Immunobiology and the Future of Myoblast Transfer Therapy

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Myoblast transfer therapy (MTT) is a cell-mediated gene transfer method aimed at the restoration of normal dystrophin expression in Duchenne muscular dystrophy (DMD). Initial clinical MTT trials were conducted amid much controversy, as they were based on very few animal studies. Unfortunately, the trials were of little therapeutic benefit. As a result, there has been a renaissance of interest in experimental studies in animal models. In MTT, myoblasts are obtained by muscle biopsy from normal, i.e., dystrophin-positive, donors, expanded in culture, and injected directly into the muscles of dystrophic recipients. The major requirement for successful MTT is the survival of injected donor myoblasts in the host environment. However, a vast majority of donor cells fail to survive for more than 1 h after injection, and very few last beyond the first week. This review on the immunological aspects of MTT focuses in particular on the roles of specific components of the host immune response, the effects of tissue culture on donor cells, and strategies under development to circumvent the problem of donor myoblast death after injection in vivo.

Key Words: myoblast transfer therapy; immunology; Duchenne muscular dystrophy; inflammatory cells; immunosuppression; tissue culture.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease characterized by a mutation in the gene encoding the muscle fiber-stabilizing protein dystrophin (1). Myoblast transfer therapy (MTT) has long been considered one potentially beneficial treatment for DMD patients. Briefly described, MTT involves transplanting muscle precursor cells (myoblasts) from normal donors into dystrophic host muscle (Fig. 1A). The subsequent dispersal and fusion of donor myoblasts are intended to result in the expression of normal functional dystrophin transcripts throughout the dystrophic host muscle. The success of MTT depends ultimately on the persistence of large numbers of donor myoblasts and their adequate dispersal and fusion throughout the host muscle.

THE PROBLEM OF DONOR MYOBLAST SURVIVAL

The major obstacle to the success of MTT is donor myoblast survival, as these cells undergo rapid and massive death after injection into host muscle (2–8) (Fig. 1B). Donor myoblast survival can be prolonged to a degree by host immunosuppression, suggesting that the host immune response is at least in part responsible for the death of the transplanted cells. Indeed, many MTT studies routinely use immunosuppressant drugs such as cyclosporine and FK506 because the prolonged survival of donor myoblasts enables other important factors in MTT (i.e., migration and fusion) to be examined (summarized in Table 1). However, FK506 and cyclosporine are known to have deleterious side effects (9, 10) and their withdrawal results in immediate donor myoblast death in MTT (11, 12). In addition, there is some evidence that these drugs do not adequately suppress the host immune response in MTT (13, 14). Other immunosuppressants have been tested in MTT (Table 1) with little success.

It is possible that the problem of donor myoblast death might be circumvented by the use of a small, immune-resistant stem cell population of injected myoblasts or delivery of myogenic stem cells via the circulation (see below). However, these approaches conceal the basic scientific challenge, i.e., elucidating the mechanisms behind the massive death of cultured donor myoblasts soon after injection.

INVOLVEMENT OF THE HOST IMMUNE RESPONSE

Several major components of the host immune response are all likely to have some part in donor myoblast death in MTT (summarized in Fig. 2).

Humoral Immunity and the Role of Complement

The speed with which donor myoblasts die following injection strongly suggests that the nonspecific humoral arm of the host immune system, the basic component of which is complement (Fig. 2, pathway A), is involved. The complement system consists of 20 complex proteins that form a proteolytic cleavage cascade that ultimately results in target cell lysis (15) (Fig. 3). This occurs via the generation of a membrane attack complex (MAC), which can lyse target cells extremely rapidly and is active for less than a second (16).

However, host complement depletion has only a slight beneficial effect on donor myoblast survival in MTT (17;
able data indicate no central role for complement in this process. Despite the rapidity of donor myoblast death, available data indicate no central role for complement in this process. Thus, it is possible that complement plays an indirect role in donor myoblast rejection as an inducer of neutrophil and macrophage chemotaxis to the injection site (17). In summary, despite the rapidity of donor myoblast death, available data indicate no central role for complement in this process.

The MHC and T Cells

The major histocompatibility complex (MHC) class I antigens (expressed by most cell types), MHC class II antigens (expressed predominantly on antigen-presenting cells and B cells), and T cells are central to the immunological rejection of transplanted organs and tissues (reviewed in (20)]. Foreign cells can interact directly with host T cells by expressing MHC antigens, which are recognized by the T-cell receptor (TCR) on T cells (21, 22) (Fig. 2, pathway B). Alternatively, an indirect route of donor cell interaction with host T cells occurs when host antigen-presenting cells (APCs) recognize and present donor MHC antigens and present them together with the host MHC to T cells (21) (Fig. 2, pathway C). The direct pathway of interaction between foreign cells and host T cells is highly responsive to immunosuppressant drugs such as cyclosporine (21). Thus, MTT studies demonstrating enhanced donor myoblast survival with the use of these drugs (Table 1) suggest a direct interaction between transplanted donor myoblasts and host T cells. Furthermore, in vitro studies have shown that myoblasts express MHC class II antigens following exposure to the inflammatory cytokine interferon-γ (IFN-γ), enabling them to function as APCs (23, 24) (Fig. 4).

One possible way to prevent the MHC class II-mediated interactions between donor myoblasts and host T cells is to ensure that donors and hosts are fully matched for this complex. However, this can still cause complications and rejection due to host recognition of antigens expressed at minor histocompatibility loci (25). Furthermore, rejection of fully MHC-matched donor myoblasts may be related to alteration of antigen expression by these cells as a result of exposure to certain tissue culture conditions, as host antibodies against donor-derived MHC antigens have been detected following MTT (26) (see below).

In addition to these concerns, myoblasts (23, 27) and myotubes (24) constitutively express MHC class I antigens in vitro, and cells with missing or altered MHC class I molecules on their surface are rapidly targeted by natural killer (NK) cells and cytotoxic T cells (28). Taken together, these observations provide several potential MHC-mediated pathways by which injected donor myoblasts may interact, either directly or indirectly, with various cell types of the host immune system and thus can be destroyed.

Do Myoblasts Participate in Costimulation?

The MHC–TCR system is an extremely potent mediator of immune responses, and this interaction is essential but not sufficient for full T-cell activation. At least one of three “costimulatory” pathways must also be initiated for this to occur, and an increasing number of studies are examining these pathways as potential targets for immunosuppression (20, 22, 29). All three pathways are mediated by the B7.1 (CD80), B7.2 (CD86), and CD40 receptors located on APCs or donor cells. The T-cell ligands for these three proteins are CD28 (for B7.1 and B7.2) and CD154 (for CD40).

Very few studies to date have examined the possibility that donor myoblasts in MTT participate in costimulation. Host treatment with CTLA4-Ig, a molecule that blocks the B7/CD28 costimulatory pathway (29), can prolong donor myoblast survival to 2 months. However, this...
effect was seen only when CTLA4-Ig was coadministered with antibodies that deplete CD4+ T cells (30). This suggests that if donor myoblasts participate in costimulation, it is not likely to be via the B7/CD28 pathway.

In a similar study using transplanted pancreatic islet cells for the treatment of insulin-dependent diabetes, host treatment with antibodies to CD154 resulted in improved donor cell survival. Nevertheless, rejection was not completely prevented and the treatment regime was more effective when combined with a host transfusion of donor lymphocytes (31). However, further studies are required to determine if the CD40/CD154 pathway is involved in the death of cultured donor myoblasts in MTT.

Another possible direct interaction between donor myoblasts and host T cells is via expression of intracellular adhesion molecule-1 (ICAM-1) (Fig. 4). ICAM-1 is a...
ligand for leukocyte function antigen-1 (LFA-1), which is expressed by most leukocytes and NK cells (32). LFA-1-mediated effects include T-cell cytotoxicity and leukocyte extravasation into sites of inflammation. ICAM-1 is constitutively expressed by myoblasts (33) and can be further upregulated by IFN-γ and tumor necrosis factor-α (TNF-α) (23, 24). Host treatment with antibodies to LFA-1 enhances donor myoblast survival in MTT, but this treatment alone did not prolong donor myoblast survival beyond 1 month (33, 34). Donor myoblast survival was prolonged up to 2 months when anti-LFA-1 treatment was combined with CD4+ and CD8+ T-cell depletion (30), suggesting that ICAM-1/LFA-1 binding is not the only interaction between donor myoblasts and host T cells.

The expression and binding of all of these molecules at the cell surface are under the strict control of several different cytokines. However, only a few studies to date have examined the roles of inflammatory cytokines in donor myoblast death in MTT.

The cytokine profile in dystrophic muscle is somewhat different than in normal muscle (35, 36), and this is believed to be related to the progression of DMD (35). It may also contribute to the death of cultured donor myoblasts following MTT. The extracellular matrix (ECM) in dystrophic muscle contains elevated levels of transforming growth factor-β (TGF-β) (35, 37), a multifunctional...
cytokine with several regulatory roles in immune responses (38, 39). Although TGF-β has not been attributed a direct role in the death of injected donor myoblasts in MTT (MTT is equally unsuccessful in normal muscle), it is possible that increased TGF-β (and other cytokines) is produced as a result of the injury caused by the injection procedure. Potential immunological effects that could result from increased TGF-β expression in the host muscle and the subsequent effects on injected donor myoblasts are depicted in Fig. 5.

Other changes in the cytokine profile in host muscle may be brought about by the injected donor myoblasts themselves. Myoblasts secrete IL-1 in vitro (40), which has a multitude of immunological functions. Although myoblast secretion of IL-1 in vivo has not been carefully examined, if this was the case the potential for these cells to initiate host cytokine signaling cascades would ultimately lead to their own demise (Fig. 3).

Several host inflammatory cell types are already present in normal and dystrophic muscle, and additional inflammatory cells infiltrate host muscle following the injection of isolated donor myoblasts (see below). Specific cytokines produced by these inflammatory cells may also contribute to donor myoblast death in MTT. TNF-α, a major mast cell product, inhibits myoblast differentiation and fusion in vitro (41) and degrades muscle cytoplasm during skeletal muscle necrosis and regeneration (42, 43). However, recent studies in our laboratory demonstrate that skeletal muscle regeneration is normal in TNF-α null and TNF-α/lymphotoxin-α null mice, suggesting a redundancy in vivo cytokine signaling systems, and that certain cytokines can mimic the effects of one another (Collins and Grounds, manuscript in preparation). Thus, it is difficult to determine if TNF-α affects donor myoblast behavior in MTT, although we are currently conducting studies using the TNF-α null mice to investigate this further.

Despite their importance, cytokines are not an attractive option in the development of new immunosuppressive strategies. One problem associated with interfering in cytokine activity is that they are multifunctional molecules. Thus, altering one function may have detrimental downstream effects on cytokine feedback systems (44). This observation may explain the observed toxic side effects of some immunosuppressants.

Host Cell Types Involved in Donor Myoblast Death

Normal skeletal muscle is populated by several different interstitial cells, including macrophages (45, 46), dendritic cells (46), and mast cells (47). In addition, untreated dystrophic muscle is characterized by the presence of T cells, B cells, and elevated numbers of macrophages (48, 49) and mast cells (47). Following MTT, host muscle is infiltrated by additional inflammatory cells, including polymorphonuclear leukocytes, lymphocytes, macrophages, and NK cells (50). Available evidence suggests that many inflammatory cell types play a role in donor myoblast death in MTT, but of key importance are host T cells, due to the evidence for their direct interaction with donor myoblasts (discussed above). Thus, considerable research interest has focused on CD4+ T cells, CD8+ T cells, and NK cells.

CD4+/CD8+ T cells. T-cell expressing CD4 and CD8 are present within murine dystrophic (mdx) skeletal muscle (49, 51, 52) and have been attributed a role in donor myoblast rejection in MTT (13, 50, 53). CD4 and CD8 are receptors for specific regions of the MHC class II and I antigens, respectively, and are believed to be important “coreceptors” in MHC–TCR-mediated T-cell activation (54). Activated CD4+ and CD8+ T cells are involved in cytokine production and cytotoxicity, respectively (55, 56), and cytotoxic T cells in particular are destructive to developing myotubes (57). Recent studies in our laboratory have demonstrated that depletion of host CD4+ and CD8+ T cells promotes the survival of donor myoblasts.
that have emigrated from sliced muscle grafts (58, 59) and after conventional MTT (7) (Fig. 1B). However, despite evidence for a role of CD4+ and CD8+ T cells in donor myoblast rejection in MTT and the initial improvement in survival when these T-cell subsets were transiently depleted (Fig. 1), the donor myoblast population within host muscle still declines over time (7). This may relate to the repopulation of the host T cells after the initial depleting regime. The fact that our treatment regimes fail to sustain the initial increase in donor myoblast survival indicates that CD4+ and CD8+ T cells are not the only effectors of the immune response against transplanted donor myoblasts.

NK cells. A more promising causative candidate for donor myoblast death may be a subset of lymphocytes called natural killer or NK cells. NK cells are present in the inflammatory infiltrate within dystrophic host muscle following MTT (50), and they can rapidly damage developing myotubes in vitro (57). NK cells can recognize foreign cells, but are believed to play only an accessory role in transplanted organ reaction (60). We have recently shown that NK1.1+ cell depletion in dystrophic (mdx) host mice results in dramatically enhanced donor myoblast survival in conventional MTT (7) (Fig. 1B). Enhanced survival (at least threefold over untreated controls) was maintained for at least 1 week (Fig. 1) despite host recovery to approximately 82% of normal NK cell activity. Long-term follow-up studies are in progress.

NK cells employ cytosisis and cytokine production as major effector mechanisms (61) and are probably activated by several cytokines and chemokines. These cells are found at high frequency in cellular infiltrates associated with xenogeneic organ rejection (62–65), and their activation appears to occur via MHC class I antigen-specific receptors (66, 67). This may enable them to interact directly with injected donor myoblasts following MTT (see above).

Macrophages, dendritic cells, and mast cells. Any role for additional inflammatory cells (e.g., macrophages, dendritic cells, and mast cells) in donor myoblast death after MTT is still uncertain. Macrophage numbers are increased in dystrophic muscle (48, 49), and they infiltrate host muscle following MTT (33, 34, 50). In vivo, macrophages play an indispensable role in skeletal muscle regeneration following injury (68), most likely by producing factors that promote myoblast chemotaxis (69) and proliferation (69–72). However, elevated levels of such macrophage-derived factors as TGF-β and basic fibroblast growth factor (bFGF) (69) within the host muscle in MTT may also have immunological effects that can lead to the death of injected donor myoblasts (see above).

Dendritic cells are APCs (73), have several similarities to NK cells, and possess cytotoxic activity (73–75). It is unknown if dendritic cells are involved in donor myoblast death following MTT, although their presence in normal skeletal muscle (46) suggests their possible involvement.

A population of resident mast cells also characterizes normal skeletal muscle, and their numbers are elevated in dystrophic muscle (35, 47). It is possible that these cells are important in skeletal muscle regeneration (76, 77), and they may play a critical role in the progression of DMD (78). Mast cells secrete several products that are likely to affect donor myoblast survival in MTT: TNF-α, in particular, which has significant effects as discussed above. Other relevant mast cell secretory products include chymase, tryptase, and heparin, which appear to be involved in myoblast activity during normal skeletal muscle regeneration (76). However, their direct effects on injected donor myoblasts have not been investigated.

The Role of the Tissue Culture Process

Donor myoblasts are grown in tissue culture in order to expand their numbers for injection in MTT experiments. The rapid death of cultured donor myoblasts occurs even when they are injected into fully histocompatible hosts (2, 4, 79), which stands in marked contrast to the excellent long-term (up to 1 year) survival of donor myoblasts derived from histocompatible whole or sliced muscle grafts (59, 80). The physical method of delivery of cultured donor myoblasts into host muscle seems to have little effect on their poor survival, as neither embedding donor myoblasts in a fibrin clot (3) nor the actual process of injection (7) enhances their survival. These observations strongly suggest that cell isolation and culture procedures (Fig. 1A) per se are relevant to the death of the transplanted cells.

Donor cell death is not a problem unique to MTT as a cell transplantation strategy. Transplantation of either isolated pancreatic islet cells in the treatment of insulin-dependent diabetes (81) or hepatocytes for treating inborn errors of metabolism (82) faces the same dilemma. Although donor islet cells can be protected to some degree from the host immune response by artificially encapsulating them prior to transplantation [reviewed in (83)], this is not an option in MTT as it is essential that donor myoblasts incorporate into host myofibers. The purity of hepatocyte preparations appears to be a key factor in the success of this form of cell transplantation (82), but most myoblast preparations used in MTT are already highly purified. In fact, reliable procedures have been developed specifically for growing enriched primary cultures of myoblasts in vitro (84).

Furthermore, the number of passages to which myoblasts are subjected in vitro does not appear to be a factor in their subsequent in vivo survival. Recent quantitative evidence demonstrates no significant difference in the survival of freshly cultured donor myoblasts (less than three passages) compared to myoblasts passed 15–20 times (7). However, a slight increase in the number of early-passage myoblasts between 2 and 7 days after injection has been seen, suggesting that myoblast proliferation occurred during this time. A related study also demonstrated increased donor myoblast numbers 2–4 days after transplantation into irradiated muscle of nude mice (2). It was concluded that these cells possibly represent a sub-
population of donor myoblasts, with stem cell properties that escape the host immune response (discussed below).

Detrimental Effects Are Reagent Specific

Culture conditions themselves, including media components, serum factors, and proteolytic enzymes, may alter donor myoblast antigenicity (53, 85). This hypothesis is strongly supported by evidence of host-derived antibodies specific for certain tissue culture reagents (e.g., fetal calf serum) soon after donor myoblast injection (53). A recent study in our laboratory demonstrated that exposure of whole muscles to tissue culture conditions prior to grafting into a histocompatible host mouse was detrimental to subsequent graft regeneration in vivo (86), most likely reflecting poor myoblast survival and/or function. This was severely affected when whole muscle grafts were exposed to serum or proteolytic enzymes. Proteases were particularly deleterious to graft regeneration and resulted in the death of most cells (including myoblasts) in the grafts by 7 days. This unexpected and lethal effect of tissue culture reagents on cells subsequently exposed to an in vivo environment has major implications for transplantation of many cell types.

Evidence for Tissue Culture-Induced Alterations at the Cell Surface

Exposure to proteolytic enzymes may cause disruption of integrins and other crucial cell-surface receptors that bind to specific ECM proteins, resulting in an inability of donor myoblasts to adhere to the ECM. The major integrins expressed by myoblasts are integrin \( \alpha_1\beta_1 \), which mediates laminin-2 binding (87), and integrin \( \alpha_4\beta_1 \), which binds fibronectin (88). Integrin-mediated interactions with ECM components are critical for myoblast proliferation, migration, and fusion during myogenesis (89–91), and a loss of cell-ECM interactions can result in a specific form of apoptotic cell death referred to as "anoikis" (92, 93). It was recently demonstrated that isolated pancreatic islet cells genetically engineered to express an anti-anoikis antibody were more viable in vitro and showed enhanced survival in vivo compared to transplanted normal islet cells (94). Furthermore, the increased migration of tumorigenic C2C12 myogenic cells into uninjured host muscle (95) and of donor myoblasts into irradiated host muscles (96) may be due, in part, to perturbed expression of integrins or ECM molecules.

Manipulating tissue culture conditions can improve donor myoblast survival (85). In particular, it has been reported that culturing donor myoblasts in the presence of growth factors such as bFGF dramatically enhances their subsequent survival within the host environment (97, 98). Unfortunately, the detection methods used in these studies did not allow for the quantitation of the total number of surviving donor myoblasts. Therefore, it is difficult to determine if the effect of bFGF was to enhance proliferation or to confer initial immunoprotection on the donor myoblasts.

Much work is still required to determine the full extent of the effects of tissue culture procedures on transplanted donor myoblasts. However, if it is true that exposure to tissue culture conditions does indeed render donor myoblasts incompatible with the host environment, this would have significant implications not only for MTT, but also for other cell transplantation strategies.

STEM CELLS AS AN ALTERNATIVE TO ISOLATED DONOR MYOBLASTS

Would delivery of myogenic stem cells via the circulation be any more likely to influence/avoid the host immune response? It is well known that myoblasts can arise from “nonskeletal muscle” cells and tissues (99–102), although such plasticity does not necessarily reflect the presence of stem cells [reviewed in (103)]. Markers have now been described for myogenic stem cells isolated from mesenchymal connective tissue (104), skeletal muscle (105, 106), and bone marrow (105). Of particular interest are observations that bone marrow-derived cells can contribute to the formation of new skeletal muscle in vivo (105, 107). Bone marrow-derived cells can be obtained (105, 107, 108) without exposure to any tissue culture reagents, and this alone may avoid many of the immunological problems (see above). However, the procedures used to purify subpopulations might themselves have adverse effects on these stem cells. In contrast, extraction of stem cells from skeletal muscle (105, 106) or other tissues (104) usually involves proteases and other culture reagents, with the associated problems already mentioned. This distinction between the two types of preparations of stem cells may have consequences when cells are exposed to the in vivo environment.

When donor cells are injected into the circulation, they are not exposed immediately to APCs such as dendritic cells and macrophages within the muscle tissue. This could allow the damaging effects of proteases or other steps in the culture procedure to be overcome with time, such as the replacement of altered surface proteins with new proteins. If this occurs rapidly while the donor cells are in the bloodstream, these donor cells might be less susceptible to the adverse host response, although it should be noted that there are no data to back this hypothesis. It has also been suggested that the bone marrow-derived myogenic stem cells might arise from cells closely associated with the vasculature (109) and this might further delay the transition of cells into the skeletal muscle compartment. Clearly, precise information on the timing of donor cells arriving in skeletal muscle from the circulation, quantitation of donor cell survival and replication, and the possible influence of the origin/preparation of the donor myogenic stem cells is required to clarify this situation. It should also be noted that available evidence indicates that only a very small proportion of injected donor bone marrow cells from the circulation contributes to the formation of new skeletal muscle in damaged muscles of adult animals (105, 108). This emphasizes the
necessity for strategies to increase the survival/recruitment and/or proliferation of such donor myogenic stem cells delivered systemically.

It has been proposed that the very few donor myoblasts that survive the massive and rapid death seen after direct injection of cultured myoblasts into host muscle may similarly represent quiescent (primitive) stem cells that are slow to become activated under the tissue culture conditions (2, 5). In light of the immunobiology, it may be that these stem cells do not express the same range of surface proteins and therefore are less immunogenic and escape the host immune response. If large numbers of such putative stem cells could be extracted and purified from skeletal muscle (or other tissues), this might represent a superior source of donor myogenic cells for direct intramuscular injection in MTT. However, if myogenic stem cells are grown in tissue culture for an extended period of time (regardless of their origins), they might become activated, increase expression of surface neoantigens, and consequently become susceptible to the same immunological problems as conventional myoblasts after intramuscular injection. Because expansion of the donor cell population is clearly highly desirable for effective MTT, an alternative possibility is for this to occur in vivo after injection. Nevertheless, despite the fact that proliferation of myoblasts has been demonstrated in irradiated host muscles, it is greatly exaggerated in this artificial mitogenic environment (2, 110), and the extent to which donor myogenic stem cells can indeed undergo extensive replication after injection into (nonirradiated) host muscles remains unknown.

**Summary**

In conclusion, striking advances are now being made in defining the mechanisms responsible for donor myoblast death in clinical MTT. As should be clear from this brief survey, this is not a simple problem that will be easily solved: there are many complex and interacting factors at play here. Available evidence identifies key areas to address as follows:

- The role of acute host immunological components that can act within minutes of challenge by “foreign” donor cells (such as the complement-activating system);
- Expression of MHC class I and II and other antigens by donor myoblasts and the specific receptor/ligand pairs that mediate direct interaction between donor myoblasts and host immune cells;
- Direct and indirect involvement of several host immune cell types, particularly T cells, NK cells, macrophages, and mast cells; and
- The strong possibility that the tissue culture process per se adversely influences the subsequent in vivo survival of donor myoblasts, probably by altering various cell-surface components.

Although the precise reasons behind the extremely rapid death of donor myoblasts in MTT are as yet unknown, we have made enough headway to be optimistic about the reemergence of MTT as a major contender for the potential treatment of DMD.

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**References**


**Review**

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