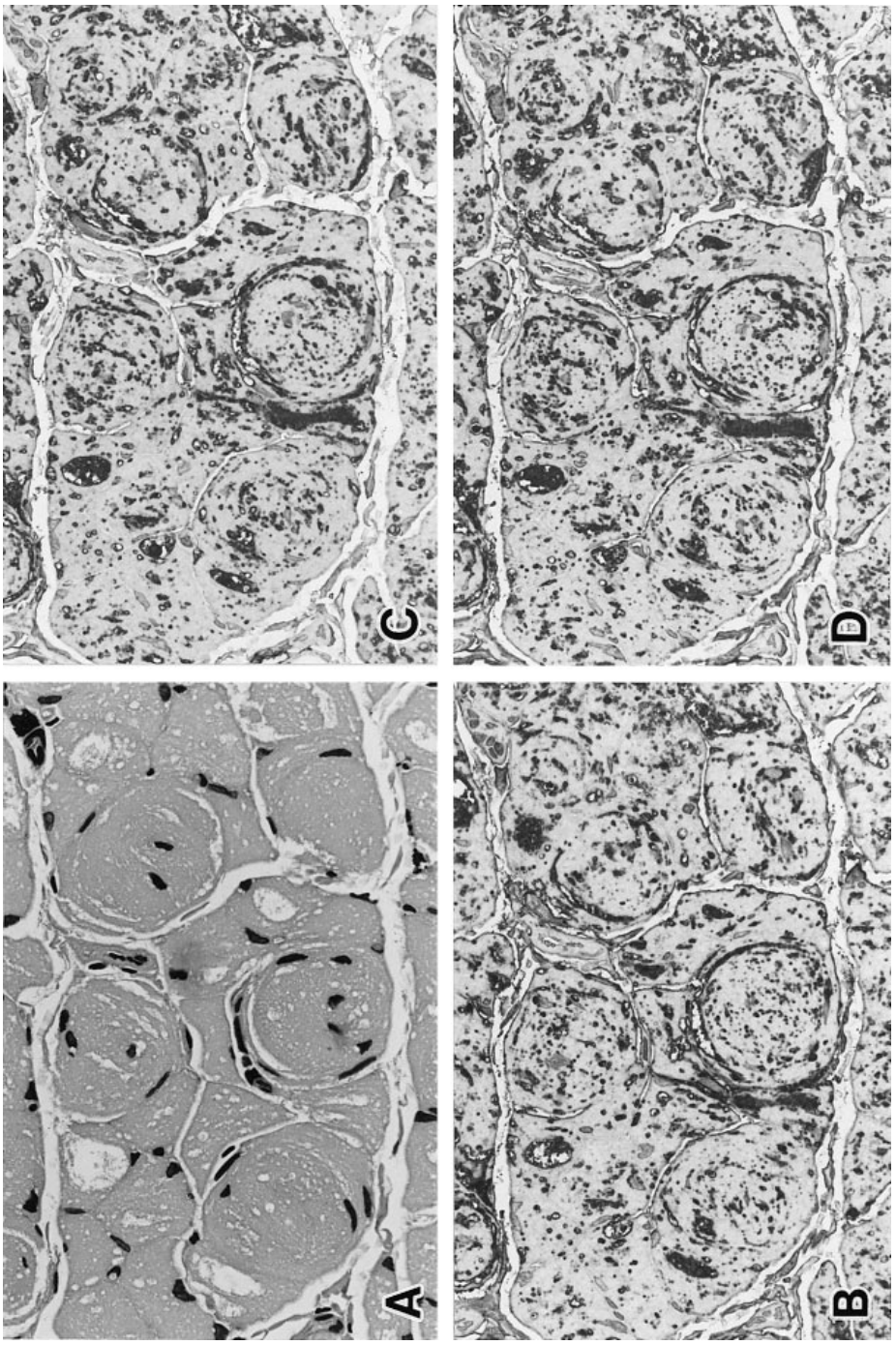

Figure 3. Photomicrographs of transverse serial sections of rat soleus muscle after 14 days of chloroquine treatment A, stained with HE, and immunostained using B, anti NT-1; C, Aβ and D, CT-2 antibodies. Note strong immunoreactivities in heterogeneous structures in B, C and D. The immunolocalizations of all the APP domains appear to be the same. $\times$470.

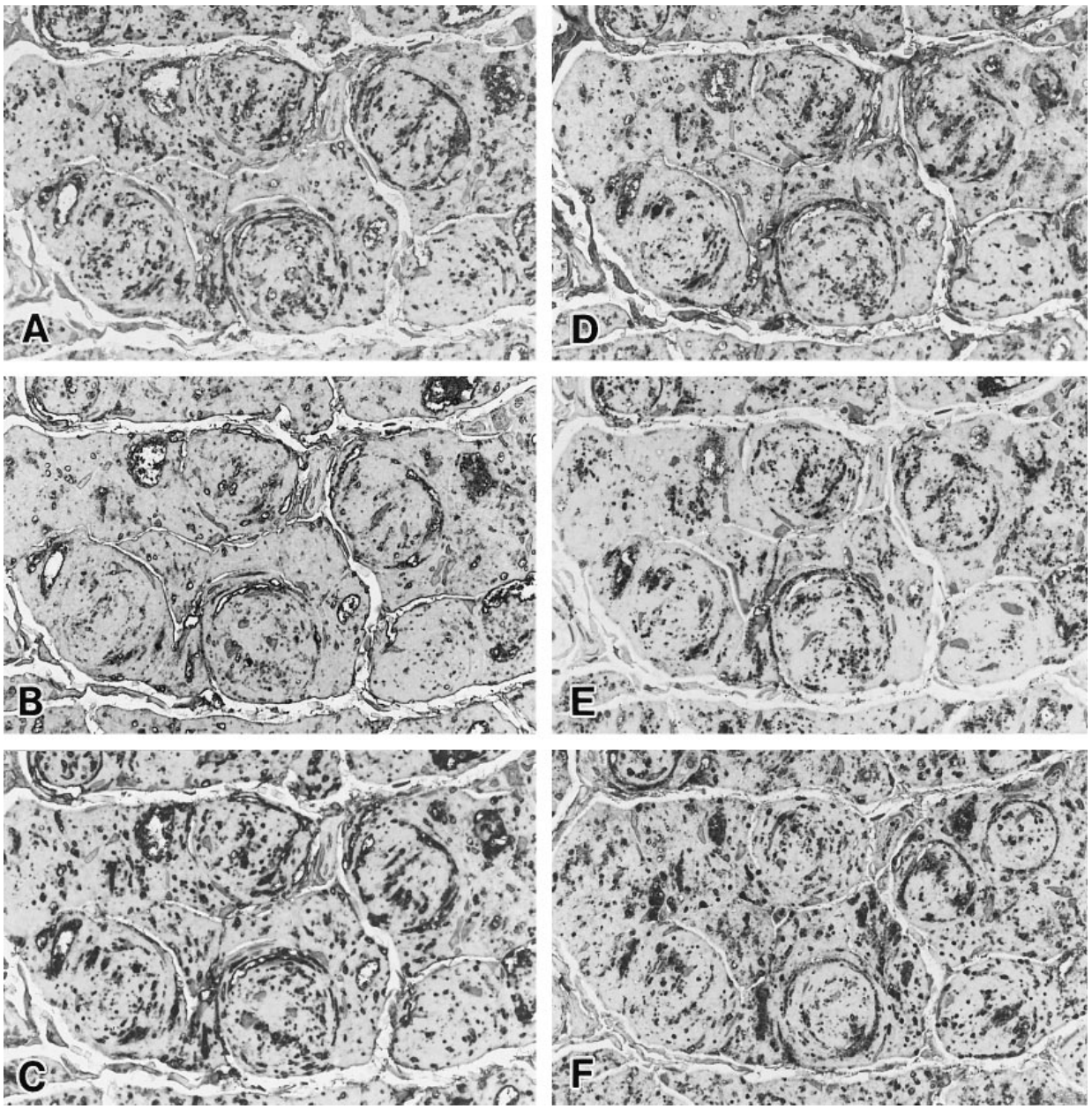

Figure 4. Photomicrographs of the same set of serial sections used in Figure 3 immunostained using A, anti apoE; B, SP-40,40; C, Aβ; D, α_1-ACT; E, ubiquitin and F, cathepsin D. Note the positive immunoreactions are located in basically the same structures when amyloid associated proteins' mcAbs are applied. $\times$420.

Discussion

Snake coiled fibres are well known because of their unique and bizarre morphology and their occurence in a wide variety of muscle diseases in humans (Dubowitz 1985; Mastaglia & Detchant 1992). To our knowledge, this study shows, for the first time, that SCF are induced in muscles of experimental animals, and that APP, Aβ, cathepsin D and amyloid associated proteins are involved in SCF. This provides the possibility that SCF in rat chloroquine myopathy may provide a peripheral model of early events occurring in Alzheimer's disease affected brain.

Pathological characteristics are described during development of the SCF. The SCF first appeared to be hypertrophied at an early stage. They then became

completely disoriented and whorled at an advanced stage. Finally, SCF became less conspicuous and were fragmented and broken apart to form large irregular or round vacuoles, which often contained unidentified red granular materials. There is a considerable amount of evidence for the involvement of autophagic degeneration in the pathogenesis of chloroquine induced myopathies. Large numbers of accumulated autophagic vacuoles, and substantial increases in lysosomal proteolytic enzyme activities were observed in the denervated muscles of chloroquine treated rats (Fedorko *et al.* 1968a,b; Kumamoto *et al.* 1993; Macdonald & Engel 1970). Our finding that cathepsin D is present immunohistochemically in SCF and vacuoles of varying size is further supporting evidence for these earlier reports.

It is an important observation that APP and many proteins other than Aβ, which are designated as amyloid associated proteins, apoE, SP-40,40, α_1-ACT, and ubiquitin are present in SCF, and large vacuoles which are assumed to be derived from degenerated SCF. These proteins apparently co-localize with Aβ in the affected muscle cells. Amyloid deposits are the invariable neuropathological feature of AD affected brain. Amyloid is known to be composed always of Aβ and amyloid associated proteins, but these proteins occur in amyloid to varying degrees (Abraham *et al.* 1988; Choi-Miura *et al.* 1992; Cole & Timiras 1987; McGeer *et al.* 1992; Namba *et al.* 1991; Perry *et al.* 1987). It should be noted that APP, apoE, and SP-40,40, share the characteristic that their expression is induced in response to a variety of cell injury (Abe *et al.* 1991; Roberts *et al.* 1991; Stephenson *et al.* 1992). APP expression, for example, is highly inducible by cell injury, ischaemia, head trauma, cell growth, and heat shock (Abe *et al.* 1991; Roberts *et al.* 1991; Siman *et al.* 1989; Stephenson *et al.* 1992). Similarly to APP itself, ApoE, and SP-40,40 are expressed constitutively at a certain level, but are highly inducible under various experimental conditions and during cell repair, such as cytotoxic injury, hormonal stimuli, or mechanical injury in experimental animals (Bandyk *et al.* 1990; Buttyan *et al.* 1989; Ignatius *et al.* 1986; Leblanc & Poduslo 1990; May *et al.* 1990; Pasinetti & Finch 1991). In fact, SP-40,40 in brain is also expressed at high levels in AD, Creutzfeldt-Jakob disease, and other neurological diseases (Duguid *et al.* 1989; May *et al.* 1990).

These amyloid associated proteins are thought to play an important role in enhancing the aggregation of Aβ and subsequent formation of amyloid plaque in AD (Strittmatter *et al.* 1993; Wisniewski *et al.* 1992) because recent studies clearly showed that soluble Aβ was known to be produced by normal physiological processes and released by cultured cells (Haass *et al.* 1992; Seubert *et al.* 1992; Shoji *et al.* 1992).

An animal model in which Aβ and amyloid associated proteins participate in the formation of lesions has not been available except for this experimental chloroquine myopathy (Murakami *et al.* 1995; Tsuzuki *et al.* 1994b; 1995). It is, therefore, an important issue whether APP and amyloid associated proteins' expression in AD is a unique phenomenon, or rather a common reaction that is not AD specific, and might be a more general phenomenon than is currently thought. This has not yet been examined in chloroquine induced myopathy, but will be. This experimental system provides a peripheral model of early events occurring in AD affected brain to shed light on basic mechanisms underlying the production of Aβ and formation of Aβ fibrils and final amyloid deposition.

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