INTRODUCTION

Development of the vertebrate neuromuscular system is thought to progress in three major steps. In the early embryo, migration and differentiation of muscle and neuronal cells occur independently. Later, at middle embryonic stages, there is a critical coordinate interaction phase, when synaptic contacts between myofibres and motor neuron endings are observed and myogenic and neurogenic programs become interdependent. In a late period of maturation and stabilization, which continues during adult life, the neuromuscular connections rectify, fit and refine themselves. It has recently become clear that cell adhesion involving cell adhesion molecules (CAMs), integrins and extracellular matrix molecules, is an essential actor in these developmental processes (Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; Edelman and Crossin, 1991; Hall and Sanes, 1993). Cell adhesion molecules are also implicated in the process of nerve regeneration and muscle reinnervation that recapitulate many aspects of normal development (Rieger, 1990; Edelman and Crossin, 1991). N-CAM, a Ca\(^{2+}\)-independent CAM of the immunoglobulin superfamily, is expressed in developing muscle and facilitates myoblast fusion by increasing myoblast-myoblast adhesion (Dickson et al., 1990; Knudsen et al., 1990a). N-CAM

N-cadherin expression in developing, adult and denervated chicken neuromuscular system: accumulations at both the neuromuscular junction and the node of Ranvier

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SUMMARY

N-cadherin, a member of the Ca\(^{2+}\)-dependent cell adhesion molecule family plays essential roles in morphogenesis and histogenesis. N-cadherin has been shown in vitro to promote myoblast fusion and neurite outgrowth. We report here the cellular localization of N-cadherin during development and regeneration of the chick neuromuscular system. N-cadherin was uniformly expressed along the surface of myoblasts and myotubes of E6 limb muscles. Later, as synaptogenesis and secondary myogenesis proceeded, N-cadherin expression was down-regulated and restricted to some large-diameter fibres, then to the areas of contact between few myofibres and subsequently disappeared by embryonic day 17, suggesting that this cadherin may be implicated predominantly in fusion of primary myoblasts and, at lower degree, of secondary myoblasts. The presence of N-cadherin in muscle during the period of nerve trunk ingrowth and its down-regulation after synaptogenesis suggests that this molecule might be implicated in both processes. N-cadherin became accumulated at the neuromuscular junction only a few days after the first synaptic contacts were established and remained at the adult neuromuscular junction, suggesting a role of this molecule in the stabilization of the mature neuromuscular junction. In sciatric nerve, the level of N-cadherin expression remained unchanged from hatching to adult life. N-cadherin was widely distributed on the surface of myelinated fibres and on myelinating Schwann cells: in addition, it was concentrated at the node of Ranvier. At the ultrastructural level, the molecule was detected inside, at the surface and in the basal lamina of Schwann cells and also associated with endoneurial collagen. These observations suggest a role of N-cadherin in the structuring and stabilization of the myelin sheaths. After nerve injury, N-cadherin continued to be expressed by proliferating Schwann cells in the distal stump providing a substratum for regenerating axons. N-cadherin reappeared at the surface of denervated muscle fibres without disappearing from the former synaptic sites. It was detected not only in the sarcolemma and on denervated muscle fibres, but also in the basal lamina and in the extracellular matrix. The reexpression of N-cadherin at the surface of denervated muscle fibres suggests a role for this molecule in muscle reinnervation. The presence of N-cadherin in basal lamina and its association with collagen fibres raise questions about the release of N-cadherin in the extracellular space and the existence of a putative heterophilic ligand for N-cadherin.

Key words: N-cadherin, cell adhesion, myogenesis, synaptogenesis, chicken neuromuscular development, nerve regeneration, muscle reinnervation

INTRODUCTION
expression is greatly down-regulated after synaptogenesis so that it only persists at synaptic sites (Rieger et al., 1985; Covault and Sanes, 1986). It reappears on the entire surface of denervated myofibres (Rieger et al., 1985; Covault and Sanes, 1985). In the peripheral nerve, N-CAM is expressed during myelination by motoneurons and Schwann cells (Daniloﬀ et al., 1986) but is restricted to the node of Ranvier after myelination (Rieger et al., 1986). After nerve injury, N-CAM is reexpressed in the proliferating Schwann cells at the site of nerve injury and in the distal stump (Daniloﬀ et al., 1986). N-CAM has been shown to play a role in muscle reinnervation by mediating proper axon-Schwann cell interactions (Rieger et al., 1988). An extracellular matrix glycoprotein, cytactin/tenascin, is also concentrated at the node of Ranvier and at the neuromuscular junction (Rieger et al., 1986). After nerve crush, cytactin expression increases in endoneurial tubes, at the motor endplates and in interstitial spaces, returning to normal levels when the muscle is reinnervated (Daniloﬀ et al., 1989). During regeneration of the frog neuromuscular system in vivo, treatment with an antibody to cytactin leads to a failure of nerve to reinnervate its muscle target suggesting that this molecule is a major nerve guidance and/or target finding cue in the neuromuscular system (Mège et al., 1992b).

In this work, we have studied the expression and ultrastructural localization of another cell adhesion molecule, N-cadherin in the developing, adult and injured skeletal muscle and peripheral nerve. N-cadherin (Hatta et al., 1988) is one of the members of a family of $\text{Ca}^{2+}$-dependent cell adhesion molecules, or cadherins, important for morphogenesis during development and for maintaining intercellular cohesion in adult tissues (Takeichi, 1991). Cadherins exhibit considerable sequence homology and are highly conserved throughout evolution (Takeichi, 1988). Their expression controls cell aggregation during the mesenchymal-epithelial transformation (Mège et al., 1988; Matsuzaki et al., 1990) and cell segregation (Nose et al., 1988; Friedlander et al., 1989). At least six members of the cadherin family have been detected in the adult or developing neuromuscular system: chicken, N-cadherin (Hatta and Takeichi, 1986; Duband et al., 1987), R-cadherin (Inuzuka et al., 1991), T-cadherin (Ranscht and Dours-Zimmermann, 1991) and B-cadherin (Napolitano et al., 1991; mouse, M-cadherin (Donalies et al., 1991) and Xenopus, EP-cadherin (Levi et al., 1991). Although the role of these cadherins during differentiation and maturation of the neuromuscular system remains to be elucidated, much evidence already supports the notion that N-cadherin may be involved in neuromuscular developmental interactions. In chicken, N-cadherin is expressed in somitic cells (Hatta and Takeichi, 1986; Duband et al., 1988), in the myotomes and in developing muscles at the surface of both myoblasts and newly fused myotubes (Hatta et al., 1987; Hahn and Covault, 1992). Innervated skeletal muscles lose N-cadherin expression before hatching (Hatta et al., 1987), as a result of nerve-induced muscle activity (Hahn and Covault, 1992). In vitro perturbations have shown that N-cadherin-mediated adhesion of myoblasts is a necessary step of myoblast fusion (Knudsen et al., 1990b; Mège et al., 1992a). N-cadherin also facilitates neurite outgrowth of various neural cells on Schwann cells and muscle cells in culture (Bixby et al., 1987; 1988; Letourneau, 1990) as well as on transfected fibroblasts (Matsunaga et al., 1988). Thus, N-cadherin may be implicated in the progression and guidance of motor axons toward their muscular target. The expression of N-cadherin in developing skeletal muscle has been documented to some extent in previous studies (Hatta et al., 1987; Hahn and Covault, 1992). However, a precise and detailed knowledge of N-cadherin expression and localization in skeletal muscle and peripheral nerve has been missing.

We present here the immunocytochemical localization of N-cadherin in developing and adult skeletal muscle and peripheral nerve. During embryonic chicken development, N-cadherin is expressed in both tissues. In the adult, N-cadherin is highly down-regulated in muscle, to persist only at the neuromuscular junction. In adult sciatic nerve, the molecule is concentrated at the node of Ranvier but is still abundant at the surface of myelinated and non-myelinated nerve fibres. We have further studied the changes in N-cadherin expression and its ultrastructural localization by immunogold electron microscopy in nerve and muscle during experimental denervation-reinnervation processes. We discuss the significance of the presence of N-cadherin in the extracellular matrix which raises the problem of the existence of a putative heterophilic ligand for N-cadherin.

**MATERIALS AND METHODS**

**Animals and surgical procedures**

For protein extractions or immunohistochemistry, hindlimbs were dissected out from 6-day, 11-day, 15-day, 19-day and 20-day-old White Leghorn embryos. For denervation experiments, young chicks (2 weeks old) were anesthetized with chloroform and the sciatic nerve was transected unilaterally by removing a 2 mm segment as previously described by Daniloﬀ and coworkers (1986). For denervation-reinnervation, sciatic nerve was crushed for 20 seconds using a smooth grips forceps which produces a 2 mm area of translucency of the nerve. Animals were killed after 4, 10 and 20 days. Injured and contralateral nerves and gastrocnemius muscles were dissected out for immunocytochemical examination. The two parts of the latissimus dorsi muscle: anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) were also examined.

**Antibodies and toxin**

Two distinct monoclonal antibodies (GC4 and FA5; Biomakor, Rehovot, Israel), reacting within the N-terminal domain of A-CAM (Volk et al., 1987, 1990) were used to recognize chicken N-cadherin which is similar or identical to A-CAM. Indeed, during chicken development, the distribution of A-CAM is exactly the same as N-cadherin (Duband et al., 1988) and, furthermore, anti-A-CAM antibodies recognize specifically N-cadherin (Matsuzaki et al., 1990) providing strong arguments to favor the idea that the two molecules are identical. The neuromuscular postsynaptic membranes were labelled with rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR).

**Gel electrophoresis and immunoblotting**

Tissue samples were homogenized in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) containing 0.3% SDS (sodium dodecyl sulfate), 1:1000 aprotinin and 10 µg/ml DNAase, centrifuged at 100 000 g for 5 minutes (Mège et al., 1992a) and the protein concentration of the supernatants determined using a Lowry modified method (Smith et al., 1985; BCA kit, Pierce, Rockford, IL). Aliquots containing 10 µg of protein for heart extracts and 75 µg of protein for muscle and sciatic nerve extracts were processed for immunoblotting as previously described (Mège et al., 1992a) except for the detection. N-cadherin
was detected by sequential incubation with the anti-N-cadherin monoclonal antibody and peroxidase-conjugated rabbit anti-mouse IgGs, revealed by chemiluminescence with the ECL kit (Amersham France SA, Les Ulis, France).

**Immunofluorescent staining**

Immunostaining procedures were performed as described elsewhere (Cifuentes-Diaz et al., 1992). Tissues were frozen in liquid nitrogen-cooled isopentane and 6 µm cryostat sections were cut at −28°C. Sections were directly incubated for 1 hour at room temperature with the monoclonal antibodies diluted in PBS containing 3% bovine serum albumin (BSA), washed in PBS, then incubated for 1 hour at room temperature with fluorescein-conjugated anti-mouse IgGs. Muscle preparations were also treated with rhodamine-conjugated α-bungarotoxin for 30 minutes. Sections were then washed, mounted in glycerol-Mowiol (Calbiochem) and observed with a fluorescence microscope (Axiophot, Zeiss).

Small bundles of muscle or nerve fibres were fixed for 1 hour in 2% formaldehyde, incubated for 1 hour in PBS−, 0.1 M glycine, then in PBS−, 0.1 M glycine and 0.5% Triton X-100. They were fixed in 3% formaldehyde plus 0.01% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 30 minutes, washed in PBS−, 0.1 M glycine for 30 minutes. Preparations were postfixed in 2% osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections were mounted on nickel grids, treated with a saturated solution of sodium periodate, rinsed in 0.2 M Tris buffer pH 7.4 supplemented with 0.2 M NaCl and 0.1% BSA and incubated for 15 minutes in a solution of 10% heat-inactivated normal goat serum in Tris buffer. They were then incubated for 12 hours at 4°C with the monoclonal antibody diluted 1:50 in Tris buffer supplemented with 1.5% BSA, washed and incubated for 1 hour with gold-conjugated anti-mouse antibodies (Auroprobe EM GAM IgG G10, Amersham) diluted 1:200 in the same buffer. Grids were washed, fixed in 2.5% glutaraldehyde for 5 minutes, rinsed and stained with uranyl acetate and lead citrate. Sections were observed in a Philips 410 transmission electron microscope.

**RESULTS**

**N-cadherin expression in developing skeletal muscle and accumulation at the adult neuromuscular synapse**

We studied the distribution of N-cadherin in chick skeletal muscles at various stages of development (Fig. 1). In agreement with previous studies (Hatta et al., 1987; Hahn and Covault, 1992), we found that hindlimb muscles from E6 and E11 chicken embryos were positive for N-cadherin (Fig. 1A,B). The staining was strong at E6, and was predominantly localized at the surface of myogenic cells identified on serial sections by ATPase staining. N-cadherin was also detected on some connective tissue cells and in cartilage. The level of N-cadherin staining dropped markedly between E6 and E11 and further decreased with increasing age of the embryos. Whereas N-cadherin was homogeneously expressed in the muscle at embryonic day 6, from embryonic day 11 until day 14, a few large-diameter fibres appeared more intensely labelled while most fibres progressively lost N-cadherin immunoreactivity. These are slow myosin-containing myotubes and probably are primary myotubes (McLenann, 1983). By embryonic day 15, all the fibres became N-cadherin negative except at a few sites of myotube-myotube contact (Fig. 1C,C′). All muscle fibres became totally N-cadherin negative by embryonic day 17-18 even at the sites of accumulation of acetylcholine receptors detected by α-bungarotoxin staining. However, by embryonic day 19, these synaptic sites were N-cadherin positive (Fig. 1D,D′). Two days after hatching, N-cadherin was not detectable on the extrasynaptic surfaces of gastrocnemius phasic muscle fibres but remained restricted to neuromuscular junctions (Fig. 1E,E′). In adult muscle, synaptic accumulation of N-cadherin staining was also observed together with blood vessels and nervous elements staining.

Colocalization of N-cadherin immunoreactivity with the typical α-bungarotoxin staining of the postsynaptic acetylcholine receptor accumulation was also observed on preparations of teased muscle fibres of gastrocnemius muscle from young adult chicks (not shown) and using confocal microscopy on preparations of posterior latissimus dorsi muscles (Fig. 1F,F′). The fast-phasic fibres of the posterior latissimus dorsi (PLD), which receive a single mononeural focal innervation (Ginsborg, 1960), displayed a precise restricted colocalization of N-cadherin, with the focal ‘en plaque’ pattern of α-bungarotoxin staining. In the anterior latissimus dorsi (ALD) constituted entirely of slow-tonic fibres which possess multiple polyneural end-plates, N-cadherin immunoreactivity was again colocalized with the α-bungarotoxin staining. However, PLD muscle fibres had undetectable levels of extrasynaptic N-cadherin staining whereas ALD fibres were clearly stained for N-cadherin on their entire surface (data not shown).

The down-regulation of N-cadherin expression in muscles during development was confirmed by immunoblotting (Fig. 2). N-cadherin was detectable in E6 leg muscle extract as a single immunoreactive species of relative molecular mass around 135×10^3 but was at the limit of detection by E11 and undetectable in post-hatching and adult muscles. It is worth noting that even at E6, the N-cadherin immunoreactivity found in skeletal muscle extracts was about two orders of magnitude lower than the N-cadherin immunoreactivity found in embryonic heart (Fig. 2, lines a and b). An N-cadherin immunoreactive band of similar relative molecular mass was also found in sciatic nerve extracts of hatching chicken (Fig. 2, line d).

**N-cadherin expression in the peripheral nerve**

The distribution of N-cadherin was determined on longitudinal sections of sciatic nerves from hatching and adult chickens (Fig. 3). At hatching, only a few small diameter fibres were myelinated (Danloff et al., 1986; Usson and Saxod, 1988). N-cadherin staining appeared in all the constitutive elements of the nerve: nerve fibres and mononucleated cells (Fig. 3A,A′). Two weeks after hatching, myelinated fibres were already evident and N-cadherin staining was as intense as at hatching (not shown). In the adult, N-cadherin staining clearly delineated the external surface and more faintly the internal surface of myelin sheaths (Fig. 3B,B′). The nodes of Ranvier and some mononucleated cells at the surface of the fibres were also clearly positive. In whole-mount preparations of sciatic nerve
Fig. 1
N-cadherin in neuromuscular system

Fig. 1. N-cadherin in developing chick skeletal muscle and at the adult neuromuscular junction. On cross-sections of leg muscles from E6 (A) and E11 (B) chick embryos, embryonic muscles were positive for anti-N-cadherin antibodies. At E11 (B), the labelling of myogenic cells was lower; however, a few large myotubes (open arrows) remained brightly stained. Transverse sections of gastrocnemius muscle from E15 (C,C'), E19 (D,D') and post-hatching (2 day old) (E,E') were doubly stained with α-bungarotoxin (C-E) and anti-N-cadherin (C'-E'). At embryonic day 15, the general level of N-cadherin staining had further decreased. Accumulations of N-cadherin were noted at some areas of contact between myotubes (arrowheads in C') but the patches of acetylcholine receptor (arrows in C) never corresponded to accumulations of N-cadherin staining. At embryonic day 19, muscle fibres were N-cadherin negative except at synapses revealed by acetylcholine receptor accumulations (arrows in D and D'). The insert shows the absence of penetration of the rhodamine fluorescence into the fluorescein wave band, in preparations stained only with the rhodaminated α-bungarotoxin. After hatching, the myofibre surfaces were N-cadherin negative except at neuromuscular junctions (arrows in E and E'). Bar for A to E', 40 µm. Whole-mount preparations of posterior latissimus dorsi muscle from 15-day-old chicks were doubly stained for the postsynaptic acetylcholine receptor (F) and for N-cadherin (F') and observed with a confocal laser microscope set up to allow the observation of 1 µm depth fields. N-cadherin staining was codistributed with α-bungarotoxin showing that the motor endplate restricted N-cadherin distribution persisted in adult. Bar for F and F', 10 µm.

Fig. 2. N-cadherin immunoblotting of skeletal muscle and peripheral nerve extracts. 10 µg of protein extract from E15 embryonic chick heart (a) and 75 µg of protein extract of leg muscles from E6 embryos (b) and hatching chickens (c) and of sciatic nerves from hatching chicken (d) were resolved by SDS-PAGE on a 6% polyacrylamide gel and immunoblotted for N-cadherin. N-cadherin was abundant in heart extract, was detectable in E6 leg muscles, but undetectable in hatching leg muscles. However, hatching sciatic nerve extract contained a detectable level of N-cadherin.

fibres from 2-week-old chicken, N-cadherin staining delineated the outer surface of myelinated fibre (Fig. 4A-B). In addition, myelinating Schwann cells identified as spindle shape cells directly adjacent to the myelin sheath were also positive for N-cadherin (Fig. 4A,A'). However, the most intense staining was observed at the node of Ranvier (Fig. 4C,C') which clearly represents a site of N-cadherin accumulation.

Fig. 3. N-cadherin distribution in post-hatching and adult sciatic nerves. Longitudinal cryostat sections of sciatic nerves from hatching (A,A') and adult chickens (B,B') were labelled with monoclonal anti-chicken N-cadherin IgGs. Preparations are shown with fluorescent (A,B) and phase (A'-B') optics. Intense N-cadherin staining was observed in both cases. It appeared as a thin line at the outer surface of myelin sheaths (arrows), at the nodes of Ranvier (arrowhead in B) and on the axons (open arrow in B). Bar, 40 µm.
Non-myelinated small diameter fibres were observed positively stained for N-cadherin in these preparations (not shown).

This localization of N-cadherin in myelinated fibres was confirmed at the ultrastructural level by immunogold electron microscopy (Fig. 5). N-cadherin revealing gold particles were mostly found on the surface of myelin sheaths and sometimes in the axoplasm (Fig. 5A). Only a few particles were associated with myelin and no particles were observed in the absence of the specific antibody (Fig. 5B). Observations at higher magnification (Fig. 5C) revealed that single particles or small aggregates were present on the external face of Schwann cells, in Schwann cell basal lamina and on collagen fibrils surrounding the nerve fibres. Some particles were also observed in the Schwann cell cytoplasm associated with vesicular elements.

**N-cadherin in injured peripheral nerve and denervated muscle**

In order to determine whether the level of expression and the cellular distribution of N-cadherin was affected by the degeneration/regeneration processes, 15-day-old chicks were unilaterally denervated either by crush or by transection of the sciatic nerve. N-cadherin expression was examined in the injured nerves and in the denervated corresponding muscles 4, 10 and 20 days after surgery. 4 days and 10 days after nerve crush or transection, the levels of N-cadherin immunoreactivity in the injured area of the nerve and in the proximal and distal regions were comparable to the level of staining in the contralateral nerve (data not shown). Endoneurial tubes of degenerated axons and groups of Schwann cells were labelled with the anti-N-cadherin antibody together with blood vessels, 20 days after transection motor axons were regenerating and fascicles of neurites surrounded by Schwann cells were present in the distal stump (Daniloff et al., 1986) and were N-cadherin positive.

The distribution of N-cadherin was also examined in denervated and reinnervated gastrocnemius muscles (Fig. 6). 4 days after denervation, the extrajunctional sarcolemma was labelled by the rhodaminated α-bungarotoxin but the staining was still brightly focalized at synaptic sites (Fig. 6A) as expected (Bennett et al., 1973; Salpeter and Loring, 1985). N-cadherin staining was also observed on the extrasynaptic surface of the denervated adult muscle fibres (Fig. 6A'). However, the accumulations of N-cadherin at the neuromuscular junctions remained evident. From 4 days after neurotomy until 10 days, the relative level of N-cadherin staining increased in the extrasynaptic areas of the denervated muscle and remained elevated for up to 20 days. 20 days after denervation by crush, nerve fibres had regenerated and gastrocnemius muscles fibres were reinnervated as indirectly shown by the restricted pattern of synaptic α-bungarotoxin staining (Fig. 6B). N-cadherin immunoreactivity was drastically decreased on extrasynaptic surfaces and was restricted to the synaptic areas delineated by the α-bungarotoxin staining (Fig. 6B').

The ultrastructural localization of N-cadherin was determined by immunogold electron microscopy in denervated gastrocnemius muscle fibres (Fig. 7). N-cadherin-binding gold particles were found in association with the sarcolemma and the basal lamina of the denervated muscle fibres. They were also found in the extracellular space associated with collagen fibres. N-cadherin was present intracellularly, associated with vesicular structures. Some particles were also found in the cytoplasm and on the plasma membrane of interstitial cells and of mononucleated cells lying between the sarcolemma and the basal lamina of the muscle fibres which could be satellite cells (not shown). In the contralateral muscle, only a few gold particles were seen in association with the surface of the fibres.

**DISCUSSION**

We report here the spatiotemporal distribution of the Ca$^{2+}$-dependent cell adhesion molecule, N-cadherin, in the chicken
N-cadherin in neuromuscular system

and its modulation after peripheral nerve injury. Our main findings are as follows: (1) N-cadherin is expressed at the surface of myogenic cells in embryonic skeletal muscle during myogenesis. (2) It becomes restricted to the neuromuscular junction during the final stages of embryogenesis and remains present in adult life. (3) N-cadherin is reexpressed extrasynaptically on denervated skeletal muscle fibres and is located in the sarcolemma, the sarcoplasm and the basal lamina of the fibres. Muscle reinnervation restores a normal motor endplate restricted N-cadherin expression. (4) N-cadherin is strongly expressed in the peripheral nerve throughout myelination, it remains in adult life and its expression is only moderately changed during nerve regeneration. (5) In the adult, N-cadherin is abundant on myelinating Schwann cells and on the inner and outer surface of myelin sheaths but is preferentially accumulated at the node of Ranvier.

In agreement with previous studies (Hatta et al., 1987; Inuzuka et al., 1991; Hahn and Covault, 1992), we found that N-cadherin is expressed in developing hindlimb muscles. N-cadherin is strongly expressed in the limb muscle territories, at embryonic day 6 (stage 28-29, Hamburger and Hamilton, 1951). At this stage and until embryonic day 9 (stage 35), intense fusion of early myoblasts generates primary myotubes (McLennan, 1983). In vitro perturbation experiments have shown that N-cadherin-mediated adhesion is a critical step in the process of chicken myoblast fusion (Knudsen et al., 1990b; Mège et al., 1992a). The expression of N-cadherin and its accumulation at the areas of contact between myogenic cells during the phase of primary myogenesis supports the notion that this molecule is important for the fusion of primary myoblasts. The fact that N-cadherin expression was highly down-regulated at embryonic day 11, a stage when the fusion of late myoblasts generates the secondary myotubes (Feldman and Stockdale, 1992), suggests that N-cadherin-mediated adhesion is less important in vivo for secondary than for primary myogenesis. These observations reinforce the idea that N-cadherin is not the only cell adhesion molecule involved in myoblast fusion and that multiple cell adhesion molecules are each contributing to the regulation of myoblast-myoblast and myoblast-myotube adhesion necessary for fusion to occur. Along this line, two cell adhesion molecules, VLA-4 and V-CAM1 have been recently impli-

Fig. 5. Ultrastructural localization of N-cadherin in adult sciatic nerve. Transverse sections of frozen sciatic nerves were immunogold reacted with a postembedding method. (A) N-cadherin-binding gold particles were present on the external surface of the myelinated fibres as single particles or small aggregates associated with Schwann cell basal lamina (triangle) and collagen fibrils surrounding nerve fibres (open arrows). Particles were also observed in Schwann cell cytoplasm (arrow) and in the axolemma (lozenges). The presence of particles in axoplasm (squares) was not always observed. (B) Control sections treated only with gold-conjugated second antibodies were depleted in particles. (C) Higher magnification of a Schwann cell showing gold particles present on cytoplasm, plasma membrane and basal lamina, some particles were present in the extracellular space. Bars, 0.3 µm.
cated in secondary myogenesis in the mouse (Rosen et al., 1992). Moreover, at day E15, N-cadherin staining was restricted to very limited sites of myotube/myotube or myoblast/myotube contact, in agreement with the observations of Inuzuka and coworkers (1991) suggesting that the selective binding of N-cadherin and of other cell adhesion molecules may lead to selective fusion between specific yet unidentified types of myoblasts. To support the hypothesis further, it will be of particular interest to study the precise expression pattern of the other muscle cadherins (B-, R-, EP-, T-, M-cadherins) during muscle development.

During the course of embryonic development of chicken thigh muscle, the major ingrowth of nerve trunks in muscles occurs at embryonic day 6 (Dahm and Landmesser, 1988). Our results show that N-cadherin is abundantly expressed in muscle territories at this stage. Since, N-cadherin in vitro facilitates neurite outgrowth on myotubes (Bixby et al., 1987), this molecule is a possible candidate to mediate the ingrowth of nerve trunks in muscle territories. The first synapses appear at stage 27-28 (day E5-E6) and their number increases until stage 36 (day E10) where 70-90% of the clusters of acetylcholine receptor are colocalized with nerve terminals (Dahm and

Fig. 6. Localization of N-cadherin in denervated and reinnervated muscles. Cross-sections of the lateral gastrocnemius muscle were doubly stained with α-bungarotoxin (A,B) and anti-N-cadherin IgGs (A’,B’) either 4 days after transection of the sciatic nerve (A,A’) or 20 days after nerve crushing (B,B’). 4 days after nerve transection, the extrasynaptic expression of acetylcholine receptor was intense (A) as a result of denervation. All of the muscle fibres were N-cadherin positive; however synaptic accumulation of both molecules remained detectable (arrows in A’). 20 days after nerve crush, a normal acetylcholine receptor staining pattern was restored as a result of muscle reinnervation (B), N-cadherin staining was also restricted to newly rebuilt neuromuscular junctions (B’). Bars, 60 µm.
Landmesser, 1991). N-cadherin was down-regulated at embryonic day 11 (stage 37), by which time, most synapses had formed. N-cadherin might be one of the cues that facilitate and determine the establishment of the first nerve-muscle contacts. It may directly mediate the adhesion between the nerve terminal membrane and the sarcolemma during the initial phase of synapse formation while these membranes are only 10 nm distant from each other.

N-cadherin became concentrated at the neuromuscular junction only a few days after the establishment of the first nerve-muscle contact. According to ultrastructural and functional criteria (Hirano, 1967; Bennett and Petitgrew, 1974), at embryonic day 19 when the first synaptic accumulations of N-cadherin are detected, the synapse is already well differentiated with a thickened postsynaptic membrane, synaptic folds and basal lamina. However, the maturation of the neuromuscular junction continues until the second week post-hatching. Thus N-cadherin might be involved in the late maturation and stabilization of the adult neuromuscular junction. A number of cytoskeletal, membraneous and extracellular proteins, including CAMs of the immunoglobulin superfamily and cytokeratin are concentrated at the adult vertebrate neuromuscular junction (Rieger, 1990; Hall and Sanes, 1993). However, N-cadherin is the first cadherin to accumulate at the synapse. The very precise colocalization of N-cadherin and acetylcholine receptor observed at the confocal microscope and the persistence of N-cadherin accumulations at denervated endplates after degeneration and phagocytosis of nerve terminal suggest that N-cadherin might be associated preferentially with muscle post-synaptic membrane and basal lamina. It might be preferentially synthesized by myofibres since N-cadherin is expressed by myoblasts in culture as well as by embryonic myotubes or denervated muscle fibres in vivo.

N-cadherin expression was up-regulated in denervated skeletal muscle (Hahn and Covault, 1992). Nevertheless, the accumulation of N-cadherin at the synaptic sites remained detectable even after 20 days of denervation, just as for the synaptic accumulation of the acetylcholine receptor. Furthermore, N-cadherin expression and distribution returned to normal after reinnervation. Since, regenerating axons preferentially reinnervate the former synaptic sites (Miledi, 1960; Letinsky et al., 1976), the persistence of N-cadherin at these sites may act as a cue for directing the precise reinnervation of the denervated fibres at the former synaptic sites. Such roles have been proposed and evaluated previously for the cell adhesion molecule N-CAM (Rieger et al., 1988) and the substrate adhesion molecule, cytotactin (Mege et al., 1992b) by antibody perturbation of nerve regeneration and muscle reinnervation. The same approach would be necessary to elucidate the role of N-cadherin in muscle reinnervation.

The present work extends previous studies by providing the ultrastructural distribution of N-cadherin in denervated muscle. N-cadherin was found associated with collagen fibrils of the extracellular space and with basal lamina of denervated fibres as well as on the sarcolemma and in the sarcoplasm. The gold particles found in the sarcoplasm of the denervated fibres were very often associated with vesicular elements which might be elements of the endoplasmic reticulum or transport vesicles and might represent N-cadherin in the process of synthesis or transport toward the surface of the denervated fibre. The presence of N-cadherin bound gold particles in the endomysium, associated with basal lamina and collagen fibres suggests

Fig. 7. Ultrastructural localization of N-cadherin in denervated muscles. 4 days after denervation, sections of gastrocnemius muscle were immunogold reacted. (A) Gold particles were found in the sarcoplasm associated with vesicular elements (circles), and at the surface of muscle fibres, associated with the sarcolemma (arrow) and the basal lamina (arrowheads). In the extracellular space, they were observed in association with collagen fibres (triangles). (B) In control preparations, no particles were observed when primary antibodies were omitted. Bar, 0.2 µm.
that this molecule may be secreted by denervated muscle fibres. This subcellular localization constitutes the first evidence for the existence of a secreted form of N-cadherin in vivo. So far, only the T-cadherin is known to be anchored in the membrane by a glycosyl-phosphatidyl-inositol tail (Ranscht and Dours-Zimmerman, 1991) and thus could be released in the intercellular space by endogenous phospholipases. N-cadherin is thought to be expressed as a unique transmembrane isofrom. However, N-cadherin is very sensitive to proteolysis and, in cultured lens cells, a large extracellular domain of N-cadherin can be released from the plasma membrane by endogenous proteases (Volk et al., 1990). Thus, N-cadherin immunoreactivity found in the endomyosium could result from proteolytic cleavage of the extracellular domain of N-cadherin (Volk et al., 1990). N-cadherin is able to mediate both homophilic and heterophilic cadherin-cadherin binding (Volk et al., 1987; Inuzuka et al., 1991) and this extracellular domain may bind in the extracellular space with itself, with intact N-cadherin or with another truncated cadherin present in the muscle, such as T-cadherin. So far only cadherin-cadherin interactions have been reported. Nevertheless, our observations open the possibility that N-cadherin may interact with collagen or other components of the extracellular matrix such as fibronectin, heparan-sulfate proteoglycan and cytotactin, which are accumulated in the basal lamina of denervated muscles (Sanes et al., 1986).

In the peripheral nerve, N-cadherin was expressed during myelination, which occurs in chicken at hatching (Usson and Saxod, 1988). This molecule may mediate the interactions between Schwann cells and motor axons during myelination (Bixby et al., 1988; Letourneau et al., 1990). In myelinating fibres, N-cadherin was detected along the inner and outer surfaces of myelin sheaths and on the myelinating Schwann cell but was particularly accumulated at the node of Ranvier. In this respect, the distribution of N-cadherin is very different from that of N-CAM and Ng-CAM which are down-regulated in the peripheral nerve as myelination occurs (Rieger et al., 1986; Daniloff et al., 1989). N-cadherin is so far the only cell adhesion molecule to be expressed constitutively all along the myelinated fibres in the adult peripheral nerve. This observation suggests that N-cadherin could ensure the cohesion of myelin sheaths. The accumulation of N-cadherin at the node of Ranvier is the only common feature with N-CAM and Ng-CAM expression in the peripheral nerve and suggests that N-cadherin may also contribute to the stabilization of this specialized structure. At the ultrastructural level, N-cadherin was found in the cytoplasm of myelinating Schwann cells, suggesting that N-cadherin is synthesized by these cells. N-cadherin was also found in the basal lamina of the myelinating Schwann cells, extending our observations of basal lamina association of this molecule in denervated muscles. A fewer gold particles were also detected in the axoplasm of a few neurons, suggesting that N-cadherin may be expressed by some motoneurons.

After peripheral nerve injury, almost no change in the level of N-cadherin expression was noted. Nevertheless, N-cadherin might provide a permissive substratum for the regrowth and migration of regenerating motor nerve growth cones. Indeed, in vitro studies have demonstrated that N-cadherin is a potent neurite outgrowth substratum (Matsunaga et al., 1988). This ‘ready to use’ neurite outgrowth substratum might further be rendered increasingly attractive or repulsive to regenerating peripheral axons by the modulation of a panel of other attractive or repulsive adhesion molecules such as N-CAM, Ng-CAM and cytotactin, which are up-regulated after neurotomy (Rieger et al., 1986; Daniloff et al., 1989; Mége et al., 1992b). Furthermore, it is also possible that N-cadherin expression in these cells could favor the adhesion on adjacent Schwann cells to constitute the bands of Büngner, in which the growth cones migrate toward their target. In conclusion, the results presented here lead to the proposal that N-cadherin might play a role at different steps of the development and regeneration of the neuromuscular system.

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