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The mdx mouse model as a surrogate for Duchenne muscular dystrophy

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Abstract

Research into fundamental principles and the testing of therapeutic hypotheses for treatment of human disease is commonly conducted on mouse models of human diseases. Although this is often the only practicable approach, it carries a number of caveats arising from differences between the two species. This article is centred on the example of skeletal muscle disease, in particular muscular dystrophy, to identify some of the principal classes of obstacle to the translation of data from mouse to man. Of these, the difference in scale is one of the most commonly ignored and is of particular interest because it has quite major repercussions for evaluation of some classes of intervention and of assessment criteria while having comparatively little bearing on others. Likewise, interspecies differences and similarities in cell and molecular biological mechanisms underlying development, growth and response to pathological processes should be considered on an individual basis. An awareness of such distinctions is crucial if we are to avoid misjudgement of the likely efficacy in man of results obtained on mouse models.

Keywords

Murine disease model; Muscular dystrophy; Interspecies translation; Effects of scale; Double mutants

Introduction

Robert Burns' deft analysis of the fundamental affinities and essential differences between the issues that afflict mice and men [1] gave early notice of questions that have beset preclinical scientific research up to the present. Failure to fully address these matters is evident in the routinely casual use of mice as models of a variety of human conditions. Mice are not men and the converse is generally true too. Yet preclinical validations of therapeutic strategies for human muscle disease are based predominantly on interventions on mouse models of homologous or analogous human conditions. Thus far, the translational flow from mouse model systems to evidence of utility in human trials has been sporadic and feeble [2–5]. A spectacular exposé of the nature of this failure was noted in a recent survey of preclinical studies to identify agents to combat ALS, where 100% (70 of 70) reports of published preclinical experiments were not reproducible in fully powered reiterations [6].

This critique attributed the blame predominantly to failure to distinguish significant effects from experimental noise, with the implication that this problem had arisen from faults in design or conduct of the original experiments, leaving in abeyance the question of the extent to which the model itself provides an accurate simulation of the human condition. Whether this is a more general issue is a matter of conjecture but the strong predisposition of journals towards publication of 'positive' results, together with the associated difficulty of recording dissenting findings and further combined with the appetite of funding organizations for therapeutic promises, are clear inducements to this and other forms of bias.

In the meantime however, it is relatively easy to identify a number of more substantive sources of disjunction between experiments conducted in murine disease models and effective therapies in their human equivalents. Several of these arise from a failure to fully accommodate to the notion that differences between the two species, if individually assessed, give valuable indications as to what is likely to be directly informative for translational purposes and what should be treated more circumspectly. The objective of this article is to identify some of the more intrusive of these issues and to point to potential ways of resolving them, where such exist. As an exemplar, it uses the mdx mouse model of muscular dystrophy, for which an instructive history of use and abuse has accumulated since its first description in 1984 [7]

Effects of Scale

Among the more obvious differences between mice and men is size; man is about 2–3,000 fold larger than the mouse at birth and this ratio difference is maintained into adulthood but the pattern of growth by which this increase is achieved is very different between the two species. Both the absolute size difference and the distinction between the two growth patterns by which this difference is maintained in postnatal life, each carries its own implications.

Square/cube rule

For a mechanical tissue like skeletal muscle, the square/cube rule is a major factor that impinges on mechanisms and relationships across the size difference between man and mouse. Its postulates place the major determinant of stress on this tissue in direct proportion to volume/weight, which is related to the cube of the linear size, while the mechanisms that cope with this stress are dependent on area, which is related to the square of the linear size [8, 9]. Accordingly, in larger animals, the area-dependent mechanisms, such as the connective tissues that transmit and absorb force, become less able to cope with mechanical stress imposed by the extra mass via its two close relatives, inertia and momentum. The resulting need to spread these forces is reflected in the thicker tendons and the presence of dense fibrous septa within the muscles of larger mammals, including man, but not in the mouse. This change in relationship also acts as a general size-related constraint on function – e.g. mice can fall from considerable heights with impunity and elephants cannot jump. A molecular correlate of this difference is the relative lack of expression in larger mammals, including man, of the 'fast' 2B myosin that is widely expressed in mice and other small mammals with fast step-cycles [10].

Such differential effects must be allowed for when considering the implications of any data derived from the mouse that involves mechanical function. Perhaps the most obvious issue is the extent to which physical stress can be regarded as equivalent between the two species. For instance, how does damage to mouse muscle induced by a given exercise regime relate to a similar exercise regime applied to man; can we rely on the mechanisms of damage or the mechanisms whereby putative therapeutic agents attenuate this damage being equivalent in the two species, especially where the effects are subtle? It is true that there is a strong resemblance between mdx mouse and DMD boys in histopathology of the muscle lesions themselves and at least some of the disease-associated changes in the proteome [11]. However, it is probably safest to use such data as indices of damage that permit comparison between therapeutic agents or regimes rather than as direct equivalents of what to expect in human trials.

Diffusion

Another phenomenon that is acutely scale dependent is molecular diffusion; the concentration of any given agent in a freely diffusing environment falls off rapidly with distance from the source. In most biological systems, there are also structural barriers to free diffusion. Of these, some, such as lipid membranes are invariant with scale while others, such as load-bearing partitions in muscles and some basement membranes which cope with the heavier mechanical load by virtue of increased thickness and perhaps denser structure, will impose more severe restrictions on diffusion in larger animals. It is important to note, in this respect, that many human muscles are divided into major compartments by dense fascia that are not present in the equivalent muscles of the mouse.

The interplay between the constraints arising from scale differences at the whole body level and the physiological limitations on cell size and intercellular spacing raises questions of varying impact across a range of situations.

In skeletal muscle, the abundant microvasculature has the potential to provide rapid access of nutrients and systemic signalling molecules as well as efficient removal of waste products and of signalling products generated endogenously within the muscle. This relationship is not very scale-dependent, for the size of muscle fibres and thus the diffusion distance between the centre of the fibre and the nearest capillary is very similar in mice and men. Likewise, within the muscle fibre, the volume of sarcoplasm maintained by each myonucleus, the nuclear domain, lies with a one fold difference across mammals of a range of sizes [12].

Migration

Skeletal muscle, is capable of very rapid and complete regeneration in response to injury [13], a feat accomplished largely, perhaps entirely, by the satellite cells [14–16] that, in the absence of injury, lie dormant between the surface of the muscle fibres and the surrounding basement membrane. The dynamics of this process are influenced by the rate of proliferation of the satellite cells, their capacity to move into sites of muscle damage, and the size of the region of damage. Rates of both myoblast proliferation and motility are very similar in man and mouse but the tasks to be accomplished are highly scale dependent. The muscle most

commonly used for studies of regeneration and for transplantation of myogenic cells in the mouse, the tibialis anterior, has a maximum cross-sectional profile of $\sim 2 \times 4$ mm. Under the best conditions used for cell transplantation so far, it is possible to populate about 70% of this area with regenerated muscle fibres of predominantly donor cell origin from a single injection of half a million myogenic cells [17]. This looks very impressive in the tiny mouse muscle but would be trivial in man. In some ways, the small size of the mouse limits its value as a species in which to explore the spread of cells from a punctate graft site. However, similarly limited yields and dispersion have been observed in grafts made in non-human primates and man [18, 19]. These limitations do not apply so markedly to therapeutic agents that can be delivered via the blood vascular system such as oligonucleotides to promote the skipping of frame-disrupting exons [20–23] expression plasmids encoding dystrophin [24, 25], or exon-skipping constructs [26–28]. For this same reason, the best hope of a cell-based therapy for diffusely distributed myonecrotic diseases such as DMD is offered by a number of classes of myogenic cell of various origins that can be delivered via the blood vascular system [29–34].

Mouse and man grow in different ways

Between birth and adulthood at ~ 12 weeks, the mouse increases in weight from 1 to 30 grams - the human neonate at 2–3 kilograms grows to an adult weight of 60 to 90 kilograms. This 30-fold increase in mass is achieved in quite different ways in the two species, with major impact on the interaction of growth with development and with disease processes.

The C57Bl/6 mouse, among the more commonly used mouse strains, establishes its full complement of muscle fibres in its limb muscles at around birth, a state that is achieved during early foetal development in man [35]. After birth, the mouse, like many other laboratory animals, grows continuously, tailing off to a plateau by around 12 weeks [36], whereas man grows in distinct phases: rapidly in the perinatal period, slowing down during childhood and rapidly again during adolescence up to around 15 years of age in girls and 18 in boys [37]. In both species, this postnatal growth involves two distinct mechanisms: increase in numbers of nuclei per fibre and increase in size of the domain around each nucleus. However the pattern of engagement of these two mechanisms across the growth period is quite different in man and mouse.

In the mouse, addition of new myonuclei to fibres by proliferation and fusion of satellite cells is limited largely to the first 3 post-natal weeks [38]. Thereafter further increase in muscle mass is achieved mainly by progressive enlargement of the domain of each nucleus. The short phase of postnatal growth involving increase in myonuclear number in the mouse is the only time during which expression of the Pax7 gene is required for proliferation of the satellite cells [39]. It also involves a distinct pattern of proliferation whereby each cell division is asymmetric, with one daughter cell fusing rapidly with the adjacent muscle fibre and the second going on to further rounds of cell division [40, 41]. Only in this way can one account for the addition of ~ 100 nuclei/fibre/week in the mouse EDL muscle by the activities of some 8–16 satellite cells [38].

In man, we have relatively little information but what we have, tells us that both mechanisms - increase in myonuclear number and increase in myonuclear domain - each operate simultaneously across the entire 15–18 year growth period [42]. We have no evidence as to involvement of Pax7 gene expression in man, but attrition of telomere length, an index of the number of divisions undergone by a cell, provides some evidence as to the pattern of cell proliferation during post-natal muscle growth. Minimum telomere repeat length diminishes continuously from ~12 to ~ 8 TFR – equivalent to some 45 cell divisions – between birth and adulthood [43]. This is fully consistent with the operation of serial asymmetric cell divisions to account for the 10–15 fold increase in muscle nuclear content between birth and adulthood [42, 44].

We must take these differences into account when comparing the effects of any agent that affects muscle growth in the two species. Similarly, superimposition of any pathological activity would be predicted to have different effects in the context of the two quite different growth patterns in mouse and man. In the specific case of dystrophin deficiency, for instance, there has been much debate about the value of the mdx mouse as a model of Duchenne muscular dystrophy in man. These commonly include the observations that ‘the mdx mouse dystrophy is milder’ - or, alternatively, that it is ‘more severe than DMD’. Since we have no objective measures of pathological severity in either species from which to draw such a comparison, neither view is rationally justifiable.

Nor, for similar reasons, is there any sensible basis for comparing clinical severity. What would be the justification for comparing rates of clinical decline, as has often been done, as a proportion of lifespan? Many processes, such as cell cycle time or rate of cell movement are very similar in absolute terms in the two species. Thus the duration of a period of development and resolution of a necrotic focus, together with associated features of these processes, such as the build-up of fibrous tissue, are also likely to be more comparable in absolute terms; it is unsurprising, therefore, that pathology in a 2 year old mdx mouse would resemble that of a 2 year old boy. Other aspects of pathology will be heavily influenced by developmental stages, which are not equivalently proportioned across lifespan in the two species. Each individual aspect of the pathology is therefore subject to a separate basis of comparison and, pending detailed investigation, there is no particular reason to favour lifespan over absolute time or developmental stage.

However, within the bounds of what we do know, it is possible to speculate sensibly on the likely effects of the different growth patterns on pathological processes in the two species. In the mouse, the Pax7-dependent program of fibre enlargement by satellite cell proliferation and fusion ends at 3 weeks; this is coincident, in the mdx mouse, with the onset of myonecrosis and repair. Whether this timing is more than a matter of coincidence has yet to be determined, but, in any event, it represents a distinct switch in the operation of the underlying myogenic mechanism. Not only is regeneration beyond this time independent of Pax7 expression but it is accomplished by a quite different pattern of proliferation and differentiation; in order for a satellite cell population of 2–4% of muscle fibre nuclei to reconstitute the mass of muscle from which they were derived within the 3 days or so of proliferation observed in acutely damaged muscle [45], they must enter a transit amplifying phase of geometric expansion followed by mass cell fusion. In the mdx mouse, the growth

phase and the subsequent period of regeneration, each based on its own regulatory mechanisms, run consecutively and should not clash with one another (see figure 1). By contrast, in DMD boys the two mechanisms run concomitantly and interference between two such different mechanisms operating in close apposition to one another seems likely to prevent either or both from operating normally. This would help to explain the difference in consequences of dystrophinopathy in the two species and is a likely contributor to the rapid decline in structure and function associated with this disease in man. Such differences should be taken into account in any translation to human trials of data gathered on the mdx mouse. It would be instructive, for instance, when considering how to boost the regenerative response in DMD, to determine which of the two myogenic programs would be best targeted; whether a given strategy should aim to augment the growth program or the regeneration program of DMD muscle. To the extent that the two processes are temporally separated in the mdx mouse, this model is likely to provide the most useful information as to the mechanisms available for intervention and whether there is likely to be interference or synergy between the two under the influence of any given agent.

Similarly, for cell-transplantation therapies, the choice of cell-type would be dictated by which myogenic process, growth or regeneration, would be most effectively targeted; again, a question that would be most readily addressed at the preclinical level in the mdx mouse.

Strictly, the square/cube rule runs on the assumption of identical proportionality across the comparison but this too varies, in a partially size dependent manner, between species. It has been observed, for instance, that the pattern of growth, especially in terms of increased length of the muscle fibres varies between muscles and between species, due to differences in proportionate growth of the skeleton with consequent differences in stress on the sarcolemma and its relationship with the adjacent connective tissue elements. This issue deserves special consideration in limb muscles, which are the objects of most function studies [46].

Differences in Cell Biology & Pathology

For preclinical research into Duchenne muscular dystrophy, the convenience and cost effectiveness of mdx mouse leaves it as the predominant model. This convenience is partly counterbalanced by the fact that the mdx myopathy is plainly not an exact reproduction of DMD. I would argue that this is not so gross an inconvenience if one becomes aware of the nature of the differences, and so can make allowance for them in the design and interpretation of any preclinical study. Indeed, the differences are themselves a source of information as to which characteristics are fundamental features of the pathological consequences of the lack of dystrophin and which are attributable to interspecies differences.

Among the more widely important questions is that of differences in pharmacological and inflammatory mechanisms between species. An illustrative example with particular relevance to myonecrotic disease is osteopontin: a multifunctional component of the interstitium that is secreted by a variety of cells, including myogenic cells and inflammatory cells. The splice-isoforms of this protein have been reported to manifest a range of effects on inflammation [47, 48], fibrosis [49] and fusion of myogenic cells [50]. Moreover, in the mdx

mouse, knockout of the osteopontin gene has been associated with a milder phenotype [49]. Puzzlingly, in DMD boys, a polymorphism in the promoter of osteopontin that is associated with a lower level of transcription of the osteopontin gene is predictive of a more severe course of disease. Still more puzzlingly, this same polymorphism is associated, in healthy women, with a greater than normal muscle growth in response to exercise [51] and, in the same vein, the osteopontin null mutation is associated with larger muscles in female mice. This complexity is exacerbated by the functional variation associated with the variety of osteopontin isoforms arising from differential splicing and post-translational modifications [52–56]. Our current lack of detailed information on the different isoform expression profiles or of their roles in the inflammatory and muscle repair processes in either man or mouse should counsel caution when translating data derived from the mouse to clinical trials in man.

Is there a perfect animal model?

Animal rights activists are fond of pointing out that animal models do not respond to potential therapeutic agents in the same way as man; they are quite correct. The nearest we have to a perfect model of a human disease is man but even man is not perfect, as demonstrated by the not infrequent withdrawal of drugs or their limitation to restricted patient subsets: reflections of the inter-individual variability within the human population. For purposes of preclinical development, apart from the intervention of ethical regulators, the main problem with man, at a practical level, is the limitation on the invasiveness of inquisitorial techniques that may be contemplated. In the case of DMD, for example, multiple serial muscle biopsies for evaluation would not pass ethical consideration as part of any practical experimental plan and, as yet, non-invasive imaging techniques lack the acuity to be adequately informative. We are left, therefore with animal models in which to explore the pathogenic mechanisms and to test potential therapies up to the point of establishing basic principles.

As a preclinical model of DMD, clinicians tend to favour the GRMD dog because it shows a severe clinical phenotype that results in premature death [57, 58]. However, the extent to which these resemblances to DMD in man reflect basic similarities in pathology requires individual evaluation. One notable difference is that a proportion of GRMD pups are severely affected at birth and die as neonates. In addition, those GRMD dogs that live beyond the first year often survive, in an ambulant state, into canine middle age [59, 60]. Neither phenomenon is reported in DMD boys. The dog also poses the practical problem that there is large inter-individual variation that makes it both time-consuming and expensive to generate sufficient numbers for the conduct of a preclinical trial. Up to now, it has been used primarily as a reference species in which to test putative therapeutic agents and protocols that have previously been shown to be of interest by virtue of demonstrations of efficacy in moderating a few standard outcome measures in the mdx mouse [21, 61].

Improvements in the usefulness of the mdx mouse have been approached by breeding it onto other genetic backgrounds. When first attempting grafts of myogenic cells into dystrophic muscle, we bred the mutation onto a nude background to minimize immune rejection problems [62]. While useful, we later discovered that the nude background, also had

profound effects on the deposition of collagen both during development in non-myopathic and mdx mice and, in the latter, over the course of the disease [63], raising questions as to the suitability of this double mutant for investigations of long-term pathogenesis.

Similarly, with the discovery of the autosomal homologue of dystrophin it was found that animals null for both the dystrophin and utrophin genes displayed a much more severe pathology than the single mdx mutant [64]. This has proven a sensitive indicator of replacement of dystrophin function, by dystrophin itself or utrophin or other surrogates in number of studies [27, 65, 66] but, again, the extent to which this animal parallels DMD in terms of the pathogenic mechanisms has yet to be ascertained.

More recently another issue, the very long telomeres in mice, which confer an almost unlimited proliferative lifespan to their stem cells, by comparison with man [67–69], has been tackled by breeding a null telomerase mutation into the mdx mouse [70]; again with the finding of a worsened pathology that is attributed to decline in proliferation potential of stem cells, but with clear indications, such as increased fragility of mature myofibres, that this is not the entire story. Each of these modifications of the mdx genotype informs us of a molecular biological complexity that counsels caution in evaluating the applicability to man of data obtained from these animals. Perhaps the most instructive example is the double MyoD-null mdx mouse, where the pathology both in skeletal and cardiac muscle is greatly exacerbated compared with the single mdx mutants. Activation of JNK-1/P38 pathways are implicated in both skeletal and cardiac muscle of the double mutants and, but for the lack of MyoD expression in the heart, we would have no *a priori* reason to separate the pathological mechanisms in the two tissues. The poor condition of the skeletal muscle is relatively simply explained in terms of disturbance of regeneration due to lack of MyoD in satellite cells; but, since MyoD is never expressed in the heart, the very severe cardiac pathology cannot entail any direct interaction of the two molecular pathways, forcing invocation of indirect, systemic pathogenic mechanisms, presumably triggered by the regenerative disturbances in the skeletal muscle [71]. It is salutary to speculate on what we would do if MyoD **were** expressed in the heart; application of the purely procedural principle of Occam's razor would content us with a hypothesis that directly implicated the lack of MyoD function in the cardiac dystrophy and the more complex possibilities of systemic interplay would remain unexplored. How often does a predisposition to interpret any interaction of genotypes in disease models in terms of direct interaction of molecular pathways leave a more comprehensive hypothesis in limbo?

For DMD research, the standard mdx mouse with a nonsense mutation in exon 23 has been by far the most used animal model. Its main defects as a model are the fact that it has an almost normal lifespan and human perception registers only minor clinical dysfunction for much of this period; perhaps another mouse might see things differently.

The popularity of the laboratory mouse as a model of human myopathic conditions is a reflection of its convenience, and of the availability of spontaneous or imposed disease-causing mutations together with genetic markers of cell type and cell provenance within the species. A particularly useful mdx mutation is the recently available knockout of exon 52 [72], which has permitted preclinical investigation of the effects of exon-skipping in the

region that is most appropriate to the majority of human DMD mutations including the holy grail of skipping exons 45–55 which is predicted to generate a very mild phenotype [73]. These advantages, combined with the relatively low maintenance costs, permit the conduct of fully statistically powered preclinical trials [74]. The main problem, as belaboured above, is the discrepancy between the two species in size, in pathophysiology and clinical outcome criteria. This is a major problem only if investigations on the mdx mouse are treated simplistically as rote procedures. It can be largely resolved by a little thoughtful analysis to distinguish those features that are common to man and mouse, those that differ in detail between the two species but may carry some transferable information and those that are likely to be peculiar to mouse or man. Even this last category, perhaps especially this category, of species-specific effects arising from lack of dystrophin may well give new and unexpected insights.

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Post-natal Muscle Growth in Man & Mouse

Figure 1. This depicts the contrasting temporal relationships between the two myogenic programs underlying post-natal growth and muscle repair in mouse (A) and Duchenne boys (B)

A. The dystrophic mouse completes the phase of postnatal growth that adds new myonuclei to muscle fibres by 21 days. This phase is dependent on expression of the Pax7 gene and involves serial asymmetric division by the satellite cells, one daughter of each mitosis leaving the cell cycle and fusing rapidly with the adjacent muscle fibre, the second remaining proliferative and going on to further asymmetric divisions. In the mdx mouse, a period of florid myonecrosis begins as the three week growth period ends. Repair of necrotic lesions must be not dependent on Pax7 expression and entail a quite different mode of cell proliferation, whereby after an early asymmetric division, there ensues a rapid phase of symmetric division within a transit amplifying population of myogenic cells largely committed to terminal differentiation.

B. In man, the increase in myonuclear number occurs throughout the childhood, juvenile and pubertal growth periods and reduces telomere length at a rate that is consistent with serial asymmetric cell division – whether it requires Pax7 expression is unknown. In DMD boys, this period of growth is coincident with the chronic repair of myonecrotic muscle fibres by the regenerative mode of cell division in which rare asymmetric divisions maintain a stem-

like subpopulation but the majority of cell divisions are devoted to a geometric, transit-amplifying pattern of expansion.

It is suggested that these two different mechanisms interfere with one another in DMD boys but not in mdx mice

Abbreviations

TRF: telomere restriction fragment.

mdx: a mouse carrying a null mutation of the dystrophin gene

GRMD: Golden Retriever Muscular Dystrophic dog, carrying a splice-site mutation in exon 7 of the dystrophin gene that causes omission of exon 7 from the dystrophin transcript, that alters the open reading frame and prevents productive translation into a protein.