J.Cell.Mol.Med. Vol 5, No 1, 2001 pp. 33-47

Invited Review

Problems and solutions in myoblast transfer therapy

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Received: March 5, 2000; Accepted: March 16, 2000

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Abstract

Duchenne muscular dystrophy is a severe X-linked neuromuscular disease that affects approximately 1/3500 live male births in every human population, and is caused by a mutation in the gene that encodes the muscle protein dystrophin. The characterization and cloning of the dystrophin gene in 1987 was a major breakthrough and it was considered that simple replacement of the dystrophin gene would ameliorate the severe and progressive skeletal muscle wasting characteristic of Duchenne muscular dystrophy. After 20 years, attempts at replacing the dystrophin gene either experimentally or clinically have met with little success, but there have been many significant advances in understanding the factors that limit the delivery of a normal dystrophin gene into dystrophic host muscle. This review addresses the host immune response and donor myoblast changes underlying some of the major problems associated with myoblast-mediated dystrophin replacement, presents potential solutions, and outlines other novel therapeutic approaches.

Keywords: myoblast • Duchenne muscular dystrophy • dystrophin gene • dystrophin • myoblast transfer therapy • stem cells • gene therapy

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Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disease that results from a mutation in the gene encoding the muscle protein dystrophin [1, 2]. The dystrophin protein is located just beneath the sarcolemma of skeletal myofibers [3], and its aberrant expression or absence in DMD results in severe and progressive skeletal muscle wasting. Many studies have attempted to correct DMD using a cell-mediated form of gene replacement therapy referred to as myoblast transfer therapy (MTT), where muscle precursor cells (myoblasts) extracted from normal (dystrophin-positive) donor muscle are injected directly into the muscles of dystrophic (dystrophin-negative) hosts (Fig. 1). Soon after dystrophin was characterized, clinical MTT studies were initiated in the USA and Italy but resulted in little or no restoration of dystrophin expression and/or muscle function [reviewed in 4]. In contrast to the majority of clinical trials, one group claimed success [5] although these studies were controversial [6] and MTT has now been discontinued in the USA (http://www.fda.gov/foi/nidpoe/n9l.pdf). For MTT to be successful, it is essential for donor myoblasts to survive, proliferate, migrate away from the transplantation site, fuse with myofibers and express a functional dystrophin molecule. However, for each of these aspects there are certain problems that must be overcome before MTT can be re-applied to the clinical situation. This review summarizes the current status of MTT. Due to space restrictions only the latest or most relevant papers are cited.

Donor myoblast survival

At this stage, the major limiting factor in MTT is that most injected cultured donor myoblasts die very rapidly (i.e. within 1 week) after being injected into either normal or dystrophic host muscle [7-10]. Donor myoblast survival is improved in hosts that are immunosuppressed or immunocompromised [10, reviewed in 11], strongly indicating a role for the host immune system in donor myoblast death. Identification of specific components of the host immune system that are involved is of central interest for improving MTT. In addition, the properties of cultured donor myoblasts that are responsible for initiating the rapid and severe host immune response must be considered. Thus, the specific factors contributing to donor myoblast death in MTT will be discussed from two perspectives; (i) the components of the host immune response, and (ii) the properties of the donor myoblasts themselves that provoke this immune response.

Host Immune Response

The death of donor myoblasts is extremely rapid and severe, with over 90% of myoblasts being eliminated within 1 hour [8] and the majority of these being killed within minutes of injection [10]. An intense, cell-mediated host immune response appears to be largely responsible for the rapid and massive death of injected cultured donor myoblasts [10-12] and while several components of the host immune system have already been investigated for their role in donor myoblast death (summarized in Table 1), many still remain to be tested. Those that are likely to be involved include components of both the cellular and humoral arms of the immune system, but the former is the main focus of this review.

Cellular immunity

The cell-mediated component of the immune system is composed of many different cell types, several of which are present in normal and/or dystrophic skeletal muscle, and others that infiltrate skeletal muscle rapidly after the injection of cultured donor myoblasts (Table 2) [reviewed in 11]. However, surprisingly few studies have examined the involvement of specific cell types in limiting the success of MTT. Depletion of host CD4+ and CD8+ T-cells, or NK1.1+ cells (a population of natural killer, or NK, cells) using specific monoclonal antibodies revealed that initial depletion of host CD4+ and CD8+ cells, or NK1.1+ cells, enhanced donor myoblast survival by up to 20-fold at 7 days [10]. There was no additional benefit of sustained depletion (i.e. continuous administration of depleting antibodies) of CD4+/CD8+ cells, NK1.1+ cells or a combination of CD4+/CD8+/NK1.1+ cells for up to 3 weeks after MTT [S. Hodgetts, manuscript under

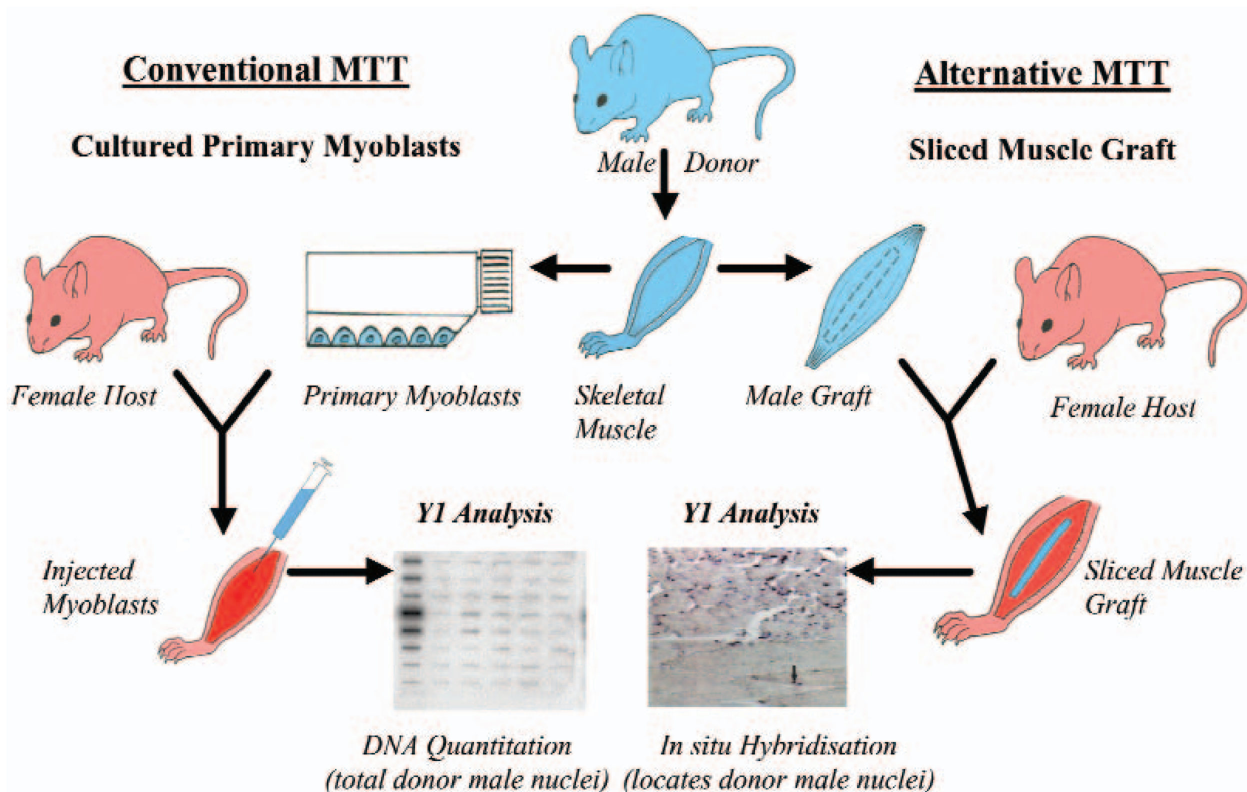

Fig. 1 Conventional (injected cultured myoblasts) and alternative (sliced muscle grafts) methodology for studying myoblast transfer therapy.

review]. Antibody depletion of host CD4+ and CD8+ cells is also more effective than the immunosuppressant FK506 at promoting the survival, migration and fusion of donor myoblasts from histocompatible sliced muscles grafted into dystrophic (mdx) host mice, and the survival of histoincompatible (major histocompatibility complex (MHC)-mismatched) donor myoblasts from sliced muscle grafts is facilitated (but significantly less than for histocompatible donors) for up to 12 weeks in host mice [13, 14]. However, it must be considered that for successful long-term survival of donor myoblasts in MTT it may be necessary to use blocking (non-depleting) antibodies in addition to the depletion regime to remove/block all T-cells at the onset, as this is required to ensure long-term (> 10 weeks) survival of skin allografted across major MHC barriers [15]. Furthermore, emerging data strongly implicate the early or immediate response inflammatory components of the host immune system in the rapid and massive death of injected donor myoblasts, and point to some cellular response even earlier than that of NK cells, since 40-50% of donor myoblasts still die within minutes in NK1.1+ depleted hosts [10].

Other depletion/blockade studies support a direct interaction between host immune cells and donor myoblasts (see also Fig. 2). Blockade of the B7-CD28 co-stimulatory pathway using CTLA4-Ig promotes the long-term survival of donor myoblasts in mice, although this is only successful when combined with CD4+ T-cell depletion [16]. This observation is not surprising, since T-cell activation (via the T-cell receptor on host T-cells and MHC antigens on target cells) requires the concomitant activation of one or more co-stimulatory pathways [reviewed in 11]. Similarly, antibody blockade of leukocyte function antigen-1 (LFA-1) on host immune cells (mainly leukocytes and NK cells), which binds to intercellular adhesion molecule-1 (ICAM-1) on the surface of target cells, slightly improves donor myoblast survival [12]. An LFA-1/ICAM-1 mediated interaction between host cells and donor myoblasts is further supported by the

Table 1 Some factors that have been tested for involvement in the rapid death of donor myoblast after injection into host muscle

Factor Host derived	**Results**	**References**
Complement	Not involved. C3 involved but not C5	[24, 25]
NK cells	NK depletion dramatically enhances donor myoblast survival	[10, 48]
Neutrophils	Massive neutrophil infiltration after MTT (and injury). Role in myoblast death unclear	[12, 21, 23]
T-cells	T cell depletion enhances cultured donor myoblast survival, as well as migration away from sliced muscle graft transplantation site	[10, 12, 14, 16]
Macrophages	Macrophage invasion follows neutrophil infiltration during inflammation	[23]
Dendritic cells	Not tested quantitatively	[19, 20]
Factor Donor-derived/Other	**Results**	**References**
ICAM-1/LFA-1	Treatment with anti-LFA-1/ICAM-1 antibodies enhances donor myoblast survival	[12, 18]
MHC I	MHC class 1 expressed on donor myoblasts - role of altered MHC expression not tested	[10, 11, 36]
Tissue culture	Massive and rapid death of donor myoblasts after MTT using (i) freshly cultured donor myoblasts (ii) those passaged numerous times in culture, or (iii) myoblasts taken from stored frozen stocks. Culture process prime candidate for myoblast antigenicity	[10]
	Exposure of muscle grafts to tissue culture components reduces graft viability and migration. Proteases and serum particularly deleterious	[39]
Injection technique	Rapid and massive death of injected cultured donor myoblasts	[10]
	Similar death after myoblast implantation in fibrin clot	[8]
	Grafts of (non-cultured) "equivalent" muscle tissue survive up to 1 year following transplantation	[38]

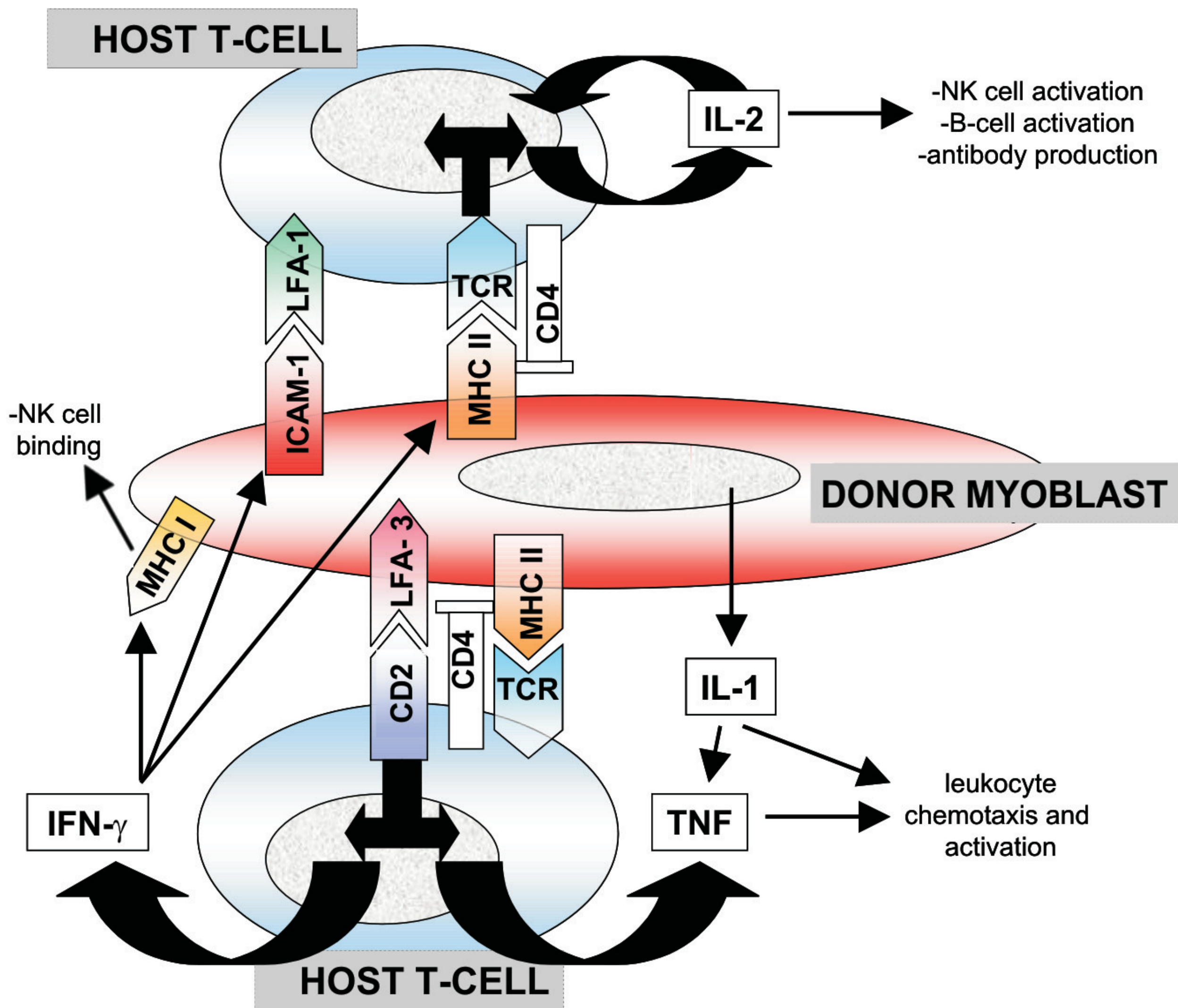

Fig. 2 Major potential mechanisms resulting in the interaction between injected cultured donor myoblasts and host T-cells. The secretion of interleukin-1 (IL-1) by donor myoblasts can have a multitude of effects including the stimulation of tumour necrosis factor (TNF) production by macrophages (not shown), and leukocyte chemotaxis. The expression of MHC class II antigens by donor myoblasts enables them to directly interact with host T-cells via the T-cell receptor (TCR), an interaction in which CD4 acts as an accessory molecule. In addition, the expression of leukocyte function antigen-3 (LFA-3) by donor myoblasts can act in a co-stimulatory capacity (by ligating CD2) to promote host T-cell activation. LFA-3/CD2 binding has been shown to induce TNF production (which can further promote leukocyte chemotaxis and activation), and interferon-γ (IFN-γ) production which may be able to upregulate MHC class I and/or II and ICAM-1 expression on donor myoblasts, providing further opportunity for interaction with host immune cells (T-cells and NK cells).

observed constitutive expression of ICAM-1 by cultured myoblasts [17] (Fig. 2). Transient immunosuppression using anti-ICAM-1/LFA-1 antibodies also enhanced survival of donor myoblasts in an earlier study [18]. Cultured myoblasts express LFA-3 at higher levels than ICAM-1, providing another mechanism by which these cells may interact directly with host immune cells [17] (Fig. 2). Several potential interactions between donor myoblasts and host T-cells via their expression of cell surface receptors are illustrated in Fig. 2.

Other candidate host cell types involved in donor myoblast death (see Table 2) include dendritic cells, neutrophils, mast cells and macrophages. Dendritic cells are present in skeletal muscle [19], and their ability to regulate NK cells, elicit specific

Table 2 Host immune cell types potentially involved in donor myoblast death following MTT

Immune Cell Type	Resident in normal skeletal muscle	Resident in dystrophic skeletal muscle	Infiltrate skeletal muscle after MTT	Role tested in MTT
Neutrophils	Circulating only [16, 87] Not resident in dogs [88]	Circulating only ?	Rapid infiltration [12]	MTT enhanced using anti-CTLA4 Ig and LFA-1 antibodies [12, 16]
NK cells	Circulating only ?	Circulating only ?	Not tested	MTT enhanced using anti-NK antibodies [10]
T cells (CD4/8)	Circulating only [11] Not in mouse [89] or dogs [88]	Circulating only ? [11, 90, 91] Not in mouse [89]	Rapid infiltration [10; 89, 91]	MTT enhanced using anti-CD4/8 antibodies [10, 13, 14]
B cells	Circulating only	Circulating only [90]	Circulating only [89]	Not tested
Macrophages	Resident [19]	Resident and increased [90]	Resident and increased [89]	Not tested
Dendritic Cells	Large population [11, 19]	Unknown - presumed present	Not tested	Not tested
Mast Cells	[11, 92]	[11, 92]	Not tested	Not tested
Eosinophils	Unknown	[93]	Not tested	Not tested

? = unknown but probably present in circulation through muscle, Resident = cells present in interstitial connective tissue rather than in vasculature, Not tested = unknown. References are indicated in square brackets

T helper cell responses and recognize and destroy a target cell within seconds [reviewed in 20] makes them a prime candidate for rapid donor myoblast death in MTT. Neutrophils are also prime suspects as they represent up to 70% of circulating leukocytes in mice and are very rapidly sequestered (within minutes) during the inflammatory response [21]. Mast cells are resident in muscle tissue, implicated in DMD disease progression [22], secrete many substances that may be highly destructive to donor myoblasts, and can degranulate (releasing these substances) within seconds of encountering a foreign entity. Macrophages are also potentially involved and are resident in muscle tissue [19], however their response is less rapid than neutrophils as they accumulate after neutrophil infiltration [23]. The roles of these cells in donor myoblast death in MTT remain to be investigated.

Humoral immunity

Complement: The rapid death (within minutes) of donor myoblasts after injection into host muscle is suggestive of a non-specific immune response, primarily the complement system (Table 1). Although one study concluded that host complement depletion did not significantly influence the outcome of MTT [24], a recent study where activated complement component C3 was depleted using cobra venom factor and complement C5 deficient (DBA-2) mice were used, demonstrated a role for host C3, but not C5, complement on the immediate and short-term survival of donor myoblasts [25]. This may be due to myoblast expression of protectin [26], which prevents activation of the C3 component of the complement cascade, however we showed that while administration of cobra venom factor depleted peripheral complement, this treatment did not significantly affect tissue, specifically muscle, complement levels [25]. It has been suggested that complement is indirectly involved, by influencing the infiltration of host immune cells (*e.g.* neutrophils) into the host muscle after injection [24].

Anti-dystrophin antibodies: Antibodies to dystrophin have also been demonstrated in dystrophic mice [27] and DMD boys [28] following MTT. This shows a host immune response specifically to donor dystrophin, which is recognized as a foreign antigen since the full-length dystrophin gene is not present in dystrophic hosts. Dystrophin is usually considered to be present only in myotubes and myofibers (where a basement membrane is formed) yet a recent paper [29] indicates that it is also expressed in myoblasts. This issue requires further investigation since the presence of this “foreign” protein in myoblasts may contribute to their rapid death. It is well documented that “revertant” dystrophic myofibers can express a truncated form of dystrophin [30, 31]. Furthermore, within individual dystrophic hosts, many different truncated isoforms of dystrophin can be present [31]. Lu *et al* [31] proposed the novel idea of producing donor myoblasts that are genetically engineered to express only truncated dystrophin isoforms (already produced by revertant myofibers within an individual host), in order to circumvent the problem of donor myoblast rejection on the basis of the presence of the full-length dystrophin protein. The successful implantation of such genetically modified donor myoblasts *in vivo* was reported [31]. Although the aim of this method is not to completely alleviate DMD symptoms, there was evidence of conversion to the less severe Becker muscular dystrophy phenotype. Non dystrophin-based alternatives to MTT are designed to avoid this immune problem (see **Alternatives and novel approaches** below). Interestingly, not only can antibodies to dystrophin be a problem, but host antibodies specific for fetal calf serum, a reagent used in the culture of myoblasts, have been detected in blood samples of mice soon after MTT [32], implicating a long-term detrimental effect of the tissue culture process. This point is addressed further in the following section.

Cytokines: The host cytokine environment may also have a major role in the death of donor myoblasts. Many host immune cells already present in normal and dystrophic muscle produce specific proinflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFNγ) [reviewed in 11]. TNFα inhibits myoblast differentiation and fusion *in vivo* [33] and degrades muscle cytoplasm during skeletal muscle necrosis and regeneration [34]. TNFα is a major product of mast cells, an important cofactor in NK cell activation, induces secretion of IFNγ by NK cells [35] and also activates T cells to produce IL-2 and IFNγ which can further activate other T (and B) cells [reviewed in 11]. Recent studies in our laboratory demonstrate that skeletal muscle regeneration

is normal in TNFα null and TNFα/lymphotoxin-α null mice, suggesting a redundancy in *in vivo* cytokine signaling so that other cytokines can compensate for the lack of TNFα [R. Collins, manuscript under review]. Studies are underway in our laboratories that specifically address the role of TNFα in the behavior of donor myoblasts in MTT.

Donor myoblasts themselves may be directly responsible for provoking the immune attack. Expression of class I MHC antigens on cultured myoblasts (see below) is altered by effectors such as IFNγ and TNFα [36]. Cultured human myoblasts also express IL-1α, IL-6, plus the chemokines IL-8 and RANTES constitutively, as well as IL-1β, TNFα and the chemokine MCP-1, after induction with the pro-inflammatory cytokines IFNγ and TNFα [37]. The secretion of such molecules by cultured donor myoblasts themselves would probably be a profound stimulus to the host immune response against these myoblasts. Strategies designed to enhance the survival of donor myoblasts may involve the abrogation of cytokine interactions, for example; cultured donor myoblasts genetically engineered to express a factor that competes with, but does not activate, the receptor for IL-1 have demonstrated an improved short-term survival rate after injection into host muscle compared with normal donor myoblasts [9].

The influence of cytokine production on MTT is undoubtedly multifactorial and complex. The potential effect of differing inflammatory cytokine profiles produced by host cells (*in vivo*) and donor cells (*in vivo* and/or *in situ*) following MTT is evident in the long term survival of sliced muscle grafts transplanted into host mice compared with the rapid death of injected isolated donor myoblasts [14].

Donor myoblasts and the question of histocompatibility

In marked contrast to the rapid death of isolated cultured injected myoblasts, the transplantation of segments or whole untreated grafts of equivalent (non-cultured) donor muscle (see Fig. 1), does not provoke this host response and the donor myoblasts survive (for at least 1 year) in histocompatible hosts [38]. This strongly suggests that the host immune response occurs because the "histocompatibility" of donor myoblasts is in some way altered as a result of the myoblast isolation and/or tissue culture.

In a recent study we demonstrated that certain steps involved in the tissue culture process can render histocompatible muscle incompatible with the host environment [39]. Whole muscle grafts were pre-incubated in various tissue culture reagents prior to transplantation into fully histocompatible hosts and grafts examined up to 7 days. Exposure of grafts to serum or proteolytic enzymes (even for 10-20 minutes) was particularly deleterious to the subsequent survival of myoblasts *in vivo.* The adverse effects of serum are further emphasized by the presence of antibodies specific to fetal calf serum in response to MTT [32]. Since protease treatments such as trypsin are routinely used to remove myoblasts from culture flasks for passaging and injection, this adverse effect of proteases on cells subsequently exposed to the *in vivo* environment provides a ready explanation for the failed clinical trials of MTT and may also be highly relevant to the transplantation of other cultured cells (*e.g.* pancreatic islet cells). Proteolytic cleavage of surface proteins may result in the expression of neo-antigens on the myoblast cell surface [36] that elicit an acute phase host reaction, and various factors such as fibroblast growth factor (FGF) [4, 40] and leukemia inhibitory factor, [41] may protect against, or modify, these deleterious effects of tissue culture when cells are transferred to the *in vivo* environment. Proteolytic disruption of integrins and other key cell surface proteins that bind the extracellular matrix (ECM) may adversely affect the behavior of transplanted donor myoblasts *in vivo*. Defects in integrin α7β1 result in clinical myopathies [42], and aberrant expression of integrin α5 disrupts myoblast adhesion [43]. It is well recognized that complex interactions with ECM molecules play a critical role in the proliferation, migration and fusion of myoblasts during myogenesis [44, 45] and such interactions may also play a crucial role in the initial survival, proliferation, migration and fusion of isolated cultured myoblasts *in vitro*.

MHC Expression: Altered or absent MHC class I expression on donor myoblasts as a direct result of tissue culture [39, 40] may provoke a response by NK cells, since NK cells recognize target cells via a mechanism that is inhibited by expression of MHC class I molecules [46]. Changes in MHC expression

have been described in muscle cells in tissue culture [36, 47]. In particular, the non-classical MHC antigen HLA-G is expressed in muscle fibers in various inflammatory myopathies, and in cultured myoblasts following treatment with IFNγ [47]. The absence or altered expression of MHC class I on myoblasts may have very serious consequences as this can lead to rapid (less than 1 minute) recognition by NK cells and subsequent destruction of donor cells within minutes [48]. It is proposed that induction of MHC overexpression using molecules such as IFNγ or insulin-like growth factor 1 (IGF-1) [47] might protect donor myoblasts from death by NK cells.

Stem Cells: There is also compelling evidence that superior *in vivo* survival occurs with a very small sub-population of cultured donor myoblasts that are potential stem cells, suggesting that the problem of donor myoblast death might be circumvented by injecting only purified preparations of such myogenic stem cells [49]. It is possible that such "quiescent" stem cells are less affected by the tissue culture process and therefore exempt from the adverse immune response. The hunt is on to find markers to distinguish different myoblast populations; useful markers identified to date are desmin [9, 50], CD34 [50], CD34 and Bcl-2 [50], and CD34 and myf5 [51]. The $CD34^{+}/Bcl\text{-}2^{+}$ muscle-derived cells were reported to have "stem cell" properties since they were capable of entering multiple tissue lineages [50]. The use of myogenic stem cells, either as a sub-population of myoblasts or derived from non-skeletal tissues such as bone marrow [52], is currently attracting much attention [53] as an alternative to conventional MTT. One crucial issue is the extent to which such myogenic "stem cells" can continue to replicate *in vivo*.

Donor myoblast proliferation and migration

Clearly, migration of normal donor myoblasts away from the injection or transplantation site will facilitate re-population of the defective host muscle by the dystrophin-positive donor muscle nuclei in MTT [4]. While most studies to date have addressed the issue of myoblast survival, attention is now also focusing on factors that influence donor myoblast proliferation and migration. These processes *in vivo* will be discussed together.

Many growth factors/cytokines are involved in the regeneration of skeletal muscle [53]. Such factors can affect the migration of myoblasts [54, 55] and also their proliferation and fusion [41]. Basic FGF is a potent mitogen for myoblasts and administration to cultured myoblasts is reported to significantly increase their survival after injection [56], although it is not clear whether this is due to modification of the cultured myoblasts (discussed above) or subsequent myoblast proliferation.

IGF-1: Another promising candidate to increase myoblast proliferation is insulin-like growth factor-1 (IGF-1), which promotes myoblast proliferation and differentiation, and a striking increase in skeletal muscle mass and strength is seen in transgenic mice that overexpress IGF-I [57]. This hypertrophy is similar to that demonstrated with viral-mediated IGF-1 overexpression [58]. The extent to which such IGF-1 overexpression could enhance conventional MTT or stem cell based therapy is under investigation.

MyoD null mice: Another approach is to use donor myoblasts that have enhanced levels of proliferation and/or migration as a result of genetic engineering. The proposal that myoblast proliferation will indeed enhance migration is supported by recent experiments using MyoD null mice. Myoblast proliferation is sustained and fusion delayed by 2-3 days in myoblasts that lack the myogenic regulatory factor MyoD [59-61]. Based on this information we have recently shown that (i) long-term new muscle formation in regenerating whole muscle grafts of MyoD null mice is not impaired [61], and (ii) there is a beneficial effect of sustained proliferation on donor myoblast migration using sliced MyoD null grafts [Smythe and Grounds, manuscript under review]. While such results support the principle that sustained myoblast proliferation will assist migration they do not endorse the use of such mutations in donor myoblasts prior to transplantation. However, they do form a strong foundation for future studies using myoblast mitogenic and/or chemotactic agents in combination with MTT.

Another approach to improving donor myoblast migration between myofibers is to reduce or modify the interstitial connective tissue barriers by expression of enzymes that degrade the ECM.

Ito and colleagues [62] demonstrated that pre-treatment of cultured donor myoblasts with concanavalin A results in enhanced migration into mouse host muscle *in vivo*: this was attributed to increased expression of matrix-degrading enzymes by donor myoblasts; however, increased donor myoblast survival and proliferation was also reported suggesting that enhanced migration might only be a secondary effect. The same group subsequently examined the effects of either engineering donor myoblasts to express matrix-degrading enzymes [63] or pre-treating host muscle with these enzymes prior to donor myoblast injection [64]. In the former study, donor myoblast fusion with host myofibers was enhanced, but there was no improvement in migration, while in the latter enhanced donor myoblast migration was only demonstrated when notexin was also used to induce host muscle degradation. Such approaches using ECM degrading enzymes or muscle injury to improve donor myoblast dissemination *in vivo* are interesting, but it is essential that the effects of such rigorous treatment on dystrophic muscle (which is highly vulnerable) be determined before such strategies are considered for clinical application.

We have also demonstrated enhanced donor myoblast migration in host mice that have been depleted of CD4+/CD8+ T-cells, or treated with FK506 [14] and this was not due to an increased overall number of donor myoblasts within the host muscle. The cellular basis for this enhanced migration is unclear but these studies indicate that such treatment regimes may have the dual role of promoting both donor myoblast survival and migration *in vivo*.

Donor myoblast fusion

The definition of "successful" MTT is dependent on the question of how much dystrophin is required to alleviate the clinical symptoms of DMD. Karpati *et al* [65] and Chamberlain [66] determined that 10-20% of normal dystrophin expression within an individual fiber is required, as compared with Hauser *et al* [67] who report that 50% of myofibers must be dystrophin-positive. These requirements are far greater than most MTT studies to date that report only very low (≤1%) numbers of dystrophin myofibers [reviewed in 52].

Nuclear domain of dystrophin expression: One parameter that will dictate how many donor myoblasts must fuse with host myofibers and the distance between donor (dystrophin-positive) nuclei within individual host myofibers, is the dystrophin nuclear domain. This is defined as the distance from the encoding nucleus that protein expression can be found, and for dystrophin was illustrated in a recent *in vivo* study using co-cultures of normal and dystrophic myoblasts [29]. This dynamic domain feature of dystrophin expression was also related to restoring the organization of dystrophin-associated proteins and acetylcholine receptors to hybrid myotubes. If dystrophin expression from the encoding nucleus is limited, as indicated by overexpression studies in transgenic mice [68, 69], then the number of donor nuclei fused with host myofibers, and the distance between them must also be considered.

Alternatives and novel approaches

Viral delivery of dystrophin: Several alternatives to classical MTT have been examined in order to circumvent the problems of limited donor myoblast survival and migration. The gene therapy revolution of recent years provided one such potential alternative by the use of viral vectors to deliver the dystrophin gene to DMD muscles. However, this approach is also problematic due to the sheer size of the dystrophin gene, and because uptake of the virus by muscle cells has proved very difficult [67]. Adeno-associated viruses are one of the safest and most efficient viral vectors, but they are restricted to an insert capacity of 5kb [70] and, at 14kb, the dystrophin gene is one of the largest in the human genome. Therefore, several studies have used adeno-associated virus-mediated transfer of truncated dystrophin gene isoforms, mini-dystrophin, to improve muscle pathology in dystrophic mice [70]. Adenovirus-mediated dystrophin gene therapy has been tested *in vivo* in mice and dogs, although this is subject to a strong host immune response specific for both the adenoviral construct and the donor dystrophin [71], and adenoviruses can have a toxic effect on muscle that cannot be prevented with immunosuppression [72].

Ex-vivo gene therapy: In order to circumvent the problem of immunological rejection of "for-

eign" donor myoblasts (and viruses), it has been suggested that autologous "genetically corrected" donor myoblasts be used [73, 74]. Myoblasts obtained from a dystrophic (mdx) mouse have been transfected "*ex vivo*" with a truncated form of the human dystrophin gene then transplanted back into mdx host muscle [74]. Although some donor myoblasts survived for up to 24 days, as determined by human dystrophin expression and beta-galactosidase localization to the donor nuclei, a similar study using human DMD myoblasts showed that the transduction efficiency with the human dystrophin minigene was extremely low [73]. Furthermore, while some of these transfected human donor myoblasts survived *in vivo*, this was only tested in immunodeficient mice, and it seems likely that the same immune problems (discussed above) apply to cultured autologous myoblasts [75]. Furthermore, autologous DMD myoblasts may be severely compromised due to an "exhausted capacity" to proliferate and fuse compared with myoblasts originating from normal skeletal muscle [76]. To date, attempts to overcome potential problems of limited proliferation capacity of DMD myoblasts by transfecting DMD myoblasts with the immortalizing telomerase gene [77] have produced little success. One great attraction of stem cell therapy is that autologous cells can be taken from other tissues (*e.g.* bone marrow, skin), rather than from already compromised skeletal muscles of DMD boys, and these autologous "myogenic stem cells" genetically corrected for subsequent MTT [78].

Another advantage of using bone-marrow derived myogenic stem cell types to replace the dystrophin is that they can be delivered through the vasculature, thus avoiding the issue of donor myoblast migration from an intramuscular injection site (discussed above). That the transplantation of normal donor bone marrow into a dystrophic host can result in small numbers of myofibers that contain donor myonuclei and express dystrophin has been demonstrated [78].

Antisense and other therapies: Another approach is to use antisense therapy to repair endogenous DNA in DMD without exogenously replacing dystrophin [79]. Antisense oligonucleotides were administered weekly by intramuscular injection to dystrophic (mdx) muscle to induce excision of the mutated portion of the dystrophin gene, and this resulted in restoration of the open reading frame so that expression of truncated dystrophin isoforms was observed for up to 5 weeks [79]. Antisense therapy has several potential advantages over gene or cell replacement therapies in that; all tissue-specific and developmental control elements of dystrophin remain intact, repeated oligonucleotide delivery should not induce an immune response and the costs are lower than that for recombinant virus in gene therapy. However, this therapy is not applicable to combat very large deletions or mutations where critical functional domains are disrupted [79]. As the powers of the biotechnology industry increase, it seems likely that many such sophisticated methods will be developed for the treatment of genetic anomalies. Another novel therapy is based on the fact that treatment of cultured cells with aminoglycoside antibiotics can suppress stop codons; administration of gentamicin to dystrophic mdx mice resulted in expression and localization of dystrophin to the cell membrane, and also provided functional protection against muscular injury [80]. A clinical trial using gentamycin is currently in progress.

Utrophin overexpression: Major activity has been directed at upregulating utrophin expression within host muscle, as an alternative to replacing dystrophin and the associated immune problems. Utrophin is a muscle protein with structural homology to dystrophin, but it is normally restricted to the cell membrane around the neuromuscular junction [81]. Adenovirus-mediated transfer of the utrophin gene produced widespread expression of the utrophin protein in mdx muscle, in addition to restoring normal expression of two dystrophin-associated proteins that are usually absent or deficient in dystrophic muscle [82]. There is much debate over whether utrophin upregulation in dystrophic muscle might be sufficient to alleviate the dystrophic phenotype. Ebihara *et al* [83] recently reported an equal improvement in muscle pathology and function in immunologically immature neonatal dystrophic (mdx) muscles after adenovirus-mediated transfer of either dystrophin or utrophin; furthermore significant utrophin upregulation was observed in immunocompetent adult mdx mice [83]. In this and many other studies, the method of testing muscle function was isometric force-generating capacity, although it has previously been reported that such testing is not sufficient to determine the efficacy of a treatment

regime in skeletal muscle, and this was suggested as one of the major shortcomings of early clinical MTT trials [84]. Utrophin gene therapy for DMD is supported by a recent *in vitro* study that demonstrated that, developmentally, utrophin appears in myogenic cells before dystrophin, and that utrophin expression alone appears adequate to anchor the remaining dystrophin-associated proteins to the cell membrane [85]. There is currently a quest to identify a substance (that ideally can be administered orally) to overcome the limited distribution of utrophin and ideally result in expression over the whole of the myofiber and substitute for the missing dystrophin [86].

Summary

Regrettably there is no proven method yet for alleviating the severe skeletal muscle wasting characteristic of DMD. However, significant recent advances have been made in understanding the mechanisms responsible for the death of injected cultured donor myoblasts in MTT, and MTT remains a biologically attractive approach for correcting the gene defect in DMD. Many other studies are examining alternative therapies to classical MTT. Therefore, the treatment of DMD is currently being confronted from many different and novel angles, with the ultimate aim of identifying some treatment that can be applied in the clinical situation.

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