Critical Review

Muscle-Derived Stem Cells: Implications for Effective Myoblast Transfer Therapy

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Summary

Stem cells have been proposed as a wonder solution for tissue repair in many situations and have attracted much attention in the media for both their therapeutic potential and ethical implications. In addition to the excitement generated by embryonic stem cells, research has now identified a number of stem cells within adult tissues which pose much more realistic targets for therapeutic interventions. Myoblast transfer therapy (MTT) has long been viewed as a potential therapy for the debilitating muscle-wasting disorder Duchenne Muscular Dystrophy. This technique relies on the transplantation of committed muscle precursor cells directly into the muscle fibres but has had little success in clinical trials. The recent discovery of a population of cells within adult muscle with stem cell-like characteristics has interesting implications for the future of such putative cell transplantation therapies. This review focuses on the characterization and application of these potential muscle-derived stem cells (MDSC) to MTT.

INTRODUCTION

In recent times stem cells have attracted a huge amount of interest in many fields of research, and have caused much excitement as potential therapeutic treatments for a variety of diseases. In the field of muscle biology the discovery of a population of cells with stem cell-like characteristics has had interesting implications for Myoblast Transfer Therapy (MTT) studies. This potential therapy for muscle wasting diseases such as Duchenne Muscular Dystrophy (DMD) takes advantage of the multinucleate nature of skeletal muscle by introducing normal muscle precursor cells (myoblasts) into muscles defective for the dystrophin gene. Fusion of donor myoblasts with multinucleated host myofibres produces a mosaic of defective and non-defective nuclei within a common cytoplasm and is highly attractive as a gene replacement strategy (1). The ideal scenario is to use autologous cells from DMD boys, derived from a source other than skeletal muscle, to genetically correct these cells and use as a source of replacement myoblasts. The early clinical trials where donor myoblasts from the father were used were controversial and unsuccessful and thus interest in MTT waned. Experimental studies in animal models by our laboratory and others subsequently demonstrated that there is a massive initial death of the donor myoblasts (2–4). Quantitation of male donor myoblast survival by Y-chromosome analysis, and more recently by real-time PCR, shows that about 40–50% of the donor myoblasts normally perish within one day after injection, and up to 80% of donor cells can rapidly die within one week. We propose that this massive initial death is due to necrosis and/or anoikis (5) of the transplanted myoblasts, likely due to exposure of the donor cells to tissue culture conditions prior to transplantation in vivo. Such quantitative studies are important for determining the efficacy of the transplantation procedure, and this scale of donor cell loss is generally not recognized in the absence of quantitative analysis. Myoblasts have also attracted much attention as supplementary cells to alleviate effects of severe heart damage; where the myoblasts provide an alternative to scar tissue but do not generally seem to convert into cardiac muscle. The clinical cardiac application has stimulated much interest in optimizing a source of autologous myoblasts for MTT (6, 7).
QUANTITATION OF DONOR CELL SURVIVAL AND PROLIFERATION

A central requirement of any transplantation study is the capacity to discriminate between donor and host cells in order to quantify donor cell engraftment. An accurate and reproducible method to determine the efficacy of MTT uses a trans-sexual transplantation model with detection of the Y-chromosome as a marker of (male) donor cell survival and proliferation in a female recipient. In comparison to other markers (e.g., β-gal, GFP), no genetic manipulation of donor cells is required, the amount of marker is not diluted as cells proliferate, gene expression is not required and the marker is not reutilized if donor cells die. Slot blot analysis of male DNA, or real-time PCR using male-specific primers, measures the total amount of donor male DNA throughout the whole injected (female) host muscle. In addition to Y-chromosome analysis, the expression of dystrophin in transplanted muscles indicates not only the survival of donor cells, but their incorporation into host muscle fibres by expression of the missing gene product dystrophin. However, immunohistochemical analysis of dystrophin is difficult to accurately quantitate as the distribution of donor cells may vary throughout the myofibre and requires sectioning and analysis of the whole TA muscle. This being said, it provides a qualitative guide to the successful incorporation of donor myoblasts and in our studies these two systems are used in conjunction to analyse the efficacy of MTT (Fig. 1). More recently, advances have been made in the field of non-invasive imaging to monitor donor cell survival, allowing repeated analysis of individual animals over time. Such quantitative techniques include Bioluminescent Imaging (8), magnetic resonance (9) and the sodium iodide symporter (10) and promise to advance the field with simplified analysis and greatly reduced animal numbers. However, at present they are in their infancy, with issues of sensitivity and accurate discrimination of donor cell location to be resolved. In spite of this, they present an exciting step forward and may be applicable to a number of stem cell investigations.

NON-MUSCLE SOURCES OF MYOBLASTS

A major focus of research groups in the field of MTT has been the type and source of cells used for injection, with much interest related to stem cells capable of forming skeletal muscle. Alternative sources of myoblasts include the thymus, thymus, thymus, thymus, thymus, thymus, thymus, thymus, thymus, thymus, thymus, thymus.

Figure 1. Myoblast transplantation therapy in a trans-sexual mouse model. Normal male donor myoblasts are isolated from C57BL/10sn (the parental strain of mdx) mice using the pre-plate technique (26) and injected into tibialis anterior muscles of female dystrophic mdx mice (that lack dystrophin). Muscles are sampled and the efficacy of the myoblast transplantation therapy is measured by DNA extraction and quantitation of male DNA using real-time PCR (to quantitate donor cell numbers) in conjunction with immunohistochemistry to observe the number of dystrophin positive muscle fibres (to measure replacement of the dystrophin gene).
dermal fibroblasts and bone-marrow derived hematopoietic stem cells (HSC) that can give rise in tissue culture to well differentiated skeletal muscle (6, 11, 12). The multi-potent nature of HSC generated much excitement with initial reports of incorporation into damaged muscle following intra-muscular injection and recruitment to sites of skeletal muscle damage from the bone marrow (12). The homing of stem cells via the blood stream is an attractive prospect, and would solve one of the major hurdles of MTT delivery and dispersion of donor cells systemically to all muscle fibres. However, bone marrow and HSC display no intrinsic myogenicity and while some integration of bone-marrow derived nuclei into myofibres has been demonstrated this occurs at a very low level unsuitable for therapeutic exploitation (13–15). Recognition that bone-marrow derived cells can fuse with myofibres without concomitant expression of muscle genes, questioned further their contribution to myogenesis and their stem cell status (13–15). The current status is that such circulating cells normally make little or no contribution to post-natal myogenesis and demonstrate only low levels of incorporation into muscle fibres (12, 13, 16, 17) substantiating conclusions from early studies where less sensitive cell markers were available (18). Theoretically, manipulations to increase recruitment and incorporation of circulating or injected cells into muscle fibres remain a possibility, and a recent study reported excellent homing and integration of injected mesoangioblasts – a type of fetal-derived mesenchymal stem cell associated with the vasculature (19) into muscle. Although some studies have demonstrated the ability of non-muscle circulating cells to localize to muscle fibres, the mechanism for committed myogenic progenitors to cross the endothelial barrier is unknown (20) and, while some studies have reported low levels of such donor cell incorporation, the number of donor-nuclei positive myofibres is so low as to be clinically irrelevant (20, 21).

MUSCLE-DERIVED STEM CELLS

Classical (committed) muscle precursor cells were originally identified based on their location beneath the basal lamina of muscle fibres, and have the potential to proliferate, self renew and repair damaged muscle (22). Recent studies have isolated a population of cells from skeletal muscle tissue, which have the ability to differentiate into both myogenic and non-myogenic lineages (23). These putative muscle-derived stem cells (MDSC) have been isolated in mice using both side population, cell surface sorting and pre-plating methods (24–26). Side population (SP) cells are able to exclude the nuclear dye Hoechst 33342 via a multi-drug resistant pump and were originally identified in bone marrow as primitive HSC (27). SP cells isolated from skeletal muscle express several surface markers associated with HSC including CD45, c-kit, Sca-1 and CD34 (16), however there is much confusion in the pattern of marker expression. These skeletal muscle-derived SP cells are able to form muscle in vivo (24), although it now appears that these cells are hematopoietic in origin (28). Similarly late pre-plate (PP6) cell populations isolated by differential adherence to collagen-coated flasks have also been shown to possess multi-potentiality in vitro with superior survival and proliferation in vivo (26). However, another study reported only a slight difference between the numbers of dystrophin positive fibres after injection of late pre-plate (PP6), or early passage primary myoblasts, although it should be noted that the MDSC were injected after many passages in vitro (29). We have recently observed increased survival and also proliferation in a population of late pre-plate cells (equivalent to MDSC) extracted from adult mice (30). Following an initial loss of 50% of male donor cells by 24 h, no further decrease was observed for up to 3 weeks post-transplantation. Of particular interest is the observation that the amount of male DNA increased by more than 2 fold by 3 months indicating proliferation of these donor cells. Subsequent, longer term analysis of the number and size of dystrophin positive myofibres up to 7 months post-MTT reveals an increase in the size of donor myofibres but not the number (Fig. 2), suggesting that donor myonuclei are persisting within these myofibres but are not migrating from the site of injury. Whether the donor myogenic cells maintain a pool of stem cells capable of repopulating the whole muscle is not clear, this might be elucidated by invoking further injury to the grafted muscle. This in vivo proliferative capacity of late pre-plate cells (compared with early passage myoblasts) supports the argument for a population of muscle-derived cells with stem cell capacity. However, while attempts to exclude immortalization of these murine cells has not revealed any chromosome abnormalities there remains the concern that small changes in gene regulation leading to immortalization may have occurred and this would accord with the relative delay in the onset of proliferation apparent only after 3 weeks. Immortalization of murine cells is common in contrast with human cells that lack telomerase and thus immortalization is less likely to be an issue for the equivalent human cells (31). Further concerns that these late pre-plate murine cells have transformed in some way comes from repeated MTT experiments in our laboratory using frozen and considerably expanded stocks of these cells, where strong dystrophin staining was evident at 3, 5 and 7 months after transplantation although little male DNA was detected by Q-PCR at any time. It was concluded that the Y-chromosome had been altered or eliminated from these donor myoblasts as a result of expansion in culture (unpublished data). It is noted that, in contrast with the late pre-plate cells obtained from mice, most cells extracted from skeletal muscles of sheep and humans appear to adhere very rapidly in culture with almost no unattached cells remaining (unpublished data from several laboratories). This raises problems for characterization of an equivalent human MDSC (as identified by a reluctance to adhere in culture, i.e., PP6 cells), for potential extrapolation to the clinical situation.
In contrast to the restricted area of donor-positive myofibres seen after injection of late pre-plate cells into host mice in our experiments, when the host muscle is irradiated prior to injection in order to ablate resident replication-competent satellite cells (myoblasts), donor myoblasts are able to repopulate most of the muscle \((32, 33)\). It appears that the altered environment of the irradiated host muscle greatly facilitates donor myoblast proliferation. These experiments using irradiated mouse muscle demonstrate that some compartment of the satellite cell population has the capacity to maintain a precursor population, like MDSC, that can extensively repair damaged myofibres and generate much new skeletal muscle.

Characterization of the potential MDSC subpopulation based on cellular markers has proven complicated. Digestion of muscle tissue yields cells from various lineages including fibroblasts, endothelial cells, macrophages, and circulating bone marrow cells and there is currently no surface marker profile from which to isolate this population of MDSC. Initial reports of increased Sca-1 and CD34 expression in late pre-plate populations seemed to support their identification as putative MDSC \((26)\), however, the level of Sca-1 expression reported varies between groups \((21, 34)\). We have observed only a small fraction (approximately 10%) of Sca-1\(^{pos}\) cells (unpublished data) in accordance with a recent study by Mitchell et al. \((2005)\) \((34)\). Their study showed a lower proliferation rate and inhibition of fusion in Sca-1\(^{pos}\) cells which suggests a functional role for this cell surface marker, but, again, conflicting reports exist \((35)\). Furthermore, Sca-1 is not confined to muscle-specific cells \((25)\) and transplantation of either single (Sca-1) or double (Sca-1 and CD34) positive populations were not as effective as predicted \((36)\). Another recently identified cell surface marker is CXCR4, a cell surface receptor for the chemokine SDF-1. This receptor has been linked to the homing of HSC from the blood stream to damaged tissues and has recently been identified on muscle precursor cells \((37)\) and implicated as a marker of MDSC \((13)\), however, in our studies we have seen only low expression of this marker.

There is increasing awareness of issues related to altered expression of cell surface markers and changes in cell properties with increasing time in culture. Other markers have

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**Figure 2.** Detection of dystrophin replacement following injection of late pre-plate myoblasts. Following injection of late pre-plate male myoblasts into female dystrophic hosts, tibialis anterior muscles were sampled at 3 weeks, 3, 5 and 7 months, sectioned and stained for dystrophin. Approximately 10 sections were examined and the section with the largest area of dystrophin positive myofibres was analysed at each time point. It should be noted that this is a semi-quantitative method of analysis which is normally used in conjunction with recovery of male DNA. Analysis of (A) the total cross sectional area (CSA) of dystrophin positive fibres, (B) the number of dystrophin positive fibres, and (C) the average CSA of dystrophin positive myofibres. \((D–G)\) Dystrophin (red) positive area at 3 weeks \((D)\), 3 months \((E)\), 5 months \((F)\) and 7 months \((G)\) post-injection. Nuclei are counter-stained with Hoechst (blue). Bar represents 100 \(\mu m\).
met with similar controversy and there does not appear to be a common link with those expressed on putative murine MDSC and human muscle precursor cells. As such, the separation of MDSC from mouse and human skeletal muscle based on cell surface markers is not yet a reality. Without a specific surface marker phenotype, the location of these multi-potential ‘stem’ cells within the muscle is yet to be fully identified. It is of particular interest to know whether they are indeed located in the satellite cell position (i.e., represent a satellite stem cell) or lie outside the muscle fibre (an interstitial stem cell), with a key question being “do they actually exist in human muscles?”.

ACKNOWLEDGEMENTS
This work was funded by the Muscular Dystrophy Association (USA) and an International Postgraduate Scholarship (UWA). We thank Hannah Radley and Marilyn Davies for technical assistance.

REFERENCES


