BIOLOGY OF SOME NEUROMUSCULAR DISORDERS

BIOLOGIJA NEKATERIH ŽIVČNOMIŠIČNIH BOLEZNI

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Abstract – *In order to understand and possibly interfere/ treat neuromuscular disorders it is important to analyze the biological events that may be causing the disability. We illustrate such attempts on two examples of genetically determined neuromuscular diseases: 1) Duchenne muscular dystrophy (DMD), and 2) Spinal muscular atrophy (SMA).*

DMD is an x-linked hereditary muscle disease that leads to progressive muscle weakness. The altered gene in DMD affects dystrophin, a muscle membrane associated proteine. Attempts were made to replace the deficient or missing gene/ protein into muscles of Duchenne children. Two main strategies were explored: 1) Myoblast and stem cell transfer and 2) Gene delivery. The possible use of methods other than the introduction of the missing gene for dystrophin into muscle fibres are based on the knowledge about the adaptive potential of muscle to different functional demands and the ability of the muscle to express a new set of genes in response to such stimuli. Stretch or overload is now known to lead to changes of gene expression in normal muscle, and the success of muscle stretch in the management of Duchenne boys is most likely to be due to such adaptive changes. Electrical stimulation of muscles is also a powerful stimulus for inducing the expression of new genes and this method too has produced beneficial effects on the progress of the disease in mice and men.

SMA is a heterogeneous group of hereditary neuromuscular disorders where the loss of lower motoneurones leads to progressive weakness and muscle atrophy. The disease subdivides into 3 forms according to the severity of the symptoms and age of onset. All three forms of SMA have been mapped to chromosome 5q11.2-13.2. Clinical features of all these forms of SMA include hypotonia shortly after birth, symmetrical muscle weakness and atrophy, finger tremor, areflexia or hyporeflexia and later contractures. In patients with SMA 1 and 2 the development of all parts of the motor unit is slower. The rate of maturation is critical for the survival of both motoneurone and muscle and that events that interfere with the time course of maturation cause both motoneurone and muscle fibre death. The proposal that the SMN gene/protein is involved in the process to developmental changes in cells and therefore crucial for their survival is put forward. The understanding of the developmental changes and their influence on motoneurone and muscle survival may help to devise therapeutic interventions. These may include a) protection of the motoneurone cell body during a critical period of its development by reducing its excitability or enhancing its defences by

Izvleček – *Živčnomišične bolezni bomo bolje razumeli in morebiti zdravili le na podlagi analize bioloških procesov, ki povzročajo prizadetost. V prispevku predstavljamo poskuse takšne analize na dveh primerih iz skupine dednih živčnomišičnih bolezni: 1) Duchennove mišične distrofije (DMD) in spinalne mišične atrofije (SMA).*

DMD je na x-kromosom vezana dedna bolezen mišic, ki povzroča napredujočo mišično šibkost. Patološko spremenjeni gen, ki povzroča DMD, določa na mišično celično membrano vezani protein distrofin. Poskusi nadomestiti patološko spremenjeni gen/protein v mišicah so bili že napravljeni z uporabo prenosa mioblastov in zarodnih celic ter neposrednega vnosa dednine/gena v mišice dečkov z DMD. Drugi smiselni terapevtski pristopi bi lahko temeljili na razumevanju adaptacijskih zmožnosti skeletne mišice na različne funkcijske zahteve in dokazane zmožnosti ekspresije novih nizov genov v mišičnih celicah v odgovor na takšne zahteve. Nateg ali prekomerna obremenitev normalnih mišičnih celic vodita v spremenjeno ekspresijo genov v mišicah, verjetno pa lahko terapevtske uspehe razteznih vaj oziroma uporabe ortoz v te namene pri dečkih z DMD pripišemo istemu mehanizmu mišične adaptacije. Tudi električna stimulacija je močan stimulus indukcije ekspresije novih genov. Njeni ugodni učinki na napredovanje mišične distrofije so že dokazani tako na živalskem modelu (miš) kot na človeku.

Spinalna mišična atrofija je klinično heterogena skupina dednih živčnomišičnih bolezni, za katero je značilna izguba spodnjih motoričnih nevronov z napredujočo mišično šibkostjo in mišičnimi atrofijami. SMA se deli v tri oblike glede na stopnjo izraženosti bolezni in na starost ob pojavu bolezni. Vse tri oblike SMA so vezane na peti kromosom (5q11.2-13.2), to je na regijo, ki določa gen SMN (»survival motoneuron gene«). Klinične značilnosti vseh oblik bolezni so zmanjšan mišični napon (hipotonija; lahko že ob ali zgodaj po rojstvu), simetrična šibkost in atrofije mišic, tremor prstov in jezika, hiporefleksija ali arefleksija tetivnih refleksov ter kasneje v poteku bolezni izrazite kontrakture. Pri pacientih s SMA1 in SMA2 je razvoj vseh delov motorične enote upočasnjen. Pravilen razvoj oziroma časovno usklajeno dozorevanje motoričnih nevronov in mišic je kritičnega pomena za njihovo preživetje, dogodki, ki posežejo v časovni potek njihovega dozorevanja, pa povzročijo smrt tako motoričnih nevronov kot mišičnih vlaken. V prispevku utemeljujemo, da je gen/protein SMN kritično udeležen v procesu razvojnih sprememb celic in tako presoden za njihovo preživetje. Razumevanje razvojnih sprememb in njihovega vpliva na preživetje motoričnih nevronov

upregulating heat shock proteins, b) stabilizing neuromuscular junctions to enhance and prolong the retrograde influences from the muscle that affect motoneurone survival, c) protecting muscle fibres from apoptosis, as well as stimulating their maturation by activity appropriate to their younger age that results from their delayed development.
These approaches should be considered in addition to or in conjunction with possible interference with the gene and its product.
In order to understand and possibly interfere/treat neuromuscular disorders it is important to analyze the biological events that may be causing the disability. In this presentation I would illustrate such attempts on two examples of genetically determined neuromuscular diseases: 1) Duchenne muscular dystrophy, and 2) Spinal muscular atrophy.

in mišic bi utegnilo pomagati pri razvoju terapevtskih intervencij. Te bi lahko bile: a) zaščita motoričnih nevronov v kritičnem času njihovega dozorevanja z zmanjševanjem njihove vzdražnosti ali krepitvijo obrambnih zmožnosti s povečano sintezo (»up-regulation«) posameznih proteinov (»heat-shock«), b) stabilizacija živčnomišičnega stika, da bi tako okrepili in podaljšali retrogradne vplive mišice na preživetje motoričnih nevronov, c) zaščititi mišična vlakna pred apoptozo, kot tudi spodbuditi njihovo dozorevanje s primerno aktivnostjo glede na stopnjo upočasnjenega razvoja.
Navedeni pristopi bi lahko pomenili dodatne ali dopolnilne terapevtske metode ob manipulaciji genov oziroma njihovih produktov.

Duchenne muscular dystrophy

Duchenne muscular dystrophy is an x-linked hereditary muscle disease that leads to progressive muscle weakness. The gene that is altered in this condition is a very large gene, dystrophin, that codes for a protein deficient in muscles of patients suffering from this disease. The mode of inheritance is illustrated in Figure 1. Since the discovery of the genetic nature of the disease attempts were made to design therapies that would replace the missing gene/protein.

Gene therapy

Duchenne muscular dystrophy was the first neuromuscular disease in which the genetic and biochemical abnormality was identified (1). Soon after the genetic abnormality was established the product of the abnormal gene was identified and named dystrophin (2). Having established these basic facts, efforts were made to replace the deficient or missing gene/protein into muscles of Duchenne children, with the hope that such treatment may halt the relentless progression of the disease. Two main strategies were explored: 1) Myoblast and stem cell transfer and 2) Gene delivery. A brief summary of these attempts follows.

Myoblast and stem cell transfer

This approach was based on the hope that normal myoblasts or myotubes when transferred into the diseased muscle may produce sufficient amounts of dystrophin to allow the diseased muscle to function (3). However, attempts to cure dystrophic muscles by myoblast transfer on children suffering from Duchenne dystrophy failed (4, 5).
More recently, attempts were made to isolate muscle derived stem cells that could be used for transfer into diseased muscle, with the hope of inducing the formation of normal muscle fibres (6). So far it does not appear that enough healthy muscle fibres can be introduced by this method to counter the progress of muscle weakness.

Delivery of genes

Efforts were made to deliver to the muscle fibres either a full-length DNA construct of the very large dystrophin gene or its truncated forms using various vectors to carry the genetic material into the cell. The lack of success of this approach may have been due to a combination of factors, such as technical problems, immunological rejection and responses of the muscle fibre itself when confronted with new, unusual proteins and vectors that were used to introduce them (7). Nevertheless, recent results based on the use of adeno-associated virus for treatment of an animal model homologous to limb girdle muscular dystrophy (LGMD) warrant some optimism (8, 9).

A slightly different approach has been used in an attempt to improve the condition of mdx mice that lack dystrophin. The expression of a protein with a similar function to dystrophin, i. e. utrophin, was upregulated and this resulted in some beneficial effects in the mdx mouse. Upregulation of utrophin in mdx mice seemed to have improved their condition while a total absence of utrophin in mdx KO mice increased the severity of the symptoms, thus indicating that utrophin may be able to compensate for the absence of dystrophin (10).
Encouraging results were recently reported by Moll et al. (11) where the introduction of an agrin minigene alleviated the symptoms in a mouse model of congenital muscular dystrophy. The rationale for this approach was that agrin binds both to the basement membrane and to alpha dystroglycans and may amend the membrane deficit responsible for the disease. These results indicate that the function of dystrophin can be replaced by other proteins.
In all these experiments introduction of new molecules or the upregulation of existing molecules used genetic manipulations, a method with many problems that are not yet fully understood.
In view of the relatively little success obtained using the methods of direct gene manipulations, it may be timely to explore alternatives where the gene expression of the muscle fibre itself is modified without introducing an alien substance into the system. Most cells, and muscle is no exception, have a tremendous adaptive potential that enables them to produce, given the right stimulus, their own alternative isoforms of proteins and molecules (12). Inducing the expression of new proteins and upregulation of some organelles such as mitochondria in the diseased muscle fibres may possibly protect them from degeneration and thus halt the progress of the disease. It is the exploitation of this avenue that is being considered. In order to achieve this aim it is important to examine the function of dystrophin.

Function of dystrophin

The cell membrane is a lipid bilayer which ensures a more or less stable internal environment of the cell by regulating the communication between the extracellular and intracellular compartment. Muscle fibres are unique, for due to their mechanical activity during contraction and relaxation unusually severe mechanical stresses are imposed upon their membrane. The fragile membrane which consists of a lipid bilayer is protected by a) the thick basal lamina on the external surface of the muscle fibre and b) a complex subsarcolemmal network on the inside of the membrane of which actin filaments are the major component. A device that keeps these two layers together is provided by dystrophin and dystrophin associated proteins (DAP) (13). In the absence of dystrophin and DAP

the lipid membrane when subjected to excessive stresses ceases to be a reliable barrier between the extra- and intracellular compartment. Thus excess calcium may enter the muscle fibre and cause damage, while other molecules that are usually not allowed to leave the muscle fibre, such as the enzyme creatinkinase, leak out in excessive amounts. Such a disturbance of the internal environment of the muscle fibre may then have disastrous consequences (14).

It could be that the undesirable consequences of the lack of dystrophin and DAP may be alleviated by inducing muscle fibres to upregulate other molecules such as agrin or intermediate filaments that can be produced by the diseased muscle fibre, for the muscles do have the genes coding for these molecules. Inducing the muscle fibres to produce these molecules by using the muscles' own resources may strengthen the muscle membrane and cytoskeleton, so as to protect it from the consequences of repeated membrane damage. In addition, the muscle fibre can be induced to increase the number of mitochondria therefore giving it a better chance to buffer Ca^{++} as well as to maintain a relatively better supply of energy, which may be reduced due to the loss of creatinkinase.

Which molecules could help to substitute the missing dystrophin? Possible candidates for such a role are peptides usually referred to as intermediate filaments (spectrin, vimentin, desmin and others). These are known to be important for the integration of various cellular structures within the sarcomere and cytoplasm as well as for connecting them to the subsarcolemmal network of actin (15). Interestingly, these molecules are abundant in skeletal muscle fibres during development and are downregulated when the muscle matures (15). It could be that this downregulation of the intermediate filament expression with age coincides in normal muscle with the upregulation of dystrophin and DAP and that the integrity of the muscle fibre then depends entirely upon this system. Such a sequence of events may explain observations on Duchenne children where muscle deterioration occurs after the first few years of life, and observations in mdx mice where a dramatic muscle degeneration can be observed during the 2nd and 3rd week of their life (16), a time when in mice neonatal isoforms of proteins are exchanged for their more mature counterparts (17). Unfortunately there are no precise data to correlate these clinical observations with the presence of intermediate filaments, or in normal muscles with the developmental changes of dystrophin.

Nevertheless it may be beneficial for muscles that lack dystrophin to prevent the downregulation of intermediate filaments that normally occurs with development as well as to find ways to upregulate the expression of these polypeptides in muscles of Duchenne patients. This may be possible to achieve by altering the activity of the muscle. Appropriate alterations of muscle activity are known to induce/upregulate specific isoforms of muscle proteins and genes coding for them.

Induction of the expression of new genes by altered muscle function

Modification of gene expression by stretch/increased load

It has long been known that increased load imposed upon skeletal muscle or muscle stretch leads to muscle hypertrophy as well as to changes in oxidative enzymes and capillary density (18–20). In addition to these changes stretch/overload also modifies gene expression, and some of these changes include alterations in structural proteins of the sarcomere such as myosin heavy chains, and possibly those involved in maintaining the structural integrity of the sarcomere (21–23). Thus, stretch has an important morphogenetic role in regulating muscle phenotype.

Modification of gene expression by contractile activity

Changing the pattern of activity of skeletal muscles has profound effects on the phenotype of skeletal muscle fibres. Reducing or abolishing continued activity typical for slow contracting postural muscles will transform them into fast contracting muscles that are activated intermittently. Moreover, applying a tonic pattern of activity on such a modified slow muscle by electrical stimulation will maintain its characteristic slow properties. These findings inspired experiments in which fast muscles that are naturally activated intermittently were subjected to electrical stimulation typical of a slow postural muscle. Results showed that this particular type of continuous activity turned fast muscles into slow muscles (24, 25).

The ability of skeletal muscle fibres to respond to altered activity has been particularly well explored in fast twitch muscles that were converted into slow twitch fibres by chronic low frequency electrical stimulation (CLFS) (for review see 12, 23). The model of CLFS of fast twitch muscles allowed the study of the adaptive potential of skeletal muscle and defines a) the various physiological, biochemical and molecular changes that occur in skeletal muscles in response to this distinct, patterned activity regime and the molecular changes that could account for the transformation, b) the changes of gene expression induced by CLFS and finally c) the mechanism that induces changes of gene expression.

The main functional changes that are induced by electrical stimulation of fast muscles are as follows: a) a change in the time course of contraction and relaxation, b) a change in the force/frequency relationship of the muscle, in that more force is developed at relatively lower rates of stimulation, c) a dramatic increase of fatigue resistance and the ability of the muscle to work for long periods of time. Various molecular changes in the different compartments of the muscle fibre are responsible for these functional alterations (Figure 2 molecular changes related to function).

The changes in the time course of contraction and relaxation induced by CLFS are likely to be linked to alterations of proteins and receptors of the sarcoplasmic reticulum (SERCA) as well as to alterations of isoforms of myofibrillar proteins, i. e. myosin heavy and light chains, and the troponin complex. The isoforms of myofibrillar proteins in typical fast muscles are gradually exchanged for their slow counterparts. This exchange involves the rebuilding of the sarcomere and a dramatic reorganization of its structure to make it more suitable for sustained and long-term activity.

An important part of the adaptation to prolonged activity is the strengthening of the cytoskeletal infrastructure of the muscle fibre. Thus a several fold increase in vimentin, desmin (26) and a change in the alpha actinin reflected also in the thickening of the Z line have been reported (27).

CLFS leads to a dramatic increase in fatigue resistance, and this may be to some extent correlated with the transition from anaerobic to aerobic metabolism as well as with the increase in capillary density and blood supply. Nevertheless other factors such as the steep increase in hexokinase II and the rise in GLUT 4, which lead to an increased capacity of glucose uptake and phosphorylation, probably also contribute to the establishment of this physiological change.

Remodelling of the muscle fibres involves altered gene expression

In the previous section some of the functional and biochemical changes that are induced by electrical stimulation were listed and discussed in relation to function. The transition from fast to slow phenotype in response to electrical stimulation involves processes of coordinated expression and repression of proteins within the same or different cellular structures or compartments.

Examination of changes at the mRNA level revealed that alterations of mRNA levels for the various proteins that are induced to change in response to electrical stimulation precede the changes in proteins. Thus there is now clear evidence that the gene expression for various isoforms of proteins of the muscle fibres can be modified by patterned electrical activity. Although the change of gene expression controlling different molecular elements of the muscle is not synchronous it can nevertheless be concluded that the gene expression for most proteins of the muscle fibre changes with activity.

Thus these experimental results taken together clearly show that it is possible to alter the gene expression of post-mitotic excitable cells by activity.

The mechanisms involved in this change in gene expression are under discussion, but it seems to be related to the duration of the Ca^{++} transients (28) possibly mediated by activation of a calcineurin and MEF2 signal NFAT (29).

These experimental results obtained on healthy muscles either by stretch or activity illustrate the ability of the muscle to activate a new programme of gene expression. They provide the background and rationale for attempting to study whether such manipulations of muscle function are beneficial for diseased muscles of mice and men.

Altered activity can protect dystrophic skeletal muscles

Since a change in gene expression of normal muscles can be induced by altering muscle function such as stretch or a different pattern of contractile activity, it may be pertinent to examine the possibility that switching on novel genes by altering muscle function may reduce the rate of deterioration of diseased skeletal muscle fibres.

Mechanical interference

Empirical observations of the effects of stretch and muscle load on the progress of the deterioration of muscles in Duchenne boys indicate that these methods of treatment are perhaps the most successful yet.

Regular stretching of muscles of Duchenne boys resulted in a slowing of the deterioration of the diseased muscles (30). The use of orthotic devices has also proved beneficial, and increased the life expectancy of the patients treated in this way (31). The use of such orthotic devices is likely to act through stretching the hamstrings and other muscle groups. In contrast to these positive effects of load and stretch, unloading the calf muscles by lengthening the achilles tendon has deleterious effects on the long-term prospect of the patients (32). It is also well recognized that assisted respiration improves the condition of the patients. While the most likely effect of this treatment is the improved respiratory function and the effect of this on the cardiovascular and other systems affected by chronic hypoventilation and ensuing lack of oxygen, a possible by-product of assisted ventilation is a stretch of respiratory muscles. This in addition to the other beneficial effects of assisted ventilation may improve the condition of the patient. These observations, though carefully documented in Duchenne children, had not hitherto had a rational explanation. However, when considering the effects of stretch on gene expression, content of oxidative enzymes and capillary density on normal skeletal muscles (19, 20, 23), these results on improved muscle function of Duchenne children induced by stretch now have a rational explanation.

Electrical stimulation

Experiments on mutant mice that suffer from severe muscle weakness (C57BL dy/2J/dy2J) that is much more severe than that of dystrophin deficient mdx mouse have shown that a particular pattern of activity imposed upon their muscles leads to reduction of the progress of deterioration of their muscles. These mutant mice have a defect of laminin M and some of the DAP molecules and there are some similarities between these mutants and congenital muscular dystrophy (CMD) (33). Electrical stimulation of leg muscles in these animals at low frequencies for several weeks led to a reduction of the rate of loss of force and muscle bulk characteristic of this disease in mice (17, 34). Moreover, electrical stimulation reverses the changes in enzyme activities caused by the disease process in muscles of these dystrophic animals (35, 36).

The encouraging results on dystrophic mice led to attempts to investigate the effects of low frequency electrical stimulation on muscles of patients with Duchenne or Becker dystrophy. The results demonstrated that electrical stimulation reduces the rate of deterioration of ankle dorsiflexors and quadriceps muscles in boys with Duchenne dystrophy, provided the stimulation was started before the muscles became excessively weak (30, 37–39). Moreover, the pattern of stimulation was critical, for only stimulation at slow frequencies (i. e. 10 Hz or less) caused improvement while stimulation at frequencies in excess of 30 Hz, which is well within the range of normal firing frequency of motoneurones to these muscles, not only did not improve the muscle function, but enhanced the deterioration of the muscle (30). It could be argued that the low frequency electrical stimulation may lead to increases of Ca^{++} levels of the stimulated muscle fibres, and it is therefore counterintuitive that this procedure would have a beneficial effect on the muscle. However, the results show that even if such increases did occur they did not prevent the improvement induced by electrical stimulation. Perhaps this is not surprising, for as our knowledge about the role of Ca^{++} in biological processes increases, it is becoming clear that the particular characteristics of the Ca^{++} dynamics, i. e. the frequency, amplitude and duration of the Ca^{++} transients, could be more important for the function of the cell than the resting concentration of this ion (see 40).

Manipulating ion channels

Recently, evidence has been presented that manipulating Cl^- channels could be beneficial to mdx mice where dystrophin is lacking (41, 42).

The firing pattern of skeletal muscle fibres is controlled by the activity that reaches them via their axons and in normal conditions each action potential in an axon elicits a single muscle action potential and a single twitch contraction. This response of the muscle fibre depends on the excitability of its membrane and is controlled by the passive membrane properties of the muscle fibre which depends on the conductance of some of the ion channels. If the conductance of the Cl^- channel is low, then the muscle fibre will be repeatedly activated by a single nerve impulse. Nature has provided us with such a model in the form of the myotonic adr mouse mutant. Recently two groups independently produced a mutant where the mdx mouse was combined with an adr mouse and surprisingly the mdx mouse benefited from this crossbreeding and showed longer life expectancy and other improvements of its performance (41, 42). The most likely explanation of this result is that the muscles of the mdx mouse were undergoing chronic stimulation, since each impulse in an axon produced several discharges in the muscle membrane. In terms of hypothetical treatment this is a very exciting result, since it shows a new way how to influence most muscles of Duchenne patients without having to stimulate each individual muscle electrically.

Spinal muscular atrophy (SMA)

Current status of SMA

SMA is a heterogeneous group of hereditary neuromuscular disorders where the loss of lower motoneurones leads to pro-

gressive weakness and muscle atrophy. The most common of these disorders is the infantile and juvenile proximal SMA, with an incidence of 1/10–15000 newborns and carrier frequency of 1/50 individuals (43). SMA represents the second most common fatal autosomal recessive disorder of children, cystic fibrosis being first. The disease usually subdivides into 3 forms according to the severity of the symptoms and age of onset (44, 45).

SMA1 is the most severe form, SMA2 intermediate and SMA3 the relatively mild form. There are some common features of all these which include hypotonia shortly after birth, symmetrical muscle weakness and atrophy, finger tremor, areflexia or hyporeflexia and later contractures. Not all muscle groups are affected to the same extent; the diaphragm, extraoccular muscles and the myocardium are usually spared, while the proximal muscles of the limbs are affected earlier than the others and the atrophy is more severe.

Molecular genetics

All three forms of SMA have been mapped by linkage analysis to chromosome 5q11.2-13.2 (46–48). Although several other genes have been localized to this region the SMN gene appears to be the most important, since around 95% of SMA patients carry homozygous deletions on this gene. The SMN gene in humans exists in 2 copies: SMN1 localized at the telomeric part of the chromosome and SMN2 at the centromeric part of the chromosome. Both copies are identical apart from 2 base pairs on exon 7 and 8 which are present only in the SMN1 copy and absent in the SMN2 copy of the SMN gene. In SMA patients the SMN1 form lacks 2 base pairs on exon 7 and 8.

There is some correlation between the number of copies of the SMN2 gene and the severity of the disease so that a higher number of copies of this gene is associated with the milder form of the disease (49). Thus the SMN2 gene, in spite of the absence of the crucial base pairs of exon 7 and 8, is able to influence to some extent the phenotype of the disease.

A further important step in elucidating the possible cause of the symptoms in SMA patients was the isolation of the protein coded for by the SMN gene. It is expressed in most tissues and is believed to be involved in mRNA metabolism, in particular pre-mRNA splicing (50–52). This cellular function indicates that the SMN protein may be involved in situations where the phenotype of cells changes, such as during development, and the finding that the expression of the SMN protein is highest during development is consistent with such a role. Thus it could be that this protein by controlling mRNA splicing and metabolism may influence and bring about developmental changes of the tissues. In systems where the timing of these changes is critical such as motoneurone and muscle, which have to achieve a certain degree of maturity to be integrated into the developing neuronal networks, this function of the SMN protein may be of particular importance.

Provided that the SMN protein is involved in bringing about developmental changes of motoneurones and muscles it could be expected that the progress of maturation of the neuromuscular system may be delayed in SMA patients.

Signs of immaturity of the neuromuscular system in SMA children

In a thorough study on a large number of SMA patients Prof. Hausmanowa's group examined all 3 components of the motor unit and showed clear signs of immaturity of all 3 components. A characteristic feature of the spinal cord taken from autopsies of patients with SMA is the absence of motoneurones in the ventral horn. Nevertheless, the remaining motoneurones show some signs of immaturity and are smaller with less developed Nissl substance than in normal fetuses of comparable age (53–55).

In addition, continuous EMG activity with a firing rate of 9–18 Hz can be recorded from muscles of SMA patients, even during rest and sleep (56–58). This altered activity of motoneurones is similar to that found in animals where maturation of motoneurones was retarded by temporarily disrupting the interaction between the motor nerve and muscle (55). These findings indicate a profound change in the excitability of the spinal motoneurones.

Consistent with the findings on motoneurones signs of delayed development have also been described for motor axons (59). In the ventral roots from SMA patients there were many multiaxonal bundles enwrapped by a single Schwann cell, a low density of axons/mm and a relatively high proportion of poorly myelinated thin axons. Small atrophic ventral roots were previously described in material from SMA patients, without signs of sparse myelination (60). In addition, axons have a prolonged latency of the response and reduced conduction velocity (61).

Finally signs of immaturity can also be seen in muscles from SMA children. In SMA1 many undifferentiated, extremely small muscle fibres were present which displayed characteristics of fetal muscles (60). There were also some myogenic cells that resembled myotubes and several of these were often enclosed in the same basal lamina. The fetal character of these very small muscle fibres was indicated by the finding that when stained with acridine orange they displayed a vivid orange colour possibly associated with the high content of RNA. Perhaps the most compelling evidence of their immaturity is the presence of desmin and vimentin, known to be downregulated during normal muscle development and absent in denervated muscles (63–65).

An interesting feature of muscles from SMA children is their different response to injury. Whereas more mature muscles respond to injury by necrosis, muscles from SMA children respond by apoptotic changes to the same insult.

In conclusion, there is strong evidence that the neuromuscular system in SMA children is immature. However, why delayed maturation should result in the specific pathology seen in SMA children needs to be explained. Such an explanation may be provided by understanding the role of accurate timing of motoneurone and muscle maturation during normal development of the neuromuscular system.

Survival of motoneurones depends on maturation

Motoneurones are among the first cells to be generated in the spinal cord. Like in other neuronal populations the initial generation of these neurones is followed by death of a proportion of these motoneurones. This event is referred to as naturally occurring cell death, and the regulation of this event is not clear.

Early studies indicated a relationship between the number of surviving motoneurones and the amount of muscle tissue available for innervation (66).

After the 'naturally occurring cell death' has been completed, motoneurones remain critically dependent on contact and active interaction with their target. If the connection between the motoneurone and target is disrupted during early postnatal development either surgically by cutting the axon and removing the target, or pharmacologically by preventing neuromuscular interactions a considerable number of motoneurones die. The extent of motoneurone death depends on the timing of the insult, i. e. when after birth it is inflicted and the duration of the interruption of neuromuscular contacts (67).

What are the changes induced by the target that allow motoneurone survival? During the developmental period when the motoneurone needs contact with its target muscle many changes occur within the CNS and spinal cord. The motoneurone is initially activated by relatively few and weak afferent inputs

which is amplified by the high excitability of the small motoneurones as well as by the electrotonic coupling of the motoneurones to each other (68). With further development the strength and efficiency of the afferent inputs to motoneurones increase. In order for the motoneurone to be able to deal with this increase of excitatory stimuli mediated by glutamate it has to acquire new properties that allow it to deal with these new functions.

Glutamate, the mediator of most excitatory afferent inputs to the motoneurone, excites the motoneurone through various receptors. The most studied of these is the NMDA receptor. The role of this receptor in excitotoxic cell death is well established. Immature motoneurones die when exposed to glutamate, and interaction with the target is necessary to induce changes that allow them to survive exposure to glutamate (69).

When neuromuscular interactions of immature motoneurones are prevented they revert to their less mature behaviour and are killed by exogenously applied NMDA (70). In view of these findings, we proposed that target deprived postnatal motoneurones fail to achieve the degree of maturity that enables them to withstand exposure to glutamate. Since glutamate is released by the ever more abundant afferent inputs that impinge upon the motoneurone, such motoneurones are then likely to die as a result of this exposure (69). Figure 3 illustrates schematically this hypothesis.

Further support for this hypothesis was provided by results that permanent survival of motoneurones destined to die after axotomy can be achieved if during the period of separation from the target muscle the cell body is protected from the excitotoxic effects of glutamate by blocking NMDA receptors (71).

These findings clearly show the importance of the precise timing of the maturation of the motoneurone in relation to the development of the rest of the spinal cord circuitry for the normal development of the system and any interference with this will have devastating effects on the immature motoneurone. The possibility that the SMN protein and its partners are important for the correct timing of these events may explain their importance for the survival of developing motoneurones.

Although these results bring us a step further and pinpoint the importance of the target for motoneurone maturation and survival, the mechanisms by which the target muscle achieves this are not clear.

It is possible that in SMA patients the motoneurone is receiving the signal from the muscle and all the messages required for its maturation but it is unable to do so for the molecules that are necessary to transform the motoneurone from an immature to a mature cell are missing. An adequate number of copies of the SMN gene and its gene product are therefore likely to be needed to carry out the changes involved in the transition of an immature to a mature motoneurone.

Critical dependence of muscle development and differentiation on continued contact with the motor nerve

Although many aspects of muscle development can proceed without innervation, and even immature muscle fibres can survive denervation for considerable periods of time, they are nevertheless critically dependent on functional innervation during early postnatal development. If the muscle is denervated during the early periods of postnatal development and then reinnervated by its own nerve about 60% of its muscle fibres die after reinnervation. The most likely explanation of this devastating effect reinnervation has on survival of immature muscles is the arrest of differentiation and maturation of these muscle fibres during the period of denervation. Such immature muscle fibres are unable to withstand the vigorous activity that is imposed upon them by the now more mature motoneurone and are destroyed by it. Thus if the timing between the maturation of the muscle and that of the motoneurone and its activity is disrupted muscle fibres are unable to survive.

Thus, like the motoneurone the immature muscle too has to become competent to cope with functions that are changing with the development of the locomotor system.

Why is the motor unit so uniquely susceptible to the lack of SMN protein?

Unfortunately, in the absence of hard evidence the answers to this problem will be largely speculative, but these speculations can nevertheless be useful since they can now be tested in the various transgenic animal models of the disease. When considering the possibility that the SMN gene and protein may play a pivotal role during changes of a cell's or neurone's phenotype the obvious question is: why is the motoneurone affected more than any other cell? To account for this unique need of motoneurones for the SMN gene it may be useful to list the particular features of the motoneurone:

a) *The motoneurone is a cholinergic cell,* i. e. the transmitter it synthesizes and releases is cholinergic. However other cholinergic cells in the CNS, even those with large projections and relatively long axons such as neurones in the Meynert nucleus, are not affected by the lack of the smn gene. Thus neither the cholinergic nature of the motoneurone nor its exceptionally large projections can account for its special need for the SMN gene.

b) *The axon of the motoneurone is outside the CNS in the periphery.* This distinguishes it from many other neurones. Nevertheless, sensory neurones too have their long processes outside the CNS and are not dramatically affected by the absence of the SMN gene. Moreover, even cholinergic cells with axons in the periphery such as many neurones of the autonomic nervous system are not critically dependent on the presence of the SMN gene and seem to survive in patients with SMA.

c) *The special relationship of motoneurone and muscle.* The last and probably most unique feature of the motoneurone is its special relationship with skeletal muscle fibres. Indeed, it appears that this feature distinguishes the motoneurone from all other neurones of the central and peripheral nervous system and therefore points to the cause of its unique involvement in SMA.

Why does a neurone that interacts with the muscle have a special requirement for the SMN gene and protein? Perhaps the answer to this question can be obtained when considering results discussing the dependence of the developing normal motoneurone on contact and interaction with the muscle. Two main points are relevant to this problem: 1) The motoneurone in order to become successfully integrated and survive the increasing excitatory inputs of the maturing spinal cord has to undergo a change of phenotype where many of the immature molecules are replaced by their more mature counterparts. 2) This transition is induced by interaction with the muscle and if it fails to occur in time the motoneurones' survival is at risk.

Conclusions

Duchenne muscular dystrophy

The possible use of methods other than the introduction of the missing gene for dystrophin into muscle fibres is discussed. These methods are based on the knowledge about the adaptive potential of muscle to different functional demands and the ability of the muscle to express a new set of genes in response to such stimuli. Stretch or overload is now known to

lead to changes of gene expression in normal muscle, and the success of muscle stretch in the management of Duchenne boys is most likely to be due to such adaptive changes. Electrical stimulation of muscles is also a powerful stimulus for inducing the expression of new genes and this method too has produced beneficial effects on the progress of the disease in mice and men. The article also illustrates that the understanding of the cellular mechanisms involved in the consequences of lack of dystrophin is essential for proposing rational treatment.

Spinal muscular atrophy

The present review provides evidence that the development of all parts of the motor unit is slower in patients with SMA1 and 2. It also argues that the rate of maturation is critical for the survival of both motoneurone and muscle and that events that interfere with the time course of maturation cause both motoneurone and muscle fibre death. The proposal that the SMN gene/protein is involved in the process to developmental changes in cells and therefore crucial for their survival is put forward.

The understanding of the developmental changes and their influence on motoneurone and muscle survival may help to devise therapeutic interventions. These may include a) protection of the motoneurone cell body during a critical period of its development by reducing its excitability or enhancing its defences by upregulating heat shock proteins, b) stabilizing neuromuscular junctions to enhance and prolong the retrograde influences from the muscle that affect motoneurone survival, c) protecting muscle fibres from apoptosis, as well as stimulating their maturation by activity appropriate to their younger age that results from their delayed development.

These approaches should be considered in addition to or in conjunction with possible interference with the gene and its product.

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References

1. Monaco AP, Neve RL, Colletti-Feener C, Bertelson CJ, Kurnit DM, Kunkel LM. Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature 1986; 323: 646–50.
2. Hoffman EP, Brown RH, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987; 51: 919–28.
3. Partridge TA. Invited review: Myoblast transfer: A possible therapy for inherited myopathies? Muscle Nerve 1991; 14: 197–212.
4. Law PK, Goodwin TG, Li HJ, Ajamoughli G, Chen M. Myoblast transfer improves muscle genetics/structure/function and normalizes the behavior and life-span of dystrophic mice. Adv Exp Med Biol 1990; 280: 75–87.
5. Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttmann R, Holland P, Koch PA, Shoubridge E, Spence D, Vanasse M, Watters GV, Abrahamowicz M, Duff C, Worton RG. Myoblast transfer in Duchenne muscular dystrophy. Ann Neurol 1993; 34: 8–17.
6. Asakura A. Stem cells in adult skeletal muscle. Trends Cardiovasc Med 2003; 13: 123–8.
7. Ferrer A, Wells KE, Wells DJ. Immune responses to dystropin: implications for gene therapy of Duchenne muscular dystrophy. Gene Ther 2000; 7: 1439–46.
8. Xiao X, Li J, Tsao YP, Dressman D, Hoffman EP, Watchko JF. Full functional rescue of a complete muscle (TA) in dystrophic hamsters by adeno-associated virus vector-directed gene therapy. J Virol 2000; 74: 1436–42.
9. Akkaraju GR, Huard J, Hoffman EP, Goins WF, Pruchnic R, Watkins SC, Cohen JB, Glorioso JC. Herpes simplex virus vector-mediated dystrophin gene transfer and expression in MDX mouse skeletal muscle. J Gene Med 1999; 1: 280–89.
10. Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, Davies KE. Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature 1996; 384: 349–53.
11. Moll J, Barzaghi P, Lin S, Bezakova G, Lochmüller H, Engvall E, Müller U, Ruegg MA. An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. Nature 2001; 413: 302–7.
12. Pette D, Vrbová G. What does chronic electrical stimulation teach us about muscle plasticity? Muscle Nerve 1999; 22: 666–77.
13. Crawford GE, Faulkner JA, Crosbie RH, Campbell KP, Froehner SC, Chamberlain JS. Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. J Cell Biol 2000; 150: 1399–410.
14. Ozawa E, Noguchi S, Hizumo Y, Hagiwara Y, Yoshida M. From dystrophinopathy: Evolution of a concept of muscular dystrophy. Muscle Nerve 1998; 21: 421–38.
15. Small JV, Furst DO, Thornell LE. The cytoskeletal lattice of muscle cells. Eur J Biochem 1992; 208: 559–72.
16. Dangain J, Vrbová G. Muscle development in MDX mutant mice. Muscle Nerve 1984; 7: 700–4.
17. Dangain J, Pette D, Vrbová G. Developmental changes in succinate dehydrogenase (SDH) activity in muscle fibres from normal and dystrophic mice. J Physiol 1984; 351: 38P.
18. Frischknecht R, Vrbová G. Adaptation of rat extensor digitorum longus to overload and increased activity. Pflügers Arch 1991;419: 319–26.
19. Frischknecht R, Belverstone D, Vrbová G. The response of adult and developing rat plantaris muscle to overload. Pflügers Arch 1995; 431: 204–11.
20. Zhou AL, Egginton S, Brown MD, Hudlická O. Capillary growth in overloaded, hypertrophic adult rat skeletal muscle: an ultrastructural study. Anat Rec 1998; 252: 49–63.
21. Goldspink G, Scutt A, Loughna PT, Wells DJ, Jaenicke T, Gerlach GF. Gene expression in skeletal muscle in response to stretch and force generation. Am J Physiol 1992; 262: R356–63.
22. Goldspink G. Selective gene expression during adaptation of muscle in response to different physiological demands. Comp Biochem Physiol B Biochem Mol Biol 1998; 120: 5–15.
23. Goldspink G. Gene expression in skeletal muscle. Biochem Soc Trans 2002;30: 285–90.
24. Vrbová G. The effect of tenotomy on the speed of contraction of fast and slow mammalian muscle. J Physiol 1963; 166: 241–50.
25. Salmons S, Vrbová G. The influence of activity on some contractile characteristics of mammalian fast and slow muscles. J Physiol 1969; 201: 535–49.
26. Baldi JC, Reiser PJ. Intermediate filament proteins increase during chronic stimulation of skeletal muscle. J Muscle Res Cell Motil 1995; 16: 587–94.
27. Eisenberg BR, Salmons S. The reorganization of subcellular structure in muscle undergoing fast-to-slow type transformation. A stereological study. Cell Tissue Res 1981; 220: 449–71.
28. Chen G, Carroll S, Racay P, Dick J, Pette D, Traub I, Vrbova G, Eggli P, Celio M, Schwaller B. Deficiency in parvalbumin increases fatigue resistance in fast-twitch muscle and upregulates mitochondria. Am J Physiol Cell Physiol 2001; 281: C114–22.
29. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. EMBO J 2001; 20: 6414–23.
30. Scott OM, Hyde SA, Goddard C, Dubowitz V. Prevention of deformity in Duchenne muscular dystrophy. A prospective study of passive stretching and splintage. Physiotherapy 1981; 67: 177–80.
31. Hyde SA, Scott OM, Goddard CM, Dubowitz V. Prolongation of ambulation in Duchenne muscular dystrophy by appropriate orthoses. Physiotherapy 1982; 68: 105–8.
32. Manzur AY, Hyde SA, Rodillo E, Heckmatt JZ, Bentley G, Dubowitz V. A randomized controlled trial of early surgery in Duchenne muscular dystrophy. Neuromuscul Disord 1992; 2: 379–87.
33. Arahata K, Engvall E, Hayashi YK et al. Defect of laminin M in skeletal and cardiac muscles and peripheral nerve of the homozygous dystrophic dy/dy mice. Proc Japan Acad 1993; 69: 259–64.
34. Luthert P, Vrbová G, Ward KM. Effects of slow frequency electrical stimulation on muscles of dystrophic mice. J Neurol Neurosurg Psychiatry 1980; 43: 803–9.
35. Reichmann H, Pette D, Vrbová G. Effects of low frequency electrical stimulation on enzyme and isozyme patterns of dystrophic mouse muscle. FEBS Lett 1981; 128: 55–8.
36. Reichmann H, Pette D, Vrbová G. Electrostimulation-induced changes in the enzyme activity pattern of dystrophic mouse muscles. Acta Histochem Suppl 1983; 28: 125–7.
37. Scott OM, Hyde SA, Vrbová G, Dubowitz V. Therapeutic possibilities of chronic low frequency electrical stimulation in children with Duchenne muscular dystrophy. J Neurol Sci 1990; 95: 171–82.
38. Zupan A. Long-term electrical stimulation of muscles in children with Duchenne and Becker muscular dystrophy. Muscle Nerve 1992; 15: 362–7.
39. Zupan A, Gregoric M, Valencic, Vandot S. Effects of electrical stimulation on muscles of children with Duchenne and Becker muscular dystrophy. Neuropediatrics 1993; 24: 189–92.
40. Berridge MJ, Dupont C. Spatial and temporal signalling by calcium. Curr Opin Cell Biol 1994; 6: 267–74.
41. Heimann P, Augustin M, Wieneke S, Heising S, Jockusch H. Mutual interference of myotonia and muscular dystrophy in the mouse: A study on ADR-MDX double mutants. Neuromuscular Disord 1998; 8: 551–60.
42. Kramer R, Lochmuller H, Abicht A, Rudel R, Brinkmeier H. Myotonic ADR-MDX mutant mice show less severe muscular dystrophy than MDX mice. Neuromuscular Disord 1998; 8: 542–50.

43. Pearn J. Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J Med Genet 1978; 15: 409–13.
44. Dubowitz V. Muscle disorders in childhood. Major Probl Clin Pediatr 1978; 16: 1–282.
45. Hausmanowa-Petrusewicz I, Fidziańska A. The clinical features of spinal infantile and juvenile muscular atrophy. In: Gamstorp I, Sarnat HB eds. Progressive spinal muscular atrophies. New York: Raven Press, 1984: 31–42.
46. Brzustowicz LM, Lehner T, Castilla LH et al. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. Nature 1990; 344: 540–1.
47. Melki J, Abdelhak S, Sheth P et al. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. Nature 1990; 344: 767–8.
48. Lefebvre S, Burglen L, Reboullet S et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995; 80: 155–65.
49. Lefebvre S, Burlet P, Liu Q et al. Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet 1997; 16: 265–9.
50. Liu Q, Dreyfuss G. A novel nuclear structure containing the survival of motor neurons protein. EMBO J 1996; 15: 3555–65.
51. Fischer U, Liu Q, Dreyfuss G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Cell 1997; 90: 1023–9.
52. Young PJ, Man NT, Lorson CL, Le TT, Androphy EJ, Burghes AH, Morris GE. The exon 2b region of the spinal muscular atrophy protein, SMN, is involved in self-association and SIP1 binding. Hum Mol Genet 2000; 9: 2869–77.
53. Malamud W. Infantile progressive muscular atrophy. In: Minckler J ed. Pathology of the nervous system. McGrow Book Company, 1968: 45–54.
54. Malinsky J. Ultrastructural changes of cell elements during the differentiation of neuroblasts and glioblasts in human spinal cord. Acta Univ Olumunic Faculty Med 1972; 64: 183–93.
55. Buchthal F, Olsen PZ. Electromyography and muscle biopsy in infantile spinal muscular atrophy. Brain 1970; 93: 15–30.
56. Hausmanowa-Petrusewicz I, Karwańska A. Electromyographic findings in different forms of infantile and juvenile proximal spinal muscular atrophy. Muscle Nerve 1986; 9: 37–46.
57. Hausmanowa-Petrusewicz I, Friedman A, Kowalski J, Skalski M. Spontaneous motor unit firing in spinal muscular atrophy of childhood. Electromyogr Clin Neurophysiol 1987; 27: 259–64.
58. Navarrete R, Vrbová G. Differential effect of nerve injury at birth on the activity pattern of reinnervated slow and fast muscles of the rat. J Physiol 1984; 351: 675–85.
59. Drac H. Histological changes in peripheral nerves in spinal Werdnig-Hoffmann muscular atrophy. Neuropatol Pol 1977; 15: 1–16 (Polish).
60. Greenfield JC, Stern RO. The anatomical identity of the Werdnig-Hoffmann and Oppenheim forms of infantile muscular atrophy. Brain 1927; 50: 652–86.
61. Krajewska G, Hausmanowa-Petrusewicz I. Abnormal nerve conduction velocity as a marker of immaturity in childhood muscle spinal atrophy. Folia Neuropathol 2002; 40: 67–74.
62. Sarnat HB. Pathology of spinal muscular atrophy. In: Gamstorp I, Sarnat HB, eds. Progressive Spinal Muscular Atrophy. New York: Raven Press, 1974: 91–110.
63. Fidziańska A, Goebel HH, Warlo I. Acute infantile spinal muscular atrophy. Muscle apoptosis as a proposed pathogenetic mechanism. Brain 1990; 113: 433–45.
64. Sarnat HB. Vimentin and desmin in maturing skeletal muscle and developmental myopathies. Neurology 1992; 42: 1616–24.
65. Goebel HH, Bornemann A. Desmin pathology in neuromuscular diseases. Virchows Arch B Cell Pathol Incl Mol Pathol 1993; 64: 127–35.
66. Hamburger V. The developmental history of the motor neuron. Neurosci Res Program Bull 1977; 15: 1–37.
67. Lowrie MB, Vrbová G. Dependence of postnatal motoneurones on their targets: review and hypothesis. Trends Neurosci 1992; 15: 80–4.
68. Navarrete R, Vrbová G. Activity-dependent interactions between motoneurones and muscles: their role in the development of the motor unit. Prog Neurobiol 1993; 41: 93–124.
69. Greensmith L, Vrbová G. Motoneurone survival: a functional approach. Trends Neurosci 1996; 19: 450–5.
70. Greensmith L, Hasan HI, Vrbová G. Nerve injury increases the susceptibility of motoneurons to N-methyl-D-aspartate-induced neurotoxicity in the developing rat. Neuroscience 1994a; 58: 727–33.
71. Greensmith L, Mentis GZ, Vrbová G Blockade of N-methyl-D-aspartate receptors by MK-801 (dizocilpine maleate) rescues motoneurones in developing rats. Dev Brain Res 1994b; 81: 162–70.