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Glycosylation pattern and enzyme activities in atrophic, angulated skeletal muscle fibres from ageing rats

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Abstract In this study enzyme activities and lectin binding patterns in skeletal muscle from very old rats were investigated in order to evaluate changes in enzyme activity or carbohydrate expression in senile muscle. Activities for adenosine triphosphatase (ATPase), succinic dehydrogenase, non-specific esterase and the binding pattern for 31 lectins were investigated in the soleus muscles from very old (36 months) and young (3 months) rats. In ageing muscles atrophic, angulated muscle fibres are frequent. In cryostat sections these fibres were mostly but not always type II defined by the myosin ATPase reaction; few showed a strong esterase activity. Some showed strong activity for succinic dehydrogenase while others were weakly reacting. A number of lectins strongly bound to the sarcoplasm in angulated fibres while the binding to normal fibres in both old and young rat muscle was much weaker or even absent. Preferential binding to the ageing, angulated fibres was seen with *Aleuria aurentia, Galantus nivalis, Caragana abborecens, Triticum vulgaris, Maackia amurensis, Sambucus nigra, Phaseolus vulgaris* erythroagglutinin, and *Phaseolus coccineus*. Samples of homogenized and centrifuged muscles were run by electrophoresis and the gels blotted to nitrocellulose paper. Subsequent lectin staining of the blots detected that two glycoproteins with molecular weights around 25000 and 21000 daltons were present in old muscle, but not in young. Aberrant or elevated expression of sarcoplasmic glycoconjugates is involved in ageing muscle atrophy.

Key words Ageing muscle · Muscular atrophy
Angulated fibres · Lectins · Glycoconjugates

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Introduction

Ageing atrophy of muscles is caused both by reduced mean fibre area and a reduction in fibre numbers (Lexell et al. 1988). The most frequent morphological manifestations are type II atrophy, small angulated fibres and fibre grouping (Tomonaga 1977).

Many processes may contribute to age-dependent atrophy of muscles such as loss of contractile material (Lexell and Downham 1992) impeded capilarization and low mitochondrial enzyme activity (Coggan et al. 1992) and chronic denervation (Lexell et al. 1988). Age-dependent changes in enzyme activities [myosin adenosine triphosphatase (ATPase)-succinic dehydrogenase (SDH] have been reported previously, indicating a change in metabolic activity in atrophic senile muscle (Armbrustmacher 1978; Griffin and Pezeshkpour 1988). The glycosylation of the muscle fibres seems to change when the function of muscle changes both under normal conditions, with specialization of muscle (Kirkeby et al. 1992a), and in muscle disease (Bonilla et al. 1980; Kirkeby et al. 1992b). Since the carbohydrate moieties in muscle fibres may be related to muscle function, the present study has been initiated to decide a possible relation between ageing of rat skeletal muscle fibres and changes in their enzyme activities and/or carbohydrate expression.

Materials and methods

Five female Wistar rats 36 months old and five female rats 3 months old were killed by cervical dislocation. The soleus muscles were removed and dissected free of fat and connective tissue and divided into halves. One part of each muscle was used for lectin histochemistry and the other part was prepared for electrophoresis and blotting.

The following biotinylated lectins (Kem-En-Tec, Copenhagen, Denmark; Sigma, St. Louis, Mo., USA; Boehringer, Mannheim, Germany) were used: L-fucose agglutinins [*Ulex europaeus I* (UEA-1), *Aleuria aurantia* (AAA), *Lotus tetragonolobus* (LTA)]; mannose and/or glucose agglutinins [*concanavalin A* (Con A), *Lens culinaris, Maclura pomifera*; *Galanthus nivalis* (GNA)]; galac-

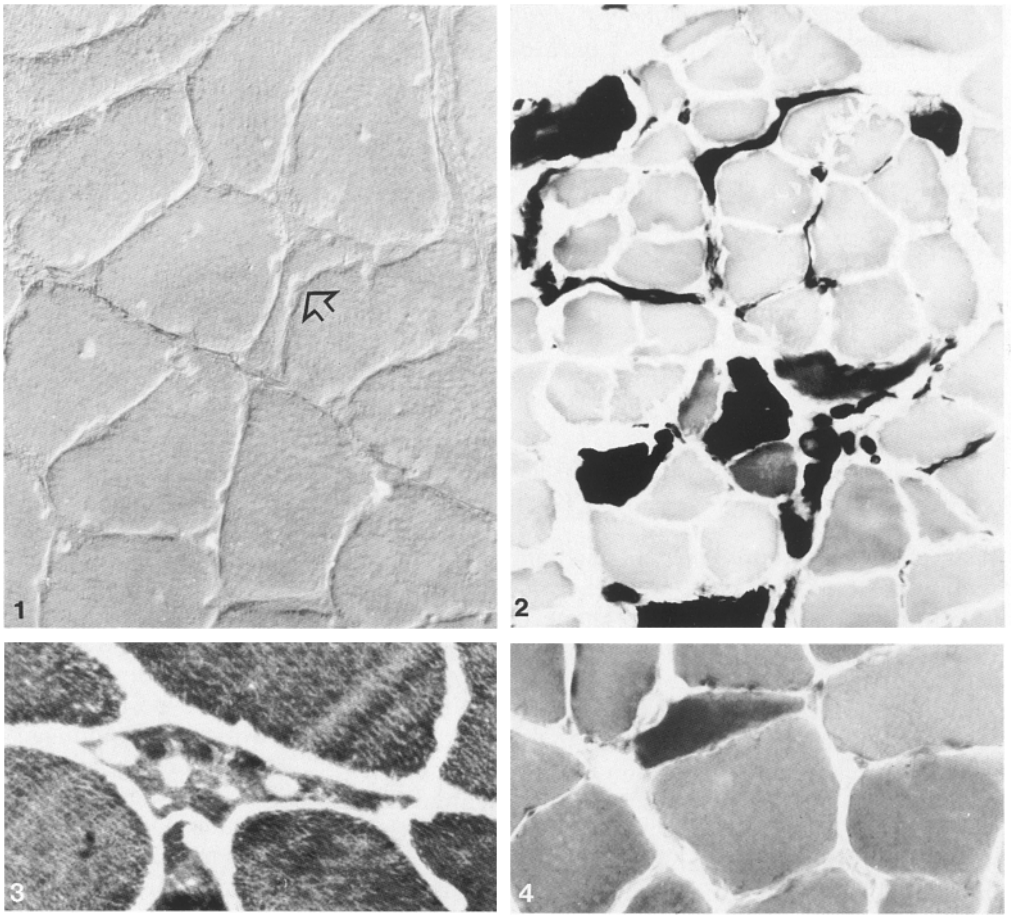

Fig. 1 Nomarski interference contrast micrograph of an unstained 12-μm-thick cryostat section of the soleus muscle from a 36-month-old rat. An atrophic and angulated fibre (*arrowhead*) appears compressed between fibres of normal size and shape. × 296

Fig. 2 Myosin adenosine triphosphatase (ATPase) activity in a cryostat section from an old soleus muscle incubated at pH 9.4. Atrophic and angulated fibres are dispersed in the section. Most of these fibres belong to type II. × 176

Fig. 3 Higher magnification of ATPase activity in an angulated fibre. The section has been preincubated at pH 4.6. Note the irregular shape of the fibre and the clear vacuoles in the cytoplasm. × 504

Fig. 4 Non-specific esterase activity in a cryostat section from an old soleus muscle. An angulated fibre shows high enzyme activity. × 344

tose agglutinins (*Arachis hypogaea, Griffonia simplicifolia, Artocarpus integrifolia* (jacalin), *Erythrina cristagalli*); *N*-acetyl-α-D-galactosamine agglutinins [*Helix pomatia, Dolichos biflorus, glycine max, Vicia vilosa, Helix aspersa, Sophora japonica, Bauhina purpurea, Phosphocarpus tetragonolobus, Caragana abborecens* (CAA), *Phaseolus liminensis*]; *N*-acetyl-β-D-glucosamine agglutinins [*Triticum vulgaris* (WGA), *Griffonia simplicifolia II, Phytolacca americana, Datura stramonium, Lycopersicon esculentum*]; sialic acid agglutinins [*Maackia amurensis* (MAA), *Sambucus nigra* (SNA)]; oligosaccharide agglutinins [*Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin]; agglutinins with unknown specificity [*Phaseolus coccineus* (PCA)].

For reviews on lectins and lectin binding specificity see Goldstein and Porez (1986), Damjanow (1987), and Spicer and Schulte (1992).

The enzyme substrates used included, adenosine triphosphate (ATP) for demonstration of myosin ATPase activity, succinic acid disodium salt for demonstration of SDH activity and α-naphthyl acetate for demonstration of non-specific esterase activity.

The muscle specimens were frozen in isopentane cooled to −150° C with liquid nitrogen and cut on a cryostat in 6 μm sections. The lectin staining of the sections was performed as described in a previous report (Kirkeby et al. 1991), using unfixed sections and avidin-alkaline phosphatase to visualize the binding sites of the biotinylated lectins, except that the concentrations of the lectins were 4 μg/ml. Localization of activity for myosin ATPase was performed according to Round et al. (1980). Routine ATPase was demonstrated after incubation at pH 9.4 and reverse ATPase after pre-incubation at pH 4.6. Activity for SDH and non-specific esterase was shown according to Kirkeby et al. (1988).

To obtain a sarcoplasmic preparation rat soleus muscle (70 mg) was homogenized in 1 ml of the following solution: 10 ml TRIS-hydrochloric acid buffer 0.1 M, pH 7.2, 20 μl Triton X-100, 1 mg digitonin, 7.8 mg mercaptoethanol, and 0.3 mg phenylmethylsulphonyl fluoride. The homogenization lasted 40 s, twice, at low speed. The homogenate was centrifuged at 30000 g for 30 min at 4° C and the resulting supernatant was used for electrophoresis.

For electrophoresis and blotting 10 μl of the supernatant was dissolved in 10 μl TRIS buffer containing dithiothreitol and sodium dodecyl sulphate (SDS). This solution was sonicated five times in 30 s bursts interspersed with 1 min cooling periods in an ice bath. The SDS electrophoresis was carried out with a Pharmacia PhastSystem on 8–25% gradient PhastGel with SDS buffer strips. The electrophoresis was performed for 65 accumulated Vh at a current of 10 MA. The gels were electrotransferred to nitrocellulose paper and stained for lectin binding according to Kirkeby et al. (1992a).

Results

When soleus muscles from young and old rats were compared, it appeared that the most prominent change in senile muscle morphology was the presence of muscle fibres that were angular in shape and compressed in the intestitial connective tissue between muscle fibres of normal morphology. These atrophic fibres could be small and triangular or they were irregular and elongated (Fig. 1) often arranged in small groups. The angulated fibres were present in the soleus muscles from all old

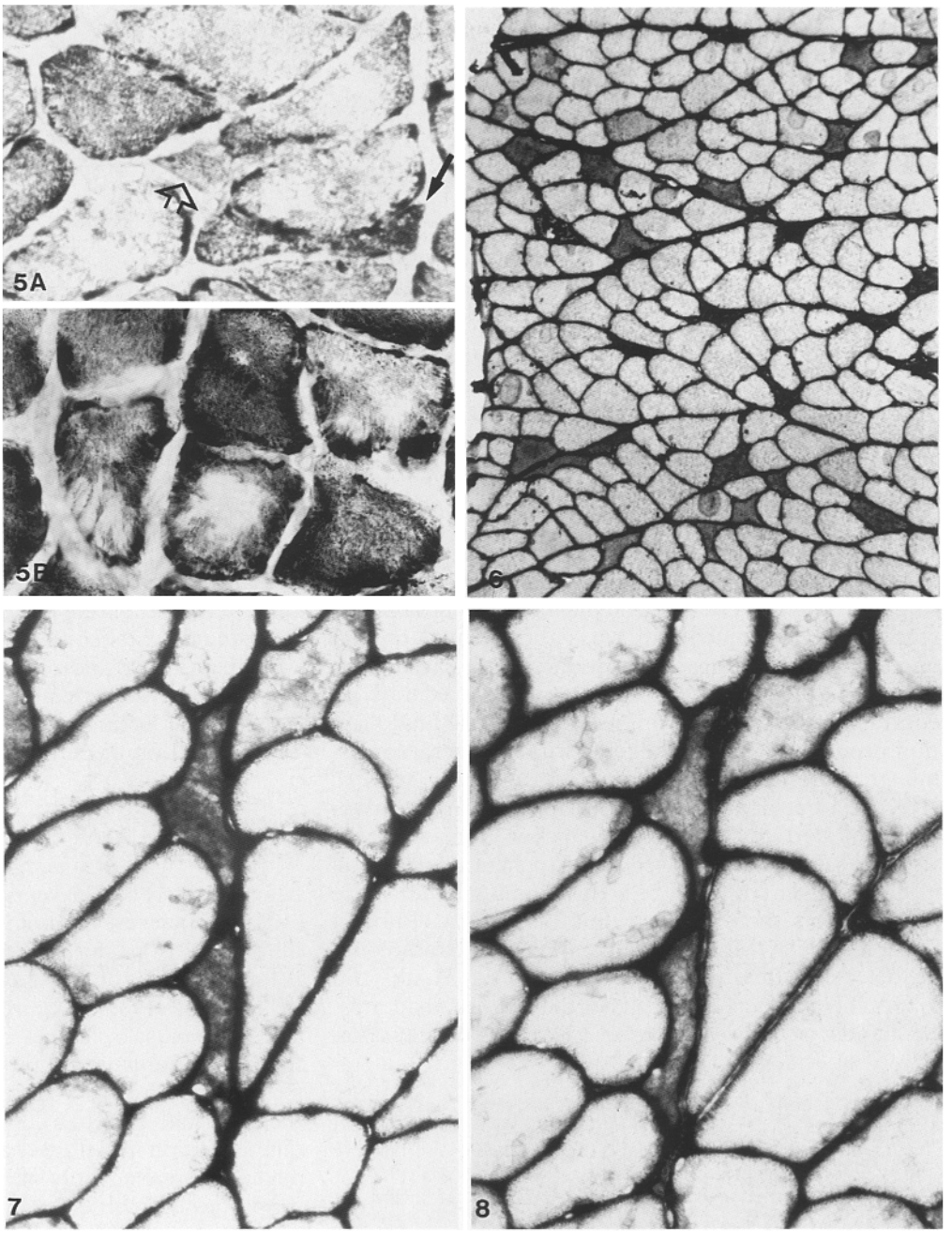

Fig. 5A, B Succinic dehydrogenase (SDH) activity in a cryostat section from an old soleus muscle. **A** The micrograph shows an angulated fibre with low SDH activity (*arrowhead*) and another angulated fibre with high SDH activity (*arrow*). ×414. **B** Target fibres with central areas without SDH activity. ×284

Fig. 6 Survey micrograph of a cryostat section of an old soleus muscle stained for *Triticum vulgaris* (WGA) binding. The angulated fibres are strongly marked though some fibres are more intensely stained than others. Note that WGA also detects the sarcoplasm of normal fibres. ×72

Fig. 7 Cryostat section of an old soleus muscle stained for *Aleuria aurantia* (AAA) binding. The sarcoplasm in the angulated fibres show affinity to AAA. The sarcoplasm in the normal fibres is unstained. ×292

Fig. 8 Serial section to that shown in Fig. 7 incubated to demonstrate *Corangana abborecens* (CAA) agglutination in the sarcoplasm of the angulated fibres. ×292

rats examined but were not found in the muscles from the young rats. ATPase staining showed that the angulated fibres could be both type I and (mostly) type II fibres (Fig. 2). They often contained clear vacuoles in the cytoplasm (Fig. 3). A few angulated fibres showed strong activity for non-specific esterase (Fig. 4), but in the majority of these atrophic fibres and in the normal fibres the esterase activity was weak. Both low activity and high activity of the oxidative mitochondrial enzyme SDH was observed in angular fibres from old rat muscles (Fig. 5A). Many fibres both angulated and non-angulated from old rats showed a central clearing after staining for SDH activity (Fig. 5B).

In the present study the binding of 31 lectins to rat skeletal muscle revealed that some lectins stained the sarcoplasm strongly in angulated fibres from very old rats. [WGA, AAA, CAA, GNA, SNA, MAA, PHA-E, and PCA (Figs. 6–10)] None of these lectins showed preferential binding to muscle fibre types defined by ATPase or SDH activities. The 8 lectins stained the fibre periphery of both normal and angulated fibres. Fibres with normal morphology in the old soleus muscles showed the same lectin staining pattern as all the muscle fibres in the soleus muscles from the young rats with little or no lectin staining of the sarcoplasm after incubation with AAA, GNA, MAA, SNA, PCA and

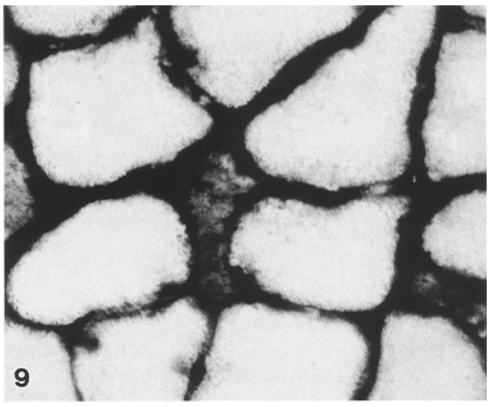

Fig. 9 Cryostat section of an old soleus muscle stained for *Sambucus nigra* (SNA) binding. The sarcoplasm of the angulated fibres is strongly stained and so is the periphery of all muscle fibres and the interfibre connective tissue. × 338

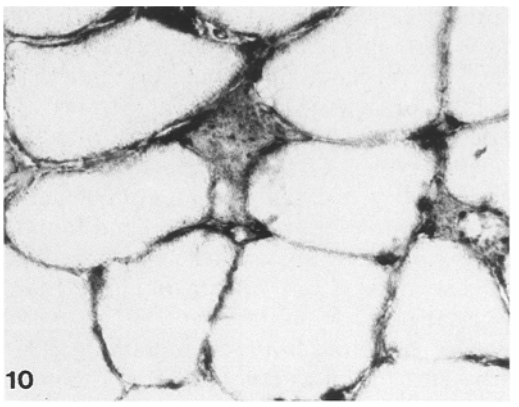

Fig. 10 Serial section to that shown in Fig. 9 incubated to demonstrate *Maackia amurensis* (MAA) binding. The angulated fibres are clearly marked although not as strong as with SNA and the staining of the muscle fibre periphery and connective tissue is much weaker. × 338

CAA and moderate staining with WGA and PHA-E (Figs. 6–10). With the histochemical method used in this study the lectin incubation brings about a fine granular and/or diffuse final reaction product in the sarcoplasm of normal muscle fibres from young and old rats. In contrast in the atrophic, angulated fibres the final reaction product was often irregular with coarse sarcoplasmic granules.

After electrophoresis the muscle preparation was electroblotted to nitrocellulose paper and incubated with the same lectins as used for histochemical detection of the angulated fibres. Lectin binding bands were present after incubation with GNA, CAA, PHA-E, SNA, and PCA (Fig. 11). No lectin positive bands could be detected after incubation with MAA, AAA, and WGA. The molecular weights of the lectin stained muscle bands were in the range 150000–21000 daltons.

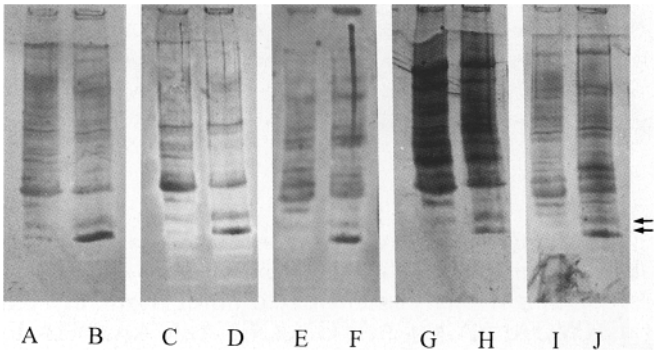

Fig. 11A–J Lectin staining of electrophoretically separated and blotted muscle proteins from young (3 months) and old (36 months) rats. **A** Young soleus *Phaseolus coccineus* (PCA); **B** old soleus PCA; **C** young soleus *Corangana aborecens* (CAA); **D** old soleus CAA; **E** young soleus *Galanthus nivalis* (GNA); **F** old soleus GNA; **G** young soleus *Sambacus nigra* (SNA); **H** old soleus SNA; **I** young soleus *Phaseolus vulgaris erythroagglutinin* (PHA-E); **J** old soleus PHA-E. The glycoprotein bands with molecular weights of 25000 and 21000 daltons that are strongly expressed in old muscles but not in young are marked by *arrows*

When the muscle preparations from the old and young rats were compared it appeared that soleus muscles from the old rats contained two bands with molecular weights of 25000 and 21000 daltons. These low-molecular-weight glycoproteins were not (or very weakly) expressed after lectin incubation of the blots from the muscle preparation of the young rats.

Discussion

Although signs of ageing atrophy in human muscles begin as early as 25 years (Bonilla et al. 1980; Lexell et al. 1988) severe pathological changes of the muscle fibres due to ageing processes are usually not evident before the age of 60 (Essén-Gustavsson and Borges 1986; Oetel 1986). According to Grimby and Saltin (1983) the muscle changes are observed at the same relative age in humans and rats. The group of old rats used in this study were 36 months of age, at the very end of their normal life span (36–38 months). In rat muscle differentiation into fibre types has finished 1 month after birth, whereafter only minor changes in contractile properties are observed (De Haan et al. 1993). The young rats used in this study were about 3 months old and their muscles thus fully developed and mature.

With increased age atrophic, angulated fibres appear in skeletal muscle (Tomonaga 1977; Poggi et al. 1987). As described by Dubowitz (1985) both type I and type II fibres (defined by the ATPase reaction) may become angulated. In the present study the majority of the angulated fibres in the muscles from the old rats were type II fibres. However, most of the type II fibres in old soleus muscle were normal in size and shape and showed the same morphology as type II fibres in the soleus from the young rats.

Angulated fibres may be evidence of denervation. Griffin and Pezeshkpour (1988) mention that in human muscle esterase-positive angular atrophic fibres represent the most frequently encountered indicator of dener-

vation. In the present study only a few of the angulated fibres in the rat muscle showed strong activity for non-specific esterase. The angulated fibres in old rat muscles showed either high or low SDH activity. Armbrustmacher (1978) describe that denervated, angular and atrophic fibres often become excessively dark with oxidative enzyme stains while Klitgaard et al. (1989) measured the activity of mitochondrial enzymes and found that it became lower in the rat soleus muscle with ageing ($\geq$24 months). The muscle fibres from old rats that showed central clearing after staining for SDH activity are termed target or targetoid fibres. This staining pattern is caused by clearing of organelles (mitochondria and sarcoplasmatic reticulum) from the central portion of the fibre. Target fibres develop when denervated atrophic fibres are reinnervated and begin to restore the intracellular structure from the outside in. Target fibres are noticed in about 50% of human muscles showing significant denervation (Armbrustmacher and Griffin 1983). Another sign of reinnervation is fibre-type grouping that is seen if two or more fibres of one histochemical type are enclosed at all points on their circumference by other fibres of the same histochemical type (Swash and Schwartz 1988). Fibre grouping was not, however, frequently observed in the senile rat soleus muscles. The atrophic, angulated fibres were thus mostly type II defined from their ATPase reaction. Some of them showed strong SDH activity and a few fibres were esterase positive.

The results of the present study indicate a consistent change of glycosylation in senile muscle sarcoplasm as detected by an altered sarcoplasmic staining pattern for some lectins. WGA might detect glycoconjugates important for muscle function. In a study on lectin binding to rat muscle Iglesias et al. (1992) noticed that WGA binds strongly to the sarcolemma and prominently labels the neuromuscular junction. Gulati and Zalewski (1985) proposed that WGA receptors may be significant in muscle fibre regeneration. In the present study WGA was found to bind much more strongly to the sarcoplasm of angulated fibres than to the sarcoplasm in muscle fibres from young rats and in seemingly normal fibres from old rats. Whether the atrophic fibres positive for WGA are in a state of regeneration cannot be decided by this study. The lectin is often used for histochemical detection of sialoglycoproteins but it binds GlcNAc and its β1,4-linked oligomers preferentially. Actually it seems that whereas other sialic acid aggutinins such as SNA and MAA bind specifically to sialic acid residues WGA binds to sialic acid based on this sugars structural similarity to GlcNAc (Shibuya et al. 1987).

Incubation with PHA-E resulted in an elevated lectin expression in angulated fibres since the sarcoplasm in all normal muscle fibres was moderately stained whereas the angulated fibres were strongly stained. Thus incubation with PHA-E provided the same staining pattern in the muscle sections as obtained after incubation with WGA. The structural determinant for binding to PHA-E is an octasaccharide containing a bisecting GlcNAc and a Galβ1-4GlcNAcβ1-2Man unit linked α-(1,6) to mannose (Goldstein and Porez 1986).

In a study on fucose expression in experimentally denervated and devascularized rat soleus muscles it was found that AAA detected abnormal sarcoplasmic reaction 18 h after the surgical intervention. In contrast, two other fucose-specific lectins (UEA-I and LTA) showed no changes in their binding pattern (Kirkeby et al. 1993). The age-dependent neurogenic changes that result in formation of angulated muscle fibres also involved a strong AAA staining in these fibres while the binding of UEA-I and LTA was unchanged. The AAA staining of normal muscle fibres was negligible. Hence, AAA may be a potential marker for neurogenic muscle disorders. The highest specificity for this lectin is fucose bound α1,6 to *N*-acetyl-β-D-glucosamine (Haselbeck et al. 1990).

Two of the lectins (PCA and CAA) that showed strong binding to angulated fibres have been paid little attention in the literature and therefore detailed information on their binding specificity is not available. PCA was isolated by Ochoa and Kristiansen (1982), who were unable to inhibit its agglutination by any sugars and therefore suggest that haemagglutination by PCA might involve cell receptors other than simple carbohydrates. CAA has been isolated and characterized by Bloch et al. (1976). The lectin is not blood group specific but shows affinity for *N*-acetyl-α-D-galactosamine.

The mannose-specific lectin GNA was isolated and characterized by Van Damme et al. (1987). Internal mannosyl residues and D-glucose and *N*-acetyl D-glucosamine do not interact with GNA. The carbohydrate specificity of GNA thus differs from other lectins of the family Leguiminosa such as Con A by only reacting with terminal α-D-mannosyl groups (Kaku and Goldstein 1989). GNA did not detect sarcoplasmic glycoconjugates in normal muscle fibres but strongly stained the angulated fibres. Con A showed sarcoplasmic reaction in all muscle fibres but revealed no increased sarcoplasmic staining of the angular fibres. The highest specificity of GNA agglutination seems to be towards α1,3-linked mannose oligosaccharides and the non-reducing terminal mannosyl residue might be the most important GNA recognizing site (Kaku et al. 1990).

Sialic acid residues hold a terminal position in sialoglycoconjugates and are frequently adjacent to β-D-galactose residues (Schulte and Spicer 1985). MAA and SNA lectins are considered as highly specific in their recognition of sialic acid. SNA was isolated by Broekaert et al. (1984) and its binding exhibit the presence of Neu5Acα2-6Gal/GalNAc sequences (Landemore et al. 1992). MAA isolated by Wang and Cummings (1988) has a definitive carbohydrate requirement of the trisaccharide sequence Neu5Acα2-3Galβ1-4GlcNAc/Glc. Unlike SNA, MAA does not require C-8 and C-9 hydroxyl groups for lectin binding (Knibbs et al. 1991). In a histochemical study of bovine submandibular gland, MAA was found to recognize serous

cells, excretory ducts and endothelial cells. SNA bound to the same structures and in addition this lectin also detected mucous cells (Sata et al. 1989). Investigating SNA binding Sata et al. (1991) noticed that malignant transformation of human colonic mucosa was accompanied by de novo expression of an α2,6 sialyl transferase.

The neurogenic changes in the senile rat soleus muscles could be caused by either loss of motor neurons in the spinal cord or a degeneration of peripheral nerve axons and/or myelin sheath (Lexell et al. 1983). Ianello and Jeffery (1992) have described denervation-induced changes in lectin binding to sarcolemmal glycoproteins and Kirkeby et al. (1993) reported novel lectin binding in the sarcolemma and sarcoplasm less than 24 h after denervation. The present results indicate that ageing angulated muscle fibres show a sarcoplasmic lectin binding different from seemingly normal fibres found in young and old animals. This novel carbohydrate expression appears both to be specific and broad – specific because only one or two lectins in a group of lectins with the same monocarbohydrate specificity showed affinity against the angulated fibres; for example, of the nine lectins with *N*-acetyl-α-D-galactosamine binding capacity tested in this study only CAA marked the angulated fibres. Broad because lectins reacting with fucose, mannose, *N*-acetyl-α-D-galactosamine, *N*-acetyl-β-D-glucosamine, sialic acid and bisected complex oligosaccharides all showed preferential binding to these fibres. Further, it seems that a few low-molecular-weight glycoproteins are pathognomonic for senile skeletal muscle and that these glycoproteins are detected by a number of lectins with different carbohydrate specificity.

The appearance of angulated fibres in ageing rat muscles might thus be the result of pathological processes that involve aberrant or elevated expression of sarcoplasmic glycoconjugates.

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