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INTEGRINS DURING MUSCLE DEVELOPMENT AND IN MUSCULAR DYSTROPHIES

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1. ABSTRACT

Cellular interactions with the extracellular matrix (ECM) have been shown to be important for a number of developmental events from the time of fertilization up till the maturation of the organism. In the following review we will discuss what is currently known about these interactions with special emphasis on the role of integrins during the formation of skeletal muscle. The importance of cell-ECM interactions will also be illustrated by a discussion of what happens when these interactions go awry, as happens in muscular dystrophies.

2. *IN VIVO* MYOGENESIS

2.1. Early vertebrate myogenesis

Vertebrate myogenesis involves a number of steps controlled by a variety of signals and is ultimately orchestrated by regulatory events at the gene level in muscle cells. Unlike many other cell types in the body the muscle fiber is multinuclear and there is evidence that transcriptional activity varies between nuclei within the muscle fiber (1-3).

The current knowledge about the complex inductive extracellular signals as well as the ensuing transcriptional events during myogenesis have been the subject of recent reviews (4-6). We will try and put some of this knowledge into the context of cellular interactions with the extracellular matrix (ECM). During vertebrate myogenesis the paraxial mesoderm becomes segmented into epithelial ball like structures named somites. Somites differentiate into sclerotome and dermomyotome. The dermomyotome will develop further into the dermatome and the myotome. With the exception of some head muscles, skeletal muscles arise from the dermomyotome. Axial muscles are derived from different regions in the myotome whereas appendicular muscles arise from a cell population in the ventrolateral part of the dermomyotome (see figure 1). The molecular cues governing these early steps of myogenensis are currently subject to intense investigation (reviewed in (7, 8). Much of these studies are currently performed in mouse. Axial structures such as the neural tube and the notochord are needed for formation of epaxial muscles (deep back and intercostal muscles) while cues from surface ectoderm and lateral mesoderm are needed for hypaxial (trunk) muscle formation. Soluble molecules that act very early in myogenesis include Wnts, sonic hedgehog and bone morphogenetic protein-4 (reviewed in (6)). The transcription factor pax-3 is needed for the formation of both axial and appendicular muscles, but not for head muscle development (9). Myogenic regulatory factors (MRFs) are transcription factors of the bHLH class and contain the members MyoD, Myf-5, myogenin and MRF-4 (10). Specific MRF members are expressed in different regions of the differentiating myotome. In the dorsal part of the myotome,which gives rise to epaxial muscles, Myf-5 is an early orchestrator of determination events. In the more ventral part MyoD appears to play an important role for hypaxial muscle formation (6, 8, 10). At these early steps a number of cell migration events occur. In Myf-5 defective embryos, myotomal cells migrate and position themselves abnormally (11). Committed myogenic cells in the ventrolateral tip of the somite which do not yet express MRFs express the c-Met receptor during the migration into the limbs (12). c-Met is a tyrosine kinase receptor for scatter factor. Recent *in vitro* data have indicated that in myoblasts an autocrine loop is active for scatter factor (13) and that the c-Met receptor signals via Grb-2 in the myogenic lineage (14). Whether an autocrine loop is also operative during myogenic migration at this step *in vivo* is not known. When the somite-derived cells have stopped migrating they start expressing MRFs and now can be identified as myoblasts. Axial and appendicular myoblast that undergo the first stage of differentiation are said to undergo primary myogenesis (15). Using the primary myotubes as a scaffold, a distinct myoblasts population-

the secondary myoblasts - line up under the basement membrane of primary myotubes, fuse with each other and form secondary myotubes (see figure 1). Subsequent to these early events a number of important reorganisation events need to take place in order to achieve the final muscle pattern. Secondary muscle fibers form an independent basement membrane. Muscles split, become innervated, achieve their final pattern and grow. At the early times of muscle development the myotube end points are thought to be important for muscle splitting and muscle growth (1, 16). Adult muscle fibers have an elaborate system for force transmission, mainly via the specialized endpoints at attachments to tendons, called myotendinous junctions.

Unlike cardiac muscle, skeletal muscle has the capacity to regenerate in response to injury. The basis for this capacity is laid down during fetal development by a distinct cell population called satellite cells. This population of myoblasts will normally not differentiate. In the case of muscle injury, part of these cells will remain as stem cells whereas others will differentiate into new muscle fibers. Recently FGF-6 and MyoD have been shown to be important for proper satellite cell activation in response to injury (17, 18).

Events during myogenesis suggested to depend on cell-ECM interactions include: somite formation, cell determination events, early cell migratory events, myogenic differentiation, basement membrane assembly, muscle positioning, myotube alignment, muscle splitting, innervation and, muscle fiber stability. With modern molecular biology tools, essentially all these events can be studied *in vivo*.

2.2. Lessons from *Drosophila* myogenesis

Drosophila has served as a useful model for studies of several complex developmental processes. No endoskeleton exists in invertebrates and the somatic musculature is used for larval crawling and later in adult insect jumping and flight. Despite the different anatomical considerations the conservation of basic molecular mechanisms during muscle formation is striking (19, 20). Recently several mutations in *Drosophila* have shed light on the complexity of the process of muscle formation (19, 21, 22). A number of mutations inactivating transcription factors, signaling molecules and cell membrane proteins, all affect muscle formation. For some of the identified proteins, corresponding orthologues have not yet been found in vertebrates. For others orthologues exist, but their involvement in vertebrate myogenesis is not clear. For those inductive mechanisms where the pathways start to pan out the emerging pattern suggest that conserved genes are used in different ways in invertebrates and vertebrates. As pointed out by Baylies *et al* (19) this might prompt us to look at vertebrate myogenesis with "new eyes" - maybe things are not what they appear to be. *Drosophila* will continue to be a valuable system to study fundamental molecular aspects of myogenesis.

3. *IN VITRO* MYOGENESIS

The formation of a syncytial myotube from individual myoblasts is a fascinating process which has attracted the interest of biologists ever since methods to culture myogenic cells became available in the 1950´s. Due to the easily observed overt changes occurring as a result of myogenic differentiation, *in vitro* myogenesis has become a popular system to study cell differentiation. As with all *in vitro* studies one must be aware of that these studies are subject to a number of limitations. When studying the differentiation of myogenic cells of a certain developmental stage into myotubes, a number of differences compared with the *in vivo* situation have to be taken into account. In the *in vitro* system, the substratum is artificial, nerves are absent, the cells grow on a planar substratum in the absence of stromal cells and the levels of growth factors are normally not reflecting the levels at the developmental stage where the process occurs *in vivo*. The finely tuned network of signaling pathways in operation *in vivo* are thus absent. Despite this, some robust programs associated with the differentiation process can still occur. The *in vitro* system has been very informative in identifying transcription factors intimately linked with the myogenic differentiation process. Thus ectopic expression of all four MRFs has been shown to be able to induce myogenic differentiation *in vitro* (reviewed in (23). Recently the use of embryonic stem cells for the generation of embryoid bodies has allowed for the study of a number of differentiation events under more *in vivo* -like culture conditions. Whereas differentiated cells will aggregate into small functional units, complex organ-like structures do not form in the embryoid bodies (24).

Recent results from myogenin-, vinculin-, and desmin-defective cells serve as good examples of differences observed between *in vitro* and *in vivo* conditions. In the case of myogenin, myoblasts from knock-out (KO) mice deficient in myogenin *in vivo* display impaired differentiation, i.e. myoblast fail to fuse into myotubes (25). When these myoblast are isolated and cultured *in vitro*, they readily fuse into myotubes. One interpretation from these results is that negative modulatory signals that prevent up-regulation of other MRFs such as MyoD are lacking *in vitro*, allowing for successful compensation.

Studies with vinculin-defective cells have indicated that while vinculin-defective ES cells *in vitro* can differentiate into many cell types including myotubes and beating cardiomyocytes, embryos lacking vinculin die early, probably due to impaired heart development (26). Interestingly, somites and limb buds are also severely defective in these embryos. The example with vinculin clearly illustrates the complexity of developmental events in a embryo setting.

In yet another series of experiments blockage of desmin expression in somatic myogenic cells by anti-sense strategy (27) and studies of myogenic differentiation of ES cells where the desmin gene had been inactivated (28), both indicated a role for desmin in myogenic differentiation, i.e both these strategies prevented myotube formation. However, in transgenic mice lacking functional desmin, myogenic differentiation is normal and the absence of desmin causes a muscular myopathy postnatally (29). One interpretation from the conflicting *in vitro* and

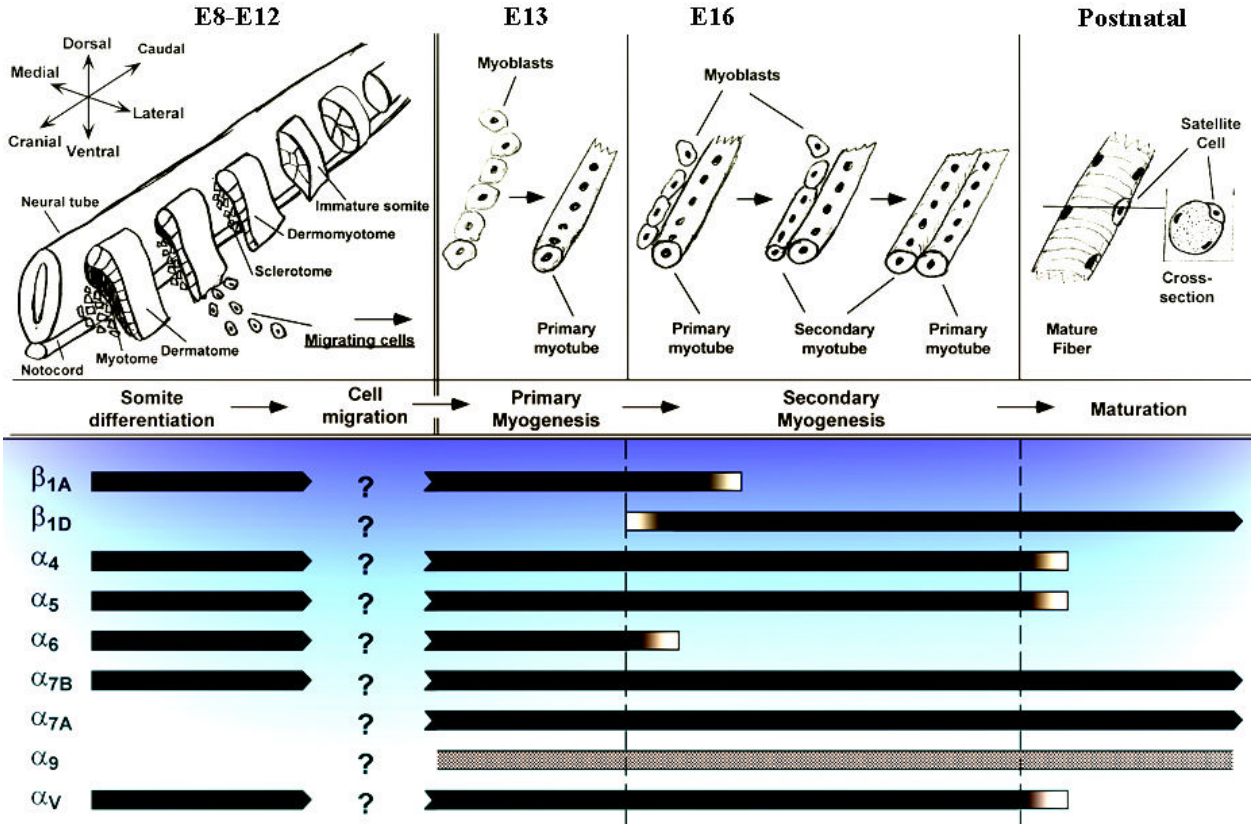

Figure 1. Schematic representation of integrin expression during different steps of myogenesis in mouse. Schematic representation of myogenesis: Skeletal muscle originate from two regions in the differentiating somite. Axial muscle form from the myotome, while appendicular muscle arise from the ventrolateral tip of the dermomyotome. At the level of the forelimb and hindlimb cells migrate out to form limb muscle. In the schematic representation of muscle formation only maturation of appendicular muscle is shown. Except for the type of cell migration observed for limb muscle, axial muscle form in similar steps. Integrin expression: Data on integrin expression during mouse muscle development has been summarized. At the somite stage, staining usually refers to myotome staining, i.e staining in the cells destined to become axial muscle.

in vivo results is again that different compensatory mechanisms are operating. One candidate compensatory molecule for desmin in the *in vivo* situation is nestin (30). These differences between *in vivo* and *in vitro* systems probably also extends to cell-ECM interactions and most likely explain some of the contradictory data observed in this field. The *in vitro* system can be used to study the influence of cell-ECM interactions on: cell growth, cell-cell recognition, cell fusion, cell differentiation, sarcomere formation and sarcomere stability.

It is thus important to keep in mind that myogenesis *in vivo* is a complex process with particular features depending on whether embryonic, fetal or adult myoblasts are involved. Therefore generalizations should be avoided since developmental stage-specific mechanisms can be at play. The limitations with the *in vitro* systems include difficulties to asses the importance of early steps of myogenesis such as somite formation, early cell migration and muscle positioning. Furthermore, systems that evaluate the importance of these interactions for mature myofiber stability, remains to be developed.

4. INTEGRINS DURING MYOGENESIS

As already mentioned the formation of a multinucleated muscle fibers is an extraordinary dynamic process involving multiple cell adhesive interactions consisting of homotypic and heterotypic cell-cell interactions as well as interactions with ECM molecules. *In vivo* these interactions are finely tuned to allow for myotube formation, alignment of myotubes and maturation of muscle fibers into different muscle groups. As integrins became prime candidates for fundamental receptors involved in cell-adhesive interactions the presence of integrins on muscle cells (31), was recognized. Early studies of integrins on myogenic cells revealed that beta1 integrins localized to focal contacts, costameres, neuromuscular junctions and myotendinous junctions (32). Following these initial findings, a number of integrin alpha chains have been identified at these sites, but the precise role of integrin heterodimers in adult muscle is still not fully understood. Antibody perturbation data have indicated a role for integrins in anchorage of muscle cells the ECM, cell migration from the somite, control of

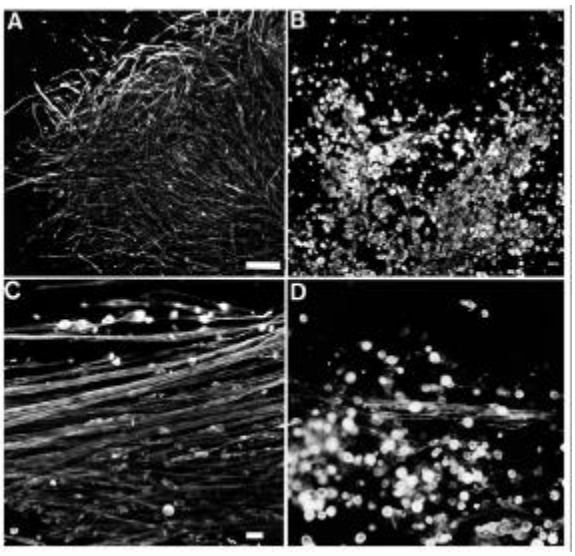

Figure 2. Myogenic differentiation into myotubes in wild type (A, C) and integrin beta1-null embryoid bodies (B, D). Normal and beta1-null ES cells were aggregated in hanging drops for 2 days, grown in suspension for 8 more days, plated for 5 days (A, B) or 15 days (C, D) on gelatin-coated glass cover slips and stained for expression of skeletal myosin heavy chain. Bar indicates 20 µm.

proliferation, cell-cell recognition preceding myoblast fusion and myogenic differentiation. Most of these collected data have been summarized in previous reviews (33-36). The expression of various integrins during different steps in mouse myogenesis is summarized in figure 1.

As a first step in analyzing the role of integrins during a certain developmental stage it is important to establish the total integrin repertoire. However, a number of factors have to be taken into consideration when analyzing the integrin repertoire on muscle cells. For the analysis of the integrin repertoire *in vivo* the heterogeneity of muscle fibers is important to take into consideration. An observed set of integrins might not distribute evenly on all types of muscle fibers in different muscle groups of different embryonic origins. Regarding *in vitro* analysis, one major consideration is that culturing myogenic cells (like many other cells) on a planar substratum in the presence of serum, changes the levels of a whole spectrum of molecules involved in cell - ECM interactions. For example, cultures of primary cells isolated from an embryo at a certain developmental stage up-regulate a number of integrin receptors not detectable at that stage *in vivo* (37). Secondly, *in vitro* studies have revealed that a number of integrins display expression patterns correlated to the differentiation state. Whereas myoblasts generally possess an interstitial matrix rich in fibronectin, myotubes lack a fibronectin matrix and instead deposit a basement membrane-like matrix. The switch in matrix is associated with a switch in integrin repertoire. A major subfamily of integrins present on myogenic cells possess the beta1 chain, capable of associating with at least 11 alpha-chains ((38) and references therein). Only a subset of possible integrin heterodimers are present on myogenic cells during different stages of myogenesis. Myoblasts *in vitro* express the alpha5beta1 integrin. The disapperance of a fibronectin matrix on avian myogenic cells during *in vitro* differentiation is reflected by a functional downregulation of alpha5beta1 ligand binding which is followed by downregulation of alpha5beta1 synthesis (39). In human muscle cells the downregulation of alpha5beta1 has been shown to be uncoupled from the differentiation event itself (40). Another integrin subfamily present on myogenic cells share the alpha v chain (37, 41). During myogenic differentiation *in vitro* the integrin beta-chain associated the with alpha v chain changes from beta3 to another, yet unidentified beta-chain (40). We previously showed that the beta5 chain appeared with time in myogenic culture suggesting this integrin beta chain as a candidate chain involved in the switch (37). Whereas loss of alpha5beta1 integrin clearly participates in loss of fibronectin matrix from the myotube surface, the functional significance of the beta-chain switch associated with the alpha v integrin chain is unknown. Recent experiments have indicated that different integrins signal through different pathways, which in turn influence proliferation versus differentiation (42). It is tempting to speculate that the integrin beta-chain switch affects intracellular signaling of the alpha v integrin heterodimer.

With the formation of a basement membrane a number of changes in the integrin repertoire occur. The laminin binding integrin alpha7beta1 is upregulated during myogenic differentiation (43). We have identified an integrin, tentatively named alpha mt, which is up-regulated during differentiation of human fetal myoblasts (44). This integrin is a candidate integrin to take part in basement membrane binding or assembly.

Another mechanism whereby the integrin repertoire changes during differentiation process is alternative splicing. During *in vitro* differentiation the beta1 integrin chain changes RNA splicing pattern from the beta1A form to the beta1D form (45). *In vitro* beta1D integrins in myotubes localize to Z-bands and muscle end points, which has prompted a great interest in the function of this integrin splice variant. During *in vitro* differentiation the alpha7 integrin splicing changes from the alpha7B form to the muscle specific alpha7A form (46) and there is an increased heterodimerization between alpha7 and beta1D. The extracellular domain of the alpha7 integrin chain is also subject to alternative splicing, generating two forms named X1 and X2 (47). These two splice variants have been shown to display different affinities for laminin-1. The functional significance of splicing events for myotube function is currently not fully understood.

In order to try and dissect out the different role of integrins during the various steps of myogenesis we have divided their functions into roles as:
4.1. cell-cell receptors,
4.2. migration receptors,
4.3. cell signaling receptors, and
4.4. mechanical links.
It is obvious that these roles are often overlapping, and this classification is merely used to present available data.

4.1. Integrins as cell-cell receptors

During early stages of myogenesis a number of cell-cell contacts are formed and disrupted. The paraxial mesoderm undergoes a mesodermal-epithelial transformation as the epithelial somite forms. As somites differentiate further the epithelial myotome will give rise to axial muscles and from the epithelial dermomyotome migratory cells arise by an epithelial-mesodermal transformation. It is likely that the integrin repertoire will change during these early changes in cell-cell interactions which also expose cells to new types of ECM.

At later stages cell-cell interactions are of prime importance for myoblast recognition leading up to fusion. Molecules implied in myogenic fusion include N-CAM (48-50), N-cadherin (49, 51-54) and M-cadherin (55-57). R-cadherin is also expressed in the myogenic lineage (58) but only M-cadherin is muscle specific during embryogenesis (59). Early *in vitro* studies indicated that antibodies to beta1 integrins inhibited myotube formation (60). Several years later studies in mice suggested that the integrin alpha4beta1 (61) was involved in a heterotypic myoblast - myotube interaction during secondary myogenesis. It is possible that the effect observed in early studies was attributable to a disturbance of the alpha4beta1 interaction with VCAM-1. However, myoblasts genetically engineered to lack integrin alpha4 still fuse *in vitro*, arguing against an essential role of alpha4 integrin in myoblast fusion (62). Furthermore, in chimeric mice with a high percentage of alpha4 integrin negative cells contributing to the myogenic lineage, secondary myogenesis takes place (62). Presumably, cell-cell recognition preceding fusion is a finely tuned mechanism involving multiple receptors and, depending on the assaying system, the effect of disturbances in one receptor system will give different results. Recent *in vitro* studies of beta1 integrin negative ES cells differentiating into muscle in embryoid bodies, and of beta1 integrin negative satellite cells isolated from chimeric embryos, have shown that myoblasts lacking beta1 integrins can fuse and form myotubes (36). However, the results are still somewhat difficult to interpret. In embryoid bodies devoid of beta1 integrins, differentiation was delayed (36) and a large number of unfused myogenic cells were observed (figure 2). It is possible that this does indeed reflect a role for integrins in the fusion process.

More recently disintegrins belonging to the ADAM family (63) have been implicated in myogenic fusion (64). It is possible that the counter-receptor for disintegrins is an integrin. The observation that ADAMs in a secreted form can stimulate myogenesis of nearby cells indicate a new role for ADAMs in myogenesis (65).

4.2. Integrins as migration receptors

Very little is known about the receptor repertoire of the myogenic cells that migrate out from the dermomyotome. Part of the difficulty in characterizing these cells lies in the lack of good immunological markers specific for migratory myogenic precursor cells. c-Met and pax-3 are expressed in these cells and in situ hybridization with probes to the mRNA for these genes has been used to mark the migratory cells. We have recently obtained a transgenic mice in which the lacZ gene has been inserted randomly (generated by Alexander Faerman, Volcani Center, Israel). The lacZ gene has integrated to an unknown locus and is active in the migrating cells leaving the somite. We are currently using this mouse strain to characterize the integrin repertoire on migrating myogenic cells. Early antibody perturbations in avian embryos indicated a role for beta1 integrins in cell migration from the somite (66). Another antibody-perturbation study has indicated that N-cadherin on migrating myogenic cells interacts with N-cadherin on mesenchymal cells to facilitate migration (51). Both N-cadherin (54) and beta1 integrin (67) KO embryos die relatively early during embryogenesis. Chimeric embryos of wild type cells and beta1 integrin-negative cells, form muscle fibers into which beta1 integrin-negative myoblasts have fused, indicating that cell migration from the somite has occurred in the absence of beta1 integrin. However, it is hard to evaluate the role of integrins for the migration event in a wild type background. Fässler *et al* have proposed a backpack model where KO cells are passively carried by wild type cells (35). In the future, selective inactivation of integrins during different stages of development in the myogenic lineage is likely to be informative in this regard. Little attention has been paid to the integrin alpha chain repertoire of migrating myogenic cells. Distribution studies in developing avian embryos indicate alpha6 expression on what appear to be migrating myogenic cells (68). Another interesting candidate integrin is the alpha7beta1 integrin which has been shown to be involved in cell migration on laminin (69). The alpha7 chain displays an intricate alternative splicing-dependent affinity modulation in the extracellular domain, which could serve a role during migration (47). Other candidate integrins involved in myogenic cell migration remains to be identified.

4.3. Integrins as signaling receptors

The concept of integrins as signaling receptors is well documented and it is becoming increasingly clear that integrins also during myogenesis play such a role. Analysis of transcription factors during myogenesis has led to an emerging picture where proliferation, fusion and differentiation are uncoupled events. Recent elegant studies with avian cells have indicated that overexpression of alpha5 integrin promotes proliferation whereas overexpression of alpha6 integrin promotes differentiation (42). It is suggested that these integrins use different signaling pathways to influence proliferation and differentiation. Further studies have indicated a cross-talk between integrins and cadherins in myogenic cells which is dependent on integrin signaling pathways (70). As mentioned earlier, a delay in the differentiation as well as an increased number of unfused cells was noted for beta1 integrin-defective myoblasts (36). Whether this reflects an effect of the balance between cell proliferation and differentiation, altered cadherin levels, or yet something else, remains an open issue requiring further investigation.

4.4. Integrins as mechanical links

Muscle progenitors first encounter an ECM in the somite. As somite forms a transient gradient of fibronectin in the forming somites is established (71). In the absence of fibronectin somites do not form (72). If this

reflects a defect already in the paraxial mesoderm stage or during the somitogenesis step is not clear. The receptors mediating the interaction with fibronectin before and during somitogenesis have not been identified. Of fibronectin binding integrins alpha4beta1 integrin is present in somites, as are alpha5beta1 and alpha v integrins (41, 73, 74). Inactivation of the alpha5 integrin gene impairs posterior somite development - identifying an indirect or direct role for alpha5beta1 in early muscle development (73).

The somite is surrounded by a basement membrane rich in the laminin isoform laminin-1 (75). The expression pattern of other laminin isoforms in the somite has not been described and knock-out mice has not yet been analyzed for somite defects due to laminin deficits. Of the laminin binding integrins alpha1beta1 (76) and alpha6beta1 (68) have been described in the avian somite, and alpha6beta1 is also prominent in mouse somites (45). We have shown that alpha7Bbeta1 is present in the mouse myotome and is concentrated at intersomitic boundaries (77). Thus, already at this step alpha7beta1 localizes to a junctional site, reminiscent of the later localization to myotendinous junction. However, in mouse embryos lacking either one of these laminin-binding integrins, muscle development is not affected. It will be a challenging task to sort out the role of different laminin isoforms and laminin-binding integrins during somitogenesis.

Studies in the late 80´s of the embryonic lethal *Drosophila* mutant myospheroid (*mys*) showed that muscle cells lacking the invertebrate homologue of beta1 integrins, betaPS integrin, formed myotubes *in vitro*, but that sarcomere integrity was impaired (78). In the *mys* mutant embryos muscles formed normally, but muscle cells came loose from their attachment points at the time of the first muscle contractions. The *in vitro* experiments were not designed to resolve whether integrins were involved in the formation of sarcomeres or in sarcomere stabilisation after formation. In an extensive genetic study of different mutants defective in the alphaPS2 integrin gene, evidence is presented for separate functions of alphaPS2 in adhesion, sarcomere integrity and morphogenetic events (79). Interestingly, in some mutants where somatic muscle sarcomere integrity was affected, somatic muscle attachments were still intact, supporting a role for alphaPS2 in sarcomere formation. Sarcomeric arrangement in visceral muscle, which in *Drosophila* is striated, was not affected in any of the alphaPS2 mutants. Other experiments in *Drosophila* have surprisingly revealed that the localisation of integrins to muscle endpoints is driven from within the muscle cell and not by binding to pre-localised ECM ligands (32). In experiments performed by Martin-Bermudo and Brown the cytoplasmic domain of the *Drosophila* integrin beta chain (betaPS integrin) was expressed as a chimeric protein with a non-integrin extracellular part and it was thus unable to bind extracellular ECM ligands. When this chimeric molecule was expressed in flies lacking the betaPS chain the chimeric molecule was still able to localize to muscle endpoints. Thus, the signals for integrin polarization comes from within the cell in *Drosophila* muscle and represent a type of inside-out signaling. It will be interesting to investigate in more detail the mechanism for this inside-out signaling. This invertebrate finding should also prompt similar analyses in vertebrate cells (chimeric molecules expressed in cells lacking integrin beta chains). Such experiments might indicate that inside-out mechanism are more common for integrin function in muscle than previously thought. Two comparative studies in *Drosophila* have tried to resolve if the two integrin alpha chains alphaPS1 and alphaPS2 have different functions during development (80, 81). From these experiments where *Drosophila* integrin mutants were rescued with various integrin chimeric molecules, a complex pictures emerges where separate integrins do have distinct roles in different organs and that this specificity resides in the extracellular part (81). In somatic muscle alphaPS1 integrin chain could not substitute for alphaPS2 whereas in the eye this was clearly the case. The suggestion is that alphaPS2 is needed for strong adhesion. Whereas alphaPS1 in the rescue experiments localized to muscle attachment sites, and most likely bound its ligand, this adhesion is apparently not strong enough, which was manifested as breakage of muscle attachments.

Vertebrates studies with rat cardiomyocytes have indicated that the localization of beta1 integrins to costameres occurs well after sarcomere formation, arguing against a role in sarcomere formation (82). Experiments with mouse cells lacking beta1 integrins have shown that cardiomyocytes in embryoid bodies have an impaired sarcomere cytoarchitecture (83). In contrast, no obvious defect was seen in skeletal myotubes formed under similar conditions (36). These data suggest that different compensatory mechanisms exists in cardiomyocytes and skeletal myotubes.

In summary, data from *Drosophila* indicate that betaPS integrins could be involved in both sarcomere formation and stabilization in a subset of striated muscle whereas in vertebrates, a limited role for beta1 integrin in sarcomere stabilization in a subset of striated muscle, seems more likely.

Following the identification of the *Drosophila mys* mutation as an integrin defect in 1988 (84) the inactivation of the mouse alpha7 integrin gene in 1997 (85) revealed a muscle phenotype with striking similarities to the *mys* mutation. An embryonic lethal phenotype was noted. The alpha7 integrin deficient mice that survived, showed a normal muscle development but did develop a muscular dystrophy postnatally, mainly affecting muscle attachment points in certain muscles.

Recent knock-out and knock-in experiments with the beta1D integrin, have shed light on the role of this integrin splice variant (86). Analysis of the highly cell selective regulated splicing event and the prominent localization to sites of force transmission had indicated a potentially important role for beta1D integrin variant in striated muscle at sites of transmission of force. The result from the knock-out experiments cast serious doubt on

these expectations. Both skeletal muscle and cardiac development were normal. The main heterodimerizing partner of the beta1D integrin chain, alpha7 integrin chain, was found to localize to myotendinous junctions and intercalated discs in the absence of beta1D, arguing against a unique role for beta1D in the localisation to these sites, since beta1A seems to be able to equally well fulfill this role. Careful analysis however revealed a mild defect in heart. The authors suggest that based on these results beta1D might be a poorer mechanotransducer than beta1A. The 13 amino acids that distinguish beta1A from beta1D clearly have specific roles during development as shown in the exon-specific knock in experiment that replaced beta1A with beta1D during development. A lethal phenotype resulted, mainly affecting the nervous system. Based on *in vitro* analysis of beta1D expressing cells a reduced expression of the beta1 integrin chain and reduced cell migration was noted. The authors suggest that it is mainly the reduced migratory capacity of cells containing the beta1D chain, instead of the normally occuring beta1A chain, that impairs development.

In summary, antibody perturbation experiments and genetic perturbations have produced different results with regard to the importance of integrins for muscle formation. Although the contribution of specific integrins to certain steps of myogenesis such as somite differentiation and myogenic cell migration has not been clarified, gene ablation experiments indicate a minor role for beta1 integrins during early steps of myogenesis (35). In the chimeric mice, myogenesis occurs in a wild type background, making these results difficult to interpret. That the chimeric model does indeed "miss" some phenotypes is illustrated by the alpha7 integrin knock-out mice. As mentioned previously these mice develop muscular dystrophy (85) indicating a role for integrins in myofiber stability. Yet this is not observed in the chimeric mice lacking all beta1 integrins on some cells. This is obviosly due to the fact that skeletal muscle is syncytial in nature and the beta1 integrin defective cells will fuse with other cells containing wild type nuclei. It is thus too early to rule a role for beta1 integrins in early steps of myogenesis. That alpha7beta1 is important for myofiber stability is clear. It is also clear that that myoblast fusion can occur in the absence of beta1 integrin. However, the large number of non-proliferating, non-apoptotic unfused myoblasts found in embryoid bodies lacking beta1 integrin indictate a, possibly indirect, role for beta1 integrins in fusion, or the steps leading up to fusion.

Cell-ECM interactions are involved in many of the processes of myogenesis. Whether integrins other than beta1 integrins or non-integrins are the primary receptors involved in these processes is an exiting area for future research.

5. MUSCULAR DYSTROPHIES

In recent years much knowledge has been gained in the field of the causes of muscular dystrophies/myopathies. A large body of evidence now point to the fact that many myopathies are caused by defects in the link between the muscle cell interior and the surrounding basement membrane (87, 88). Molecules currently known to be involved in this link include collagen type VI, laminin alpha2 chain, components of the dystroglycan associated glycoprotein complex, alpha7 integrin chain and dystrophin. It is unclear if the recently identified protein fukutin, defective in Fukuyama muscular dystrophy (89), is also part of this linkage. In muscular dystrophies a primary defect causes muscle necrosis, regeneration and a progressive degeneration of the muscle tissue. Much of the data on secondary events in muscle disease have been obtained through the studies of Duchenne Muscular Dystrophy patients and mdx mice (both lacking dystrophin in skeletal muscle). Two competing situations in the diseased muscle will be discussed: muscle regeneration, and fibrosis.

5.1. Muscle regeneration

In the diseased muscle satellite cells are activated, they replicate, migrate on basement membranes and fuse to form new muscle fibers (90). Different growth factors and cytokines such as FGFs (reviewed in (18)), TGF-betas (91) and HGF/SF (92) released from muscle cells and invading inflammatory cells are thought to be of importance for generating this response. Activated satellite cells express MyoD at an early stage (93, 94). MyoD-deficient (17) and FGF-6-deficient (18) mice are severely deficient in their muscle regeneration capacity. This appears to be due to increased muscle stem cell renewal and reduced satellite cell differentiation. In FGF-6 deficient mice satellite cells form normally, but upon injury their activation to proliferating cells fail (18). This results in fibrosis and myotube degeneration.

In a moderate injury the satellite cells use the basement membranes of necrotic fibers as a scaffold to ensure a similar position of the new muscle fibers. Likewise, components in the basement membrane during "normal" muscle damage guide the formation of neuromuscular junctions. As a late step in muscle regeneration the basement membrane of the necrotic fiber is phagocytized. In muscular dystrophy the response to injury is different. The invasion of inflammatory cells is much greater and the basement membranes of necrotic muscle fibers are removed before they have had a chance to act as scaffolds for the generation of new muscle fibers and to guide innervation. The result is a muscle tissue with abnormal muscle fiber arrangement.

5.2. Fibrosis

The growth factors and cytokines released in a dystrophic muscle also stimulate the proliferation of fibroblasts which with time will produce an accumulating fibrotic interstitial extracellular matrix (91). Fibrosis is a major obstacle to all types of gene therapy in muscle disease.

A schematic view of the involvement of fibrosis and muscle regeneration in the pathology of muscular dystrophy is shown in **figure 3**.

5.3. Integrins as cell-cell receptors

During the regeneration event the satellite cells go through a reiteration of a differentiation program with certain features similar to secondary

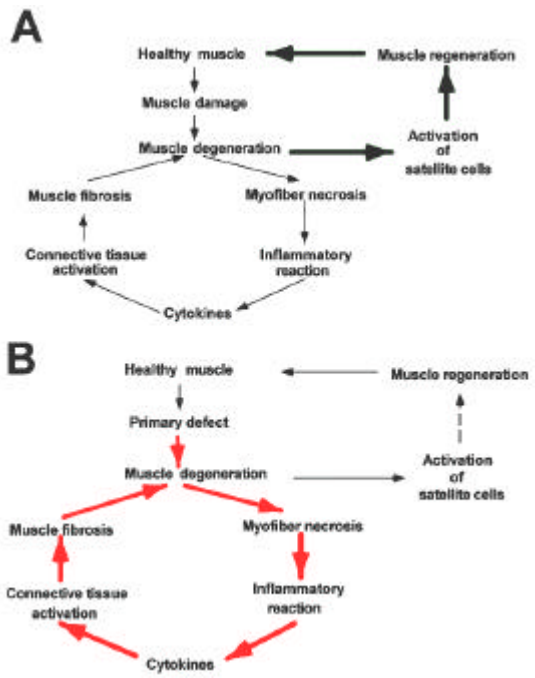

Figure 3. Schematic illustration of the relationship between fibrosis and regeneration in a moderate muscle injury versus muscular dystrophy. A. In a healthy muscle, muscle fiber damage leads to a moderate activation of connective tissue cells. Satellite cells are activated and replace the damaged muscle with new muscle fibers. B. In a dystrophic muscle, the primary defect leads to continous myofiber degeneration which leads to massive activation of connective tissue cells, resulting in fibrosis. Muscle regeneration is unable to replace the damaged muscle with new muscle fibers.

myogenesis. The same cell-cell recognition molecules as those used during fetal myogenesis are likely to be used at this step. Thus, VCAM-1 is expressed on satellite cells and regenerating muscle fibers (95). It has been suggested that this VCAM-1 expression enables alpha4beta1 integrin positive leukocyes to adhere to muscle cells. Release of cytokines from recruited leukocytes could lead to activation of HGF/SF release, further augmenting the regeneration process.

5.4. Integrins as migration receptors

It is likely that integrins play an important role for cell migration during satellite cell migration. The mechanism used for cell migration in these steps is largely unknown. One candidate receptor of importance for this step is the alpha7beta1 integrin (69).

5.5. Integrins as signaling receptors

Little is known about the role of integrins during activation of satellite cells during regeneration events. A number of restriction points exist during satellite cell activation. Some satellite cells are responsible for self-renewal of the stem cell population whereas others differentiate to new muscle fibers. The recent findings of cross-talk between integrins and cadherins in muscle cell lines indicate the possibility that synergistic signals from integrins and cadherins play a role (70). During fibrosis integrins on fibroblasts are likely to regulate matrix synthesis and to be involved in matrix assembly.

5.6. Integrins as mechanical links

At least two receptor systems for basement membranes in muscle have been identified. The dystroglycan complex and the alpha7beta1 integrin both bind laminins. It is notable that no receptor for collagen IV has been identified in skeletal muscle. In the dy/dy mice and in mersoin-negative congenital muscular dystrophies laminin alpha2 chain is defective. Since laminin alpha2 chain is not only present around muscle cells but is also expressed in the nervous system it is unclear how the lack of laminin alpha2 in the nervous system contributes to the disease phenotype. Electron microscopy analysis of basement membranes in laminin alpha2 defective patients has revealed intact basement membranes on Schwann cells (96). Immunostochemical analysis however reveal strongly reduced laminin alpha2 immunoreactivity on peripheral nerves in dy/dy mice (97). Finally, the restoration of laminin alpha2 in muscle of laminin alpha2 defective mice does not revert the complete disease phenotype, supporting an neurological component in disease caused by laminin alpha2 defects (98).

Two recent studies have yielded somewhat conflicting results on whether the alpha7beta1 and dystroglycan complex regulate each other in disease situations in the muscle. From immunohistochemical analysis of mice and men deficient in laminin alpha2 by the group of Engvall (99) two implications arise:

1. the distribution of dystroglycan complex and beta1D integrins are separately regulated,
2. laminin alpha2 containing laminins are the major ligands for alpha7beta1 integrins.

These data also raise some intriguing questions. Why is not dystroglycan distribution changed in the absence of laminin alpha2 chain? Is the dystroglycan complex so firmly anchored from within the cell that changes in the ligand distribution goes by unnoticed? Alternatively, can the laminin isoforms that are up-regulated in dystrophic muscle serve as efficient ligands as laminin-2, or are there non-laminin ligands for dystroglycan? Conversely, do these data indicate that the laminin isoforms induced in dystrophic muscle for some reason are unfit to serve as ligands for alpha7beta1 or alternatively, are these laminins deposited in such a way in the basement membrane that they are not accessible for alpha7beta1 integrins?

In a separate study it was found that in muscle from mdx mice and DMD patients the alpha7beta1 integrin is upregulated when the dystroglycan complex fails to localize to the sarcolemma(100), suggesting that alpha7beta1 and the dystroglycan complex are interdependent on each other. In dy/dy mice there appeared to be a reduced levels of alpha7beta1 integrins as well as reduced alpha7 integrin mRNA levels.

It is clear from the studies of Mayer *et al* (85) that in certain muscle groups in mice the absence of

alpha7 integrin causes a muscular dystrophy. Recent analysis of 117 Japanese patients with uncharacterized congenital myopathies resulted in the identification of 3 patients with reduced or absent levels of alpha7 integrin mRNA and protein (101). A number of nonsense mutations were identified in both ITGA7 alleles of these patients. Based on the absence of the morphological/histological hallmarks of muscular dystrophy the authors suggest that in humans, as well in mice, the absence of alpha7 integrin causes a congenital myopathy rather than muscular dystrophy. A number of points need to be investigated before this issue can be settled. Firstly, in some muscles of the alpha7 KO mice there is clear evidence for muscular dystrophy, although not as severe as in some other animal models. Secondly, there are a number of examples where there are major differences between the human and mouse phenotype. Mechanisms explaining these differences include: different expression patterns in the two species and different compensation mechanisms such as gene duplications in mouse and man. This has become obvious in emerging animal models for a number of muscle diseases. Caution should thus be used when comparing mice and men. Thirdly, a more thorough analysis of different human muscle groups, as well as analysis of more patients, including uncharacterized congenital muscular dystrophies, might be needed before confining the alpha7 integrin defects in humans to the dystrophy or myopathy group.

6. PERSPECTIVE

Remaining questions in the area of muscle formation and muscle diseases include:

1. Which receptors are involved in early steps of myogenesis such as somite formation and differentiation? In embryos lacking fibronectin somites do not form. Analysis of chimeric mice with a population of myogenic cells lacking beta1 integrins have not addressed this question. Are beta1 integrins, integrins from other subfamilies, a combination of integrins from different integrin families, or non-integrin receptors needed in these early steps?
2. Why is *in vitro* myogenic differentiation delayed in embryoid bodies lacking beta1 integrins? Does this reflect a role for integrins in growth regulation (cell signaling) or fusion (cell-cell recognition)?
3. What is the relative importance of integrins for basement membrane assembly? How does impairment of muscle basement membranes at early stages of myogenesis affect further growth, positioning and maturation?
4. In *Drosophila*, mouse, and man, defects in integrin genes have indicated an important role for integrins in maintaining the structural integrity of muscle. The specific molecular mechanism whereby integrin stabilisation influence muscle integrity remains to be elucidated.
5. A recent study has identified mutations in the alpha7 integrin chain in patients (101). In the future it will be interesting to determine if disease - causing mutations also are to be found in other integrins and basement membrane components involved in the interaction between muscle cells and the basement membrane. We have recently identified a novel integrin alpha-chain on human fetal muscle cells (44) ; the role of this integrin during muscle development and disease is unknown.
6. The importance of cell ECM interactions during other aspects of muscle disease is less evident. Which integrins mediate the migration over basement membranes in the case of muscle injury? Are integrins needed as accessory molecules during satellite cell fusion, how does the satellite cell derived myotube mature - which integrins are needed at early steps? Are integrins transmitting signals that determine whether satellite cells will proliferate and ultimately contribute to new myofibers or whether they should stay dormant? Which integrins are involved in contribution to the fibrotic response?

It is obvious that this exciting field is full of questions waiting for answers. Whether known, or yet uncharacterized integrins, are major part of the answers remains to be determined, as the saga of cell-ECM interactions in muscle continues to unravel.

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