Age-related changes in expression of the neural cell adhesion molecule in skeletal muscle: a comparative study of newborn, adult and aged rats

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Neural cell adhesion molecule (NCAM) is expressed by muscle and involved in muscle–neuron and muscle–muscle cell interactions. The expression in muscle is regulated during myogenesis and by the state of innervation. In aged muscle, both neurogenic and myogenic degenerative processes occur. We here report quantitative and qualitative changes in NCAM protein and mRNA forms during aging in normal rat skeletal muscle. Determination of the amount of NCAM by e.l.i.s.a. showed that the level decreased from perinatal to adult age, followed by a considerable increase in 24-month-old rat muscle. Thus NCAM concentration in aged muscle was sixfold higher than in young adult muscle. In contrast with previous reports, NCAM polypeptides of 200, 145, 125 and 120 kDa were observed by immunoblotting throughout postnatal development and aging, the relative proportions of the individual NCAM polypeptides remaining virtually unchanged at all ages examined. However, changes in the extent of sialylation of NCAM were demonstrated. Even though the relative amounts of the various NCAM polypeptides were unchanged during aging, distinct changes in NCAM mRNA classes were observed. Three NCAM mRNA classes of 6.7, 5.2 and 2.9 kb were present in perinatal and young adult skeletal muscle, whereas only the 5.2 and 2.9 kb mRNA classes could be demonstrated in aged muscle. This indicates that metabolism of the various NCAM polypeptides is individually regulated during aging. Alternative splicing of NCAM mRNA in skeletal muscle was studied by Northern blotting using DNA oligonucleotide probes specifically hybridizing to selected exons or exon combinations. Exon VASE, which has previously been shown to be present in both brain and heart NCAM mRNA, was virtually absent from skeletal muscle at all ages studied. In contrast, the majority of NCAM mRNA in postnatal skeletal muscle was shown to contain extra exons inserted between exons 12 and 13. Of the various possible exon combinations at this splice site, the combinations 12-a-AAG-13 and 12-a-b seemed to be prevalent in postnatal skeletal muscle. No significant change in the relative proportion of these two exon combinations occurred during aging. The observed upregulation of NCAM protein in aged muscle supports the assumption that an increasing proportion of muscle fibres are denervated in aged muscle. Selective upregulation of the 5.2 and 2.9 kb mRNA forms have previously been demonstrated in muscle cell lines and in primary cultures of muscle cells during formation of myotubes *in vitro*, and this switch in NCAM mRNA classes has been suggested to correlate with myogenesis. However, the selective upregulation of the 5.2 and 2.9 kb mRNA classes is even more pronounced after muscle denervation [Covault, Merlie, Goridis and Sanes (1986) J. Cell Biol. **102**, 731–739] as well as in the age-related NCAM upregulation reported here. We therefore suggest that upregulation of the 5.2 and 2.9 kb NCAM mRNA forms in muscle may be correlated with lack of innervation of the myofibre rather than to myogenesis *per se*.

INTRODUCTION

The neural cell adhesion molecule (NCAM), is a cell surface glycoprotein which mediates cell–cell and cell–substratum binding [for review, see Linnemann and Bock (1989)]. NCAM-mediated cell adhesion plays a role in several morphogenetic events, including muscle formation and innervation (Rutishauser et al., 1983; Knudsen et al., 1990; Dickson et al., 1990). Involvement of NCAM in these processes is thought to depend on temporal and spatial regulation of transcription, translation and post-translational modulation of the molecule. Changes in the surface density of NCAM have been shown to have a pronounced effect on binding efficiency. Thus, a doubling of NCAM concentration increases binding rates 30-fold (Hoffman and Edelman, 1983). The carbohydrate structure of NCAM also changes during development. Embryonic brain NCAM is heavily glycosylated, with most of the carbohydrate moiety consisting of polysialic acid residues with an average chain length of approximately 25 sialic acids or more (Frelinger and Rutishauser, 1986). Later in development, NCAM polysialylation is reduced (Linnemann et al., 1985). Even though polysialic acid residues do not participate directly in NCAM binding, they diminish the binding affinity of the molecule (Sadoul et al., 1983; Moran and Bock, 1988).

NCAM is the product of a single-copy gene, which consists of at least 25 exons (Owen et al., 1987; Small et al., 1987; Gower et al., 1988; Thompson et al., 1989; Santoni et al., 1989). By alternative splicing and polyadenylation, five NCAM mRNA classes with sizes of 7.4, 6.7, 5.2, 4.3 and 2.9 kb are generated in rodents (see Figure 1). Exons 1–14 seem to be expressed in all NCAM mRNAs. Alternative usage of the 3′-end exons 15–19 produces mRNAs encoding three major NCAM polypeptide species with different C-termini. In the 7.4 kb mRNA class, exon 14 is followed by exons 16, 17, 18 and 19. This mRNA class encodes a transmembrane NCAM species with a long cytoplasmic domain. The 6.7 kb mRNA class contains exons 16, 17 and 19,

Abbreviations used: NCAM, neural cell adhesion molecule; PVDF, polyvinylidene difluoride; WB, washing buffer; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; poly(A)$^{+}$, polyadenylated; PMSF, phenylmethanesulphonyl fluoride.

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but lacks exons 15 and 18. As a result this mRNA class encodes a transmembrane NCAM species with a relatively short cytoplasmic domain. Both the 5.2 and 2.9 kb mRNA classes contain exons 1–15, but lack exons 16–19. These two mRNA classes are both considered to encode an NCAM species that is attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor (Owen et al., 1987; Barthels et al., 1987). The difference in size between the 5.2 and the 2.9 kb mRNA classes is due to alternative polyadenylation (Barbas et al., 1988). The protein product of the 4.3 kb mRNA has not yet been identified, but this mRNA class has been shown to contain exons encoding intracellular sequences (Small et al., 1987; Andersson et al., 1990). Evidence for yet another NCAM polypeptide species comes from the identification of a human NCAM cDNA clone, containing an alternatively spliced exon termed SEC. SEC may be inserted between exons 12 and 13 and contains a stop codon 63 bp downstream. This means that NCAM mRNA containing exon SEC encodes a truncated NCAM species which lacks any membrane attachment, and hence is a soluble NCAM species (Gower et al., 1988). However, this secreted isoform has not been demonstrated in mouse muscle by the PCR technique (Hamshere et al., 1991).

NCAM is a member of the Ig gene superfamily (Hemperly et al., 1986; Cunningham et al., 1987). Members of this family all share a common extracellular polypeptide domain called the Ig homology unit [for a review, see Salzer and Colman (1989)]. Ig homology domains have been divided into constant (C) and variable (V) domains, which differ with regard to the number of antiparallel β-strands (seven and nine respectively) and amino acid sequence patterns around cysteine residues. NCAM and other CAMs of the Ig superfamily contain homology units with features intermediate between C and V domains. This domain type is termed C2 (Williams and Barclay, 1988). NCAM contains five C2 Ig homology domains, which constitute the *N*-terminal part of the molecule (Hemperly et al., 1986). The cell–substratum and cell–cell binding sites are presumed to be located in Ig domains two and three respectively, and Ig domain five contains the attachment site for polysialic acid-containing carbohydrate chains (Frelinger and Rutishauser, 1986).

Alternative splicing is known to occur in Ig domain four. A 30 bp exon called VASE (variable-domain, alternatively spliced exon) may be inserted between exons 7 and 8, which together encode this Ig domain (Small et al., 1987; Small and Akeson, 1990). NCAM mRNA containing VASE is expressed in both brain and heart, and VASE is present in all five NCAM mRNA classes. The fraction of NCAM mRNA that contains VASE increases with age from 3 % and 10 % in embryonal day 15 brain and heart respectively to approximately 50 % in adult brain and heart (Small et al., 1988; Small and Akeson, 1990; Andersson et al., 1990; Reyes et al., 1991). Neural and muscle cell lines express no or very little VASE (Small and Akeson, 1990). The functional significance of the expression of VASE is not clear. Insertion of the ten amino acids encoded by VASE changes Ig domain four from a C2 to a more V-like Ig domain. Variable Ig domains are involved in antigen binding, and it is tempting to assume that the insertion of VASE-encoded sequences in Ig domain four may introduce a binding site in this domain.

Another position of alternative splicing in the extracellular part of NCAM is located C-terminally to the Ig domains, close to the transmembrane part of the molecule. At this site, which is located between exons 12 and 13, a complex alternative splicing pattern is displayed. Besides the above mentioned exon SEC, four exons, designated a, b, c (formerly MSD1a–c) and AAG of respectively 15, 48, 42 and 3 bp, may be inserted here. When present simultaneously, these four exons encode a presumed muscle-specific NCAM domain termed MSD1 (Dickson et al., 1987). The MSD1 domain is predominantly contained in GPI-anchored NCAM forms, and the joint expression of the four exons is thus mainly associated with the 5.2 and 2.9 kb mRNA classes [for a review, see Walsh and Dickson (1989)]. However, other combinations of these four exons have been demonstrated in muscle, heart and brain (Santoni et al., 1989; Andersson et al., 1990; Reyes et al., 1991; Hamshere et al., 1991). The expression of exons b and c is mainly restricted to skeletal and cardiac muscle, whereas exons a and AAG are also expressed in brain (Reyes et al., 1991). The significance of the variable polypeptide sequences encoded by different combinations of these four exons on NCAM function is still unknown. Their localization distant from the suggested binding sites in the Ig domains may indicate that they are only indirectly involved in intermolecular interaction. Expression of exon a introduces a stretch of four proline residues, which has been suggested to induce a flexible 'hinge' region into NCAM (Walsh and Dickson, 1989). Furthermore, expression of the MSD1 domain has been shown to introduce an attachment site for O-linked glycosylation in the NCAM molecule (Walsh et al., 1989).

In embryonic muscle, NCAM is uniformly present on the surface of myotubes, but, as the myotubes mature, NCAM is downregulated and postnatally NCAM becomes concentrated in synaptic regions (Covault and Sanes, 1986). However, after denervation or during disease-associated muscle degeneration, NCAM expression is again upregulated (Moore and Walsh, 1985; Covault et al., 1986; Cashman et al., 1987). NCAM in rat skeletal muscle is known to be encoded by three 6.7, 5.2 and 2.9 kb mRNAs and expressed mainly as a transmembrane 140 kDa and a GPI-anchored 125 kDa NCAM polypeptide (Covault et al., 1986; Moore et al., 1987). The 140 kDa NCAM isoform is expressed throughout embryonic and postnatal development, whereas the 125 kDa isoform is upregulated perinatally (Covault et al., 1986). Alternative splicing of NCAM mRNA at the exon 12–13 junction has been investigated in mouse skeletal muscle using the PCR technique (Hamshere et al., 1991) By this technique the exon combinations 12–13, 12-AAG-13 and 12-a-b-c-13 were found to be prevalent in NCAM mRNA from adult muscle. The expression of VASE in normal skeletal-muscle tissue has not previously been studied. neither has the expression of NCAM in aged rat skeletal muscle been described before.

We here report a study on NCAM expression in aging skeletal rat muscle. We observed that significant changes in NCAM expression, both at the mRNA and protein level, occurred. In aged muscle a considerable increase in NCAM occurred compared with young adult muscle. Furthermore, selective upregulation of certain NCAM mRNA classes was observed.

MATERIALS AND METHODS

Tissue

Hindleg bulk muscle, brain and liver were dissected from Wistar rats. Biopsies were obtained from Wistar rats at the following ages: postnatal day 1 (P1), 10 (P10), 40 (P40), 270 (P270, corresponding to a 9 month ± 10 day-old-rat), and 730 (P730, corresponding to a 24 month ± 10-day-old rat).

Oligonucleotides

Oligonucleotides (30–40 nt) specific for different NCAM mRNAs were constructed using published sequences of NCAM cDNA clones (see Figure 1). The following oligonucleotide probes were constructed from the rat NCAM cDNA sequence published by Small et al. (1987); probe E7 covering nt 1187–1217, which

hybridizes to part of exon 7 contained in all NCAM mRNA classes; probe EVASE covering nt 1271–1300 and probe E7/8 covering nt 1257–1270+1301–1315, which are specific for NCAM mRNA that either possesses or lacks VASE respectively; probe E16 covering nt 2502–2541, which hybridizes to part of exon 16. From the murine NCAM cDNA sequence published by Santoni et al (1989), oligonucleotide probes specific for exon a followed by AAG (probe E12/a/AAG/13, nt 1946–1959+a+AAG+1960–1969) and mRNA lacking alternatively spliced exons between exon 12 and 13 (probe E12/13, nt 1946–1976) were constructed.

From the human NCAM cDNA sequence published by Dickson et al. (1987) probe E12/a/b covering nt 316–347 was constructed. This probe was expected to recognize mRNA containing exon 12 followed by exons a and b. However, when the sequences of exons b and c in rat were subsequently published (Reyes et al., 1991), it became apparent that this probe may also recognize mRNA containing exons 12-a-13 and to a lesser extent exon 12-a-c. Oligonucleotides were synthesized on a Biosearch 8750 DNA synthesizer and labelled with [^{32}P]dATP (New England Nuclear) using a DNA-tailing kit from Boehringer-Mannheim Biochemica. The specificity of the oligonucleotide probes has previously been described in Andersson et al. (1990).

Northern-blot analysis

Northern-blot analysis was performed as described by Andersson et al. (1990). In general, prehybridization (2 h) and hybridization (18–20 h) were performed at 60–70 °C in a solution containing 4 × SSC (1 × SSC = 0.15 M NaCl + 0.015 M sodium citrate), 0.1 % SDS, 0.1 % Denhardt's solution [2 % Denhardt's solution = 2 % Ficoll + 2 % BSA + 2 % poly(vinylpyrrolidone)], 0.2 mM EDTA, 200 μg poly(A)/ml, 0.06 % tetrasodium diphosphate. After hybridization, filters were washed in 1 × SSC/0.1 % SDS at hybridization temperature. Results are based on three to five independent RNA preparations of each age group.

An RNA standard from BRL was applied to every gel. Poly(A)$^+$RNA from brain and liver were used as positive and negative controls respectively. The amount of poly(A)$^+$RNA on blots was evaluated by ethidium bromide staining.

Antibodies

Polyclonal rabbit anti-(rat brain NCAM) antibodies were prepared as described by Rasmussen et al. (1982). For immunoblotting experiments, these antibodies were purified as follows: brain membrane proteins from adult rats were submitted to SDS/PAGE and electroblotted on to nitrocellulose paper (Millipore Corporation) as described below. Bands containing the 190 and 135 kDa NCAM isoforms were excised, blocked with 2 % Tween 20 (Sigma) and incubated with anti-NCAM antibodies. The nitrocellulose was washed five times for 10 min in washing buffer (WB) consisting of 50 mM Tris/HCl, pH 10.2, 150 mM NaCl, 0.1 mM phenylmethanesulphonyl fluoride (PMSF) and 0.05 % Tween 20. Bound antibodies were eluted with a solution containing 0.1 M sodium citrate buffer, pH 2.6, 0.5 M NaCl, 1 % BSA and 0.05 % Tween 20 for 2 min. Eluted antibodies were adjusted to pH 10.2. From the same nitrocellulose paper, other parts not containing NCAM were incubated with anti-NCAM antibodies and treated as described above. These eluates were used as control antibodies.

The monoclonal mouse antibody OB11 was a generous gift from Dr. Harry Langbeheim, Biomakor, Israel. This antibody is directed against a cytoplasmic epitope common to the rat 190 and 135 kDa NCAM isoforms in brain (Neill and Barnstable, 1990). All other antibodies used were obtained from Dakopatts.

Immunoblotting

Total muscle homogenates contain a substantial amount of myosin which interferes with electrophoresis and may give rise to unspecific binding of antibodies in immunoblotting. In order to reduce this technical problem, muscle tissue was fractionated as follows. Muscle tissue was homogenized in 50 mM Tris/HCl, pH 7.4, 15 mM NaN_3 and centrifuged at 18000 ***g*** for 15 min. The supernatant was subsequently centrifuged for 1.5 h at 100000 ***g*** to produce a high-speed supernatant. The membrane fraction was solubilized by sonication in a Triton-solubilization buffer (TB) consisting of 2 % Triton X-100, 15 mM NaN_3, 10 mM Tris/barbital buffer, pH 8.6, and 100 units/ml aprotinin (Bayer) followed by centrifugation for 15 min at 18000 ***g*** to produce a Triton X-100-soluble membrane fraction. The Triton X-100-insoluble fraction was subsequently washed three times in TB. All fractions (high-speed superantant, TB-soluble membrane fraction, TB washing fractions and the TB-insoluble membrane fraction) were boiled in SDS/PAGE sample buffer containing 5 % 2-mercaptoethanol and applied to 7.5 % gels essentially as described by Laemmli (1970).

Separated proteins were electroblotted on to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation). Non-specific binding was blocked by incubation with 2 % Tween 20 in WB (see above). Primary antibodies were diluted in WB. Alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin was used as secondary antibody. Results are based on a minimum of four rats in each age group. The three major NCAM isoforms from adult rat brain of 190, 135 and 115 kDa were used as molecular-mass markers.

When incubation with OB11 was carried out, it was necessary to immunopurify NCAM before SDS/PAGE. CNBr-activated Sepharose 4B beads (Pharmacia) were coupled to polyclonal rabbit anti-(rat NCAM) antibodies as described by the manufacturer. Solubilized proteins were incubated with antibody-coupled beads for 30 min at 4 °C. Beads were subsequently washed twice in PBS, once in PBS containing 0.1 M NaCl and finally in 10 mM Tris/barbital buffer, pH 8.6. Beads containing bound NCAM were boiled in SDS/PAGE sample buffer and, after centrifugation at 18000 ***g*** for 5 min, supernatant now containing NCAM was subjected to SDS/PAGE as specified above. After electrotransfer to a PVDF membrane, non-specific binding was blocked in PBS containing 10 mM NaCl and 2 % BSA. OB11 washing buffer consisted of PBS with 0.1 % BSA, and the antibody was diluted in PBS containing 2 % BSA and 15 mM NaN_3. Alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin was used as secondary antibody.

Enzyme treatment

Endosialidase N was a generous gift from Dr. Jürgen Roth, University of Basel. For endosialidase N treatment, membrane fraction or high-speed supernatant was mixed with 4×10^9 units of endosialidase N/ml of sample (10^9 units/ml digests 100 μg of polysialic acid after 24 h at 37 °C) and incubated for 30 min at 37 °C. Control samples were incubated without enzyme for 30 min at 37 °C or at 4 °C. As a control for enzyme activity, polysialylated samples from newborn brain were treated with endosialidase N and analysed by immunoblotting, converting diffuse polysialyated zones into distinct desialylated bands.

E.l.i.s.a.

Quantification of NCAM was performed by an e.l.i.s.a. as described by Ibsen et al. (1983). Tissues were homogenized in 70 mM Tris/barbital buffer, pH 8.6, containing 1% Triton X-100, 0.1 mM PMSF and 15 mM NaN_3. Quadruplicate determinations were performed on four rats in each age group. Total protein was determined by the method of Lowry et al. (1951). Differences in NCAM amount were statistically tested by Student's t test. $2P < 0.05$ were considered to be statistically significant.

RESULTS

NCAM mRNA expression in aging skeletal muscle

The NCAM mRNA expression in skeletal muscle during aging was examined by Northern-blot analysis with various DNA oligonucleotide probes. The probes were designed to specifically recognize certain exons or exon combinations (Figure 1). Probe E7 was used as a general NCAM probe. Using this probe, hybridization to three NCAM mRNA classes of 6.7, 5.2 and 2.9 kb was observed in skeletal muscle (Figure 2a). All three mRNA classes were expressed from postnatal day 1 (P1) to 40 (P40) (Figure 2a, lanes 2–4). At P1 and P10, the three NCAM mRNAs were abundant and present in approximately equal amounts (Figure 2a, lanes 2 and 3). In P40 muscle, the total NCAM mRNA amount was greatly decreased and at this age the 5.2 and 2.9 kb mRNAs were slightly more abundant than the 6.7 kb mRNA (Figure 2a, lane 4). In P270 (9 months old) muscle, NCAM mRNA was hardly detectable, whereas in P730 (24 months old) muscle, the 5.2 and 2.9 NCAM mRNA classes were upregulated and clearly expressed (Figure 2a, lanes 5 and 6). The 6.7 kb mRNA was not upregulated and hardly detectable at this age (lane 6). Thus, during aging, not only the total NCAM mRNA amount but also the expression of individual mRNA classes changed.

Hybridization with probe E16 was performed in order to determine which NCAM mRNAs in skeletal muscle encode transmembrane NCAM forms. This probe is complementary to part of exon 16, which encodes transmembrane and cytoplasmic regions of NCAM. Probe E16 specifically hybridized to the 6.7 kb mRNA in skeletal muscle (for P10, see Figure 2b, lane 2). Thus translational potential for both transmembrane and GPI-anchored NCAM species was present in perinatal and young adult muscle, whereas in aged muscle mainly mRNAs encoding GPI-anchored NCAM species could be demonstrated by this technique.

Expression of VASE in skeletal-muscle NCAM mRNA

The alternative splicing between exon 7 and 8 in skeletal-muscle NCAM mRNA was studied using probes E7/8 and EVASE. Probe E7/8 only hybridizes to NCAM mRNAs that lack alternatively spliced sequences inserted between exons 7 and 8. Probe EVASE, on the other hand, is complementary to VASE. The majority of NCAM mRNAs in skeletal muscle lacked alternative splicing between exon 7 and 8 at all ages examined (compare Figures 3a and 3b). Using probe EVASE, only barely detectable levels of VASE could be observed in the 5.2 and 2.9 kb NCAM mRNA in perinatal as well as aged muscle after prolonged exposure times (Figure 3b, lanes 2 and 3). Compared with the expression of VASE in adult brain (Figure 3b, lane 1) the expression in skeletal muscle was negligible.

Alternative splicing between exon 12 and 13 in skeletal-muscle NCAM mRNA

Several combinations of the a, b, c, AAG and SEC exons may be inserted between exons 12 and 13 (Dickson et al., 1987; Santoni

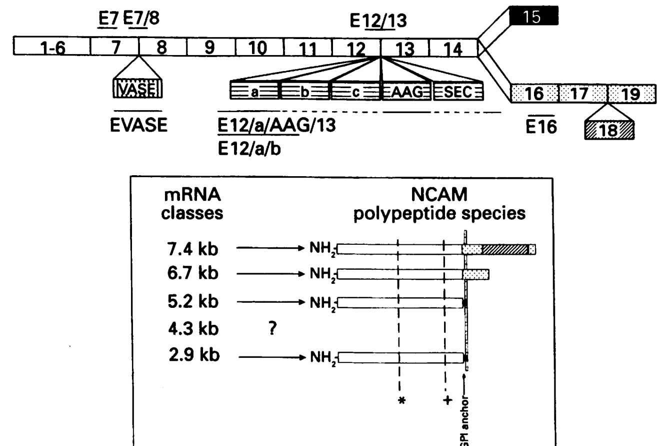

Figure 1 Schematic presentation of the known NCAM exons

Exons common to all NCAM species are white. Exons common to transmembrane NCAM species are light grey, whereas the exon specific for the largest transmembrane NCAM isoform is hatched (▨). The black exon is specific for mRNA encoding GPI-anchored NCAM isoforms. Alternately spliced exons encoding extracellular sequences are striped. The approximate localization of the employed oligonucleotide probes (see the Materials and methods section) is indicated by solid lines. All exons on the figure are of equal size. In reality the sizes vary considerably. Insert: Diagram of the three major polypeptide species encoded by NCAM mRNAs, and their association with the cell membrane. White, light-grey and hatched regions are encoded by exons with the corresponding signature. The black segment represents the GPI anchor. The sequence encoded by exon 15 constitutes a signal sequence for GPI linkage, which is presumed to be removed on attachment of the GPI anchor. Hence, the exon 15-encoded sequence is presumably not present in mature GPI-anchored NCAM isoforms (He et al., 1987). Dotted vertical lines represent the two sites of alternate RNA splicing in the extracellular part of NCAM. *, Exon 7–exon 8 splice site; +, Exon 12–exon 13 splice site.

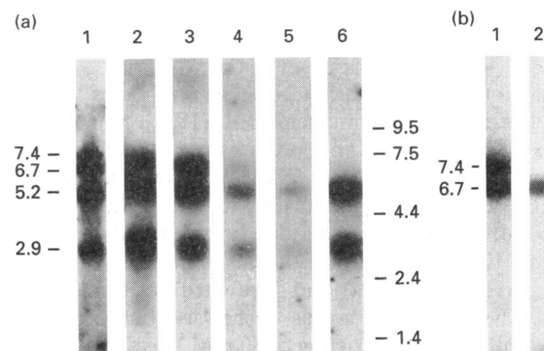

Figure 2 Northern-blot analysis of NCAM mRNA expression in normal rat skeletal muscle during aging.

In (**a**) the general NCAM probe, E7, was hybridized to poly(A)$^+$RNA from adult brain (lane 1), postnatal day (P) 1 muscle (lane 2), P10 muscle (lane 3), P40 muscle (lane 4), P279 muscle (lane 5) and P730 muscle (lane 6). Lanes 2–6 were from the same autoradiogram. In (**b**), probe E16 specific for mRNA encoding transmembrane NCAM species was hybridized to adult brain (lane 1) and P10 muscle (lane 2). Lane 1 in both (**a**) and (**b**) contained 10 μg of poly(A)$^+$RNA and all other lanes contained 15 μg of poly(A)$^+$RNA. The sizes of the hybridizing species are indicated on the left hand side (kb). In (**a**), the positions of standards are indicated on the right hand side.

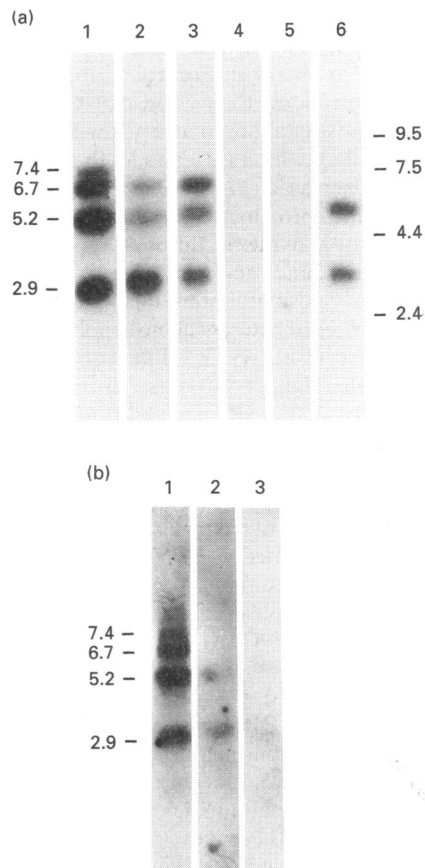

Figure 3 Northern-blot analysis of splicing of NCAM mRNA at the exon 7–exon 8 junction in normal rat skeletal muscle

In (**a**), probe E7/8 was hybridized to adult brain (lane 1), postnatal day (P) 1 muscle (lane 2), P10 muscle (lane 3), P40 muscle (lane 4), P270 muscle (lane 5) and P730 muscle (lane 6). All lanes were from the same autoradiogram. In (**b**), probe EVASE was hybridized to adult brain (lane 1), P1 muscle (lane 2) and P730 muscle (lane 3). Lanes 1 and 2 were from the same autoradiogram, lane 3 was from a comparable autoradiogram. Lane 1 in both (**a**) and (**b**) contained 10 μg of poly(A)$^+$RNA and all other lanes contained 15 μg of poly(A)$^+$RNA. The sizes of the hybridizing species are indicated on the left hand side in (kb). The positions of RNA standards are indicated on the right hand side in (**a**).

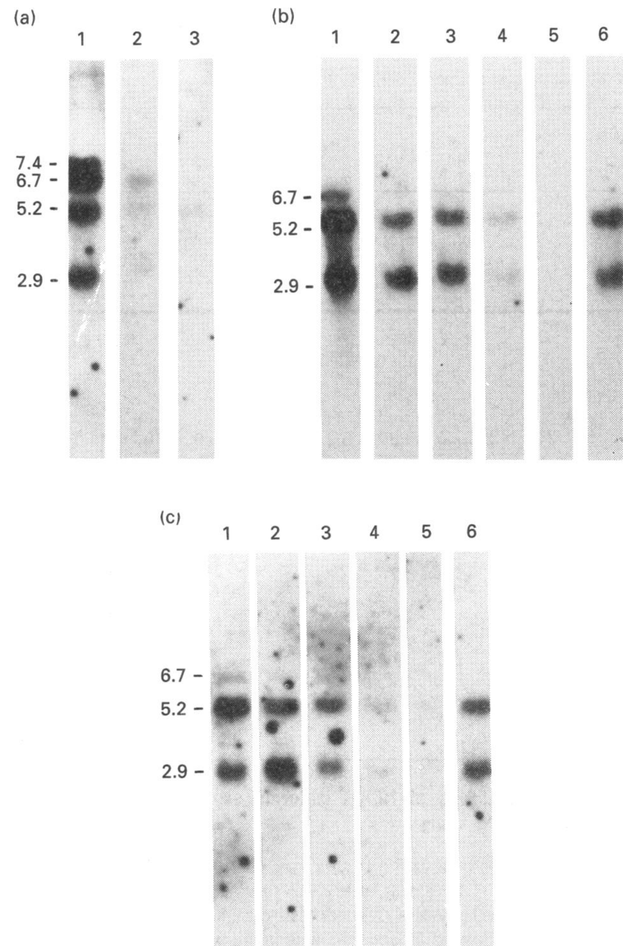

Figure 4 Northern-blot analysis of splicing of NCAM mRNA at the exon 12–exon 13 junction in normal rat skeletal muscle

In (**a**), probe E12/13 is hybridized to adult brain (lane 1), postnatal day (P) 10 muscle (lane 2) and P730 muscle (lane 3). Lanes 1 and 3 were from the same autoradiogram, lane 2 was from a comparable autoradiogram. Hybridization of probe E12/a/AAG/13 and E12/a/b is shown in (**b**) and (**c**) respectively. Lane 1, adult brain; lane 2, P1 muscle; lane 3, P10 muscle; lane 4, P40 muscle; lane 5, P270 muscle; lane 6, P730 muscle. All lanes were from the same autoradiogram. Lane 1 contained 10 μg of poly(A)$^+$RNA and all other lanes contained 15 μg of poly(A)$^+$RNA. The sizes of the hybridizing species are indicated on the left hand side (kb).

et al., 1989; Andersson et al., 1990; Reyes et al., 1991). Collective expression of exon a, b, c and AAG is believed to be restricted to the 5.2 and 2.9 kb mRNA classes in muscle. However, little is known about the distribution of other combinations of these exons in the 6.7, 5.2 and 2.9 kb mRNA classes or about the frequency of alternative splicing at this site in muscle mRNAs. By means of probes E12/13, E12/a/AAG/13 and E12/a/b, we examined the prevalence of alternative splicing between exons 12 and 13 in normal skeletal muscle and attempted to relate the expression of some possible exon combinations to the different NCAM mRNA classes. According to the nomenclature of the probes, probe E12/13 recognizes NCAM mRNA lacking alternatively inserted sequences between exons 12 and 13, probe E12/a/AAG/13 recognizes NCAM mRNA containing exons a and AAG inserted between exons 12 and 13 and probe E12/a/b recognizes NCAM mRNA containing exon 12 followed by exons a and b, with the reservations mentioned in the Materials and methods section.

In all three mRNA classes, expression of both NCAM mRNAs containing and lacking inserted exons between exons 12 and 13 was observed (Figure 4a–4c). The 6.7 kb mRNA class in muscle consisted mainly of mRNA lacking alternative splicing between exons 12 and 13. Hybridization to this mRNA class could, however, be faintly observed with both probes E12/a/AAG/13 and E12/a/b with prolonged exposure (not shown). The 5.2 and 2.9 kb mRNA classes consisted mainly of mRNA isoforms containing additional exons between exons 12 and 13 (Figure 4b and 4c). The fraction of NCAM mRNA containing alternative splicing between exons 12 and 13 in skeletal muscle did not seem to change significantly compared with total NCAM mRNA during aging (compare Figure 2a and Figure 4b and 4c). Hybridization with probe E12/a/AAG/13 showed that expression of the exon combination 12-a-AAG-13, which has previously been demonstrated in brain and heart (Andersson et al., 1990; Reyes et al., 1991), also occurs in postnatal skeletal-muscle NCAM (Figure 4b). This indicates that the expression of exon a in skeletal muscle, as well as in brain and heart, is regulated independently of exons b and c. Indeed, this exon combination seemed to be present in skeletal-muscle mRNA

Table 1 Exons and exon combinations demonstrated in adult and aged rat skeletal muscle by Northern-blot analysis

Parentheses indicate a weak reaction not seen in all experiments.

mRNA classes	7	7/8	VASE	12/13	12/a/ AAG/13	12/a/b	16
6.7 kb	+	+	−	+	(+)	(+)	+
5.2 kb	+	+	(+)	+	+	+	−
2.9 kb	+	+	(+)	+	+	+	−

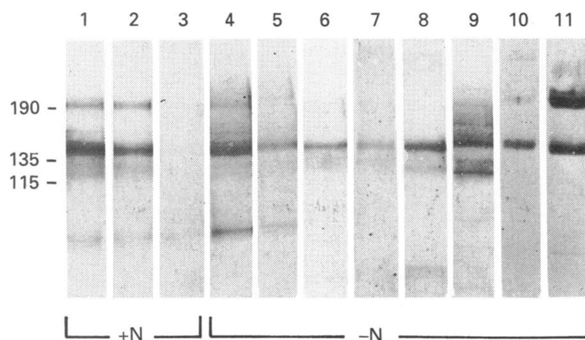

Figure 5 Immunoblotting of NCAM isoforms expressed in normal rat skeletal muscle during aging

Muscle membranes from postnatal day (P) 1 (lanes 1 and 4), P10 (lanes 2 and 5), P40 (lanes 3 and 6), P270 (lane 7), P730 (lane 8), muscle high-speed supernatant from P1 (lane 9), immunopurified NCAM (see the Materials and methods section) from P1 muscle (lane 10) and P40 rat brain (lane 11). Samples in lanes 1–3 were treated with endosialidase N (+N). Samples in lanes 4–11 were untreated (−N). Lanes 1–9 were incubated with retroblotted polyclonal NCAM antibodies. Lanes 10 and 11 were incubated with the monoclonal antibody OB11, which reacts with an epitope on the cytoplasmic domain of NCAM. Lanes 1–5 were run on the same gel, lanes 7–9 and 10 and 11 likewise. In all experiments, newborn muscle membranes and P40 brain membranes served as internal controls. Total protein applied per lane was 13 μg (lanes 1–5, 7 and 8), 60 μg (lane 6) and 110 μg (lane 9). The positions of the three major 190, 135 and 115 kDa NCAM isoforms from P40 rat brain are indicated on the left-hand side.

encoding GPI-anchored NCAM isoforms in substantial amount. Hybridization with probe E12/a/b showed that the exon combination 12-a-b was also substantially expressed in muscle mRNA encoding GPI-anchored NCAM (Figure 4c). Hybridization signals obtained using probe E12/a/b possibly consisted of various NCAM mRNAs with exon b followed by different exons such as, e.g., exon c, exon AAG or exon 13.

The hybridization results are summarized in Table 1.

NCAM polypeptide composition in aging skeletal muscle

NCAM protein expression was examined in skeletal muscle from P1, P10, P40, P270 and P730 rats by immunoblotting using retroblotted polyclonal anti-NCAM antibodies that recognize all NCAM isoforms. Tissue homogenates were separated into a buffer-soluble high-speed supernatant, a Triton X-100-soluble membrane fraction and a Triton X-100-insoluble membrane fraction as described in the Materials and methods section.

In the Triton X-100-soluble skeletal-muscle membrane fraction, four polypeptides of 200, 145, 125 and 120 kDa were observed at all ages investigated, with the 145 kDa polypeptide being predominant (Figure 5, lanes 4–8). Occasionally a 165 kDa polypeptide was also observed. Control antibodies did not react with NCAM polypeptides. Expression of NCAM polypeptides decreased during postnatal life to a very low level in P40 muscle (Figure 5, lanes 1–3). In aged muscle the level of NCAM polypeptides had increased (Figure 5, lanes 3 and 6–8, note that the amount of applied protein is higher in lane 6 than in the other lanes). In early postnatal muscle, diffuse staining extended above the 200 and 145 kDa bands and above the 120–125 kDa area. In embryonal and early postnatal brain this pattern is known to be caused by the presence of heterogeneously polysialylated NCAM polypeptides (Finne et al., 1983; Linnemann et al., 1985). In order to investigate whether this was also the case in skeletal muscle, membranes of P1, P10 and P40 muscles were treated with endosialidase N which removes polysialic acid residues. The enzyme treatment removed the diffuse staining leaving only well-defined bands (Figure 5, lanes 1–3). In contrast, controls incubated without enzyme at either 4 °C or 37 °C were unchanged (not shown). This indicated that NCAM expressed in early postnatal skeletal muscle contained polysialic acid. At older ages no diffuse staining was observed, indicating that no or only minor polysialylation of NCAM occurred in young adult and aged skeletal muscle.

Transmembrane NCAM polypeptides were identified using the monoclonal antibody OB11 which reacts with a cytoplasmic epitope common to the transmembrane 190 and 135 kDa brain NCAM isoforms (Figure 5, lane 11). Immunoisolated NCAM polypeptides from skeletal-muscle membranes of 200 and 145 kDa reacted with OB11 (Figure 5, lane 10), indicating that these two polypeptides were carrying the cytoplasmic OB11 epitope.

In order to study the expression of soluble NCAM polypeptides in muscle, a high-speed supernatant was prepared from the buffer-soluble fraction and analysed by immunoblotting. Soluble NCAM polypeptides of 200, 145, 125 and 120 kDa comparable in size with the polypeptides observed in the Triton X-100-soluble membrane fraction were observed at all ages examined [Figure 5, lane 9; only one age (P1) is shown]. The pattern of soluble NCAM polypeptides did not seem to change during aging (not shown). Diffuse staining was observed extending above the NCAM polypeptide bands in the high-speed supernatant at early postnatal ages. Treatment with endosialidase N removed this diffuse staining, indicating that soluble NCAM polypeptides also contained heterogeneous polysialylation at these ages (not shown).

In the Triton X-100-insoluble fraction, NCAM polypeptides of 145, 125 and 120 kDa were observed (not shown). In addition, a large smear at approx. 200 kDa, presumably representing unspecific binding to myosin, was observed. The relative amount of the 145, 125 and 120 kDa polypeptides was apparently the same as in the Triton X-100-soluble fraction. The ratio of the amount of NCAM in the membrane fractions to that in the high-speed supernatant seemed to remain constant at all ages investigated.

Amount of NCAM in aging skeletal muscle

The amount of total NCAM protein in P1, P10, P40, P270 and P730 skeletal muscle was determined using an e.l.i.s.a. (Figure 6). Skeletal muscle at P1 contained 1.21 ± 0.44 μg of NCAM/mg of total protein (mean ± S.E.M., $n = 4$). The amount of NCAM decreased to a minimum of 0.063 ± 0.008 μg/mg of total protein ($n = 4$) around P40. In aged muscle the NCAM amount increased again to 0.37 ± 0.11 μg/mg of total protein ($n = 4$) in P730 rats.

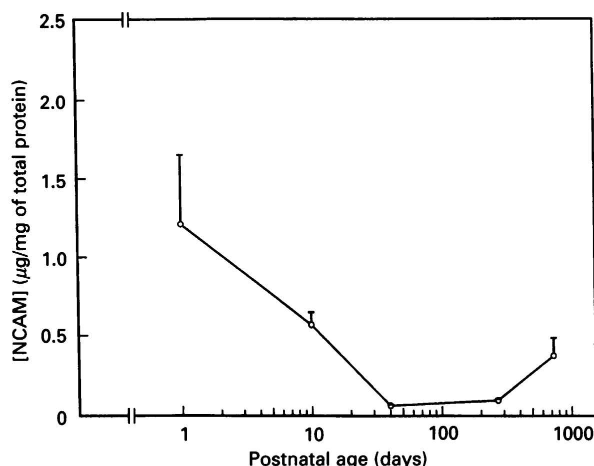

Figure 6 Amount of NCAM in normal rat skeletal muscle determined by e.l.i.s.a. and expressed as μg/mg of total protein

The age is indicated in days (270 days = 9 months, 730 days = 24 months). Values are given as means ± S.E.M. ($n = 4$).

The level of NCAM in P730 muscle was statistically significantly higher than the level in P40 and P270 muscle ($2P < 0.05$).

DISCUSSION

NCAM mRNA expression in rat skeletal muscle

The data presented here show that significant changes in the NCAM mRNA pattern occur in skeletal muscle during aging. Thus three NCAM mRNAs of 6.7, 5.2 and 2.9 kb were expressed during early postnatal development, being downregulated in young adult muscle. In aged skeletal muscle the 5.2 and 2.9 kb NCAM mRNAs were specifically upregulated, whereas the 6.7 kb mRNA remained unchanged.

The exon VASE was found to be virtually absent from postnatal skeletal muscle at all ages. In contrast, VASE is expressed in both brain and heart in increasing amounts during postnatal development (Small et al., 1988; Andersson et al., 1990; Small and Akeson, 1990; Reyes et al., 1991). Thus expression of NCAM isoforms containing the VASE-encoded domain is not only developmentally regulated but also regulated in a tissue-specific manner.

A major fraction of the 5.2 and 2.9 kb NCAM mRNAs was shown to contain various combinations of exons inserted at the exon 12–13 junction, whereas only a relatively small proportion of the 6.7 kb mRNA contained inserted exons at this site. The exon combinations 12-a-AAG-13 and 12-a-b were both found to be abundant in postnatal skeletal muscle at all ages studied. No change in the relative proportion of NCAM mRNA lacking or containing extra exons inserted at the exon 12–13 junction seemed to occur during aging (compare Figures 2a and 4b and 4c). The significance of these various exon combinations for NCAM function remains to be clarified. We found that the majority of NCAM mRNAs contained exons inserted between exons 12 and 13 in adult and aged muscle, whereas Hamshere and co-workers (1991) found that the exon combination 12–13 was the prevalent mRNA form. Moreover, these authors found that the exon combination 12-a-AAG-13 was not expressed in normal muscle and only expressed at a low level in the muscle cell line C2, whereas we observed substantial amounts of this exon combination in NCAM mRNA from muscle. This discrepancy may be due to species differences or different techniques employed.

Expression of NCAM protein in aging skeletal muscle

In agreement with previous observations (Rieger et al., 1985; Covault et al., 1986), we observed a downregulation of NCAM during early postnatal development to a very low level in young adult skeletal muscle. However, in aged rat, muscle NCAM protein level increased considerably. NCAM protein concentration in aged skeletal muscle reached a level corresponding to that previously reported for denervated rat muscle by Covault et al. (1986). It has been suggested that a slow on-going denervation–reinnervation process takes place in muscle throughout life (Wernig and Dorlöchter, 1989). In histopathological studies of aged striated muscles, alterations indicating a process of deterioration of both myogenic and neurogenic origin have been observed, the neurogenic type being predominant (Tomonaga, 1977). Furthermore, aged muscle and denervated young muscle exhibit similar changes in myofibrillar properties (Ansved and Larsson, 1989). During aging, the number of muscle fibres and motor units decreases in rodent and man (Caccia et al., 1979; Edström and Larsson, 1987). It has been suggested that the decreased number of muscle fibres is due to loss of entire motor units and incomplete reinnervation of denervated muscle fibres by the remaining motor neurons (Caccia et al., 1979; Edström and Larsson, 1987) resulting from impaired axonal regeneration in the aged rat (Drahota and Gutmann, 1961). The increased levels of NCAM in aged skeletal muscle reported here support this assumption.

Although the proportions of the different NCAM mRNA classes changed during aging, no apparent change in the proportion of NCAM polypeptides occurred. The increased amount of the 145 kDa transmembrane NCAM form in aged muscle as compared with that in young adult muscle was not accompanied by an increase in the 6.7 kb mRNA class. This may imply that the catabolism of the 145 kDa polypeptide is decreased in aged muscle or, alternatively, that the translational rate of the 6.7 kb mRNA is enhanced compared with the 5.2 and 2.9 kb mRNA classes. The 145 kDa polypeptide was the predominant NCAM isoform observed at all ages examined. Antibodies against a cytoplasmic epitope of NCAM showed that both the 200 and 145 kDa polypeptides in muscle membranes were transmembrane NCAM isoforms, indicating that the 200 kDa NCAM polypeptide is the muscle homologue to the 190 kDa NCAM polypeptide observed in brain. A 180 kDa NCAM polypeptide presumably corresponding to the 200 kDa polypeptide observed in this study has recently been observed in primary cultures of mouse muscle cells (Tassin et al., 1991). Failure to detect the 7.4 kb mRNA class encoding this NCAM polypeptide in muscle at any age may be attributed to a very low amount of this mRNA class, indicating either a low catabolism of the 200 kDa NCAM polypeptide or an enhanced translational rate of this mRNA class in muscle. Postnatal developmental changes in the amount of the 200 kDa NCAM polypeptide correlated with developmental changes in the total amount of NCAM in muscle, indicating that expression of the 200 kDa NCAM polypeptide is subject to the same regulation as the other NCAM isoforms in skeletal muscle and thus may be expressed by the same cells.

The 120–125 kDa NCAM isoform observed in this study in the buffer-soluble fraction of muscle homogenate may be a GPI-anchored NCAM isoform, spontaneously released from the membrane. It has been demonstrated that a 125 kDa NCAM polypeptide can be released from the surface of myotubes in

primary culture when treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (Moore et al., 1987; Tassin et al., 1991). GPI-anchored NCAM isoforms are also spontaneously released from the membrane of C6 cells and muscle cells in primary cultures (He et al., 1987; Tassin et al., 1991). An additional PI-PLC-sensitive 155 kDa NCAM polypeptide has been described in the mouse muscle cell lines C8-1 and C2 by Moore et al. (1987). In this study neither a membrane-associated nor a soluble 155 kDa polypeptide could be demonstrated. Neither was this 155 kDa NCAM isoform observed in primary cultures of mouse muscle cells by Tassin et al. (1991). The origin of the soluble NCAM polypeptides of 200 and 145 kDa observed in this study is uncertain. Soluble NCAM isoforms of similar size have been demonstrated in rat brain and cerebrospinal fluid (Krog et al., 1992).

Previous studies on rodent muscle expression of NCAM polypeptide isoforms have given conflicting results. In normal postnatal skeletal muscle, a major NCAM isoform of 140/145 kDa is generally observed (Rieger et al., 1985; Covault et al., 1986; Daniloff et al., 1986). In contrast, a 125 kDa NCAM polypeptide seems to predominate in postfusion muscle cell lines and in primary cultures of myotubes (Covault et al., 1986; Moore et al., 1987). These muscle cell cultures lack neuronal stimulation. Since previous studies on denervated and paralysed muscle have shown that neuronal stimulation regulates the level of NCAM expression in skeletal muscle (Covault and Sanes, 1985; Moore and Walsh, 1986), we suggest that neuronal stimulation may also regulate expression of individual NCAM isoforms. Thus, the discrepancy between the major NCAM isoforms observed in normal postnatal skeletal muscle tissue and in *in vitro* systems lacking innervation may be due to the fact that myotubes express different NCAM isoforms depending on their state of innervation, indicating that the different NCAM isoforms in muscle serve different functions.

Currently, new tools for the investigation of individual NCAM isoforms are being produced. Thus, the availability of isoform-specific antibodies and NCAM cDNA clones containing different combinations of alternately spliced exons will enable further studies of the distribution and function of specific NCAM isoforms. As more is learned about the role of individual NCAM isoforms in muscle development and muscle–nerve interaction, elucidation of changes in muscle NCAM expression during aging may contribute to an increased understanding of the aging processes in muscle.

Note added in proof (Received 18 December 1992)

Recently, a paper describing increased NCAM expression in aging murine muscle has been published by Kobayashi et al. (1992).

This work was supported by The Foundation of 17-12-1981, The Højmosegård Foundation, The King Christian X Foundation, The Ferdinand and Ellen Hindsgauls Foundation, The Danish Research Academy (V890168) and The Lundbeck Foundation (9/89 and 30/90).

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Received 24 July 1992/18 September 1992; accepted 30 September 1992