Perspective Article

The physiology of stem cells:
Potential for the elderly patient

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Introduction

In 1999, Beardsley wrote "A flurry of startling discoveries in stem cell biology in past months has shattered preconceptions about how cell specialization is controlled in the body and has boosted the field to the top of scientific, political and commercial agendas. The excitement has raised hopes that the long sought goal of being able to regenerate human tissues may be closer than had been thought"[1]. Five years later, stem cell therapy research is still in its early stages, however, promising results with experimental transplants have been achieved, and first clinical applications are expected in 5-10 years. What is stem cell therapy? It is a potentially revolutionary new way to repair damaged and diseased tissues with healthy new cells provided by stem cell transplant. It offers an opportunity to treat many degenerative diseases arising as a consequence of death, destruction or malfunction of specific cells, in the context of the body's inability to repair or restore them. Currently, the only interventions available to treat wasting disorders include transplant surgery, or therapeutics to delay the onset and relieve the symptoms of the underlying malady.

Stem cell biology

Stem cells are specialized cells with the unique potential of self-renewal and cell specification following stimulation with appropriate biochemical/biophysical cues. Initially uncommitted, following specific signals, these cells have the capacity to differentiate into lineage-committed cells. Three broad categories exist to classify these cells: **1.** Following fertilization of an egg, the zygote replicates numerous times, ultimately giving rise to the 216 different cell types, which comprise the human body. The first three divisions of the zygote, however, yield 8 cells, each of which is capable of developing into a human being. These cells are referred to as **totipotent** stem cells. **2.** As the cells continue to divide from the 8 cell stage, the number of stem cells yielded increases, however, their capacity to trans-differentiate into different cell types becomes more limited. Five days post-fertilization, the blastocyst forms. The outer cell layer generates the placenta and the inner cell mass of approximately 50 cells creates the embryo. These latter cells are designated **pluripotent** embryonic stem, or ES cells. Although each is capable of generating most embryonic cell types, they are not capable, individually, of creating a human being. **3.** As the embryo continues to develop, the cells become more and more specialized and commit to specific cell types. In order for this differentiation to occur, as the embryo develops, genes necessary for earlier stages of development "switch off", until only those required for a specific tissue function/phenotype remain active. However, a small number of only partially differentiated stem cells persist in some adult tissues, and are referred to as **multipotent** stem cells. These are capable of forming a limited number of specialized cell types, and generally function locally to replace fully differentiated cells lost through depletion or damage.

Sequencing of the human genome has contributed to rapid advances in cell biology, improving our understanding of the physical cues and biological signals that control cell phenotype, and will potentially enable us to manipulate stem cells in culture and begin to unravel and utilize the healing process in patient tissues. Indeed, a key goal of stem cell research, whether in adult or embryonic stem cells, is to understand how differentiation is controlled and to learn how to direct its progression both *in* and *ex vivo*. Because of the perceived difficulties in working with adult stem cells (low numbers obtainable and problems of senescence), many argue that studying pluripotent ES cells will expedite the development of stem cell therapies. These cells are capable of generating more cell types than the adult variants, they

grow more quickly and are easier to differentiate, they are more abundant and easier to isolate and ultimately ES research may speed the development of adult stem cell therapies. However, despite the potential benefits of utilizing ES cells (e.g., 1. surplus blastocysts created during IVF therapy, 2. the extraction of cells destined to become eggs or sperm from aborted/miscarried fetuses or 3. the production of tailor-made ES cells using cloning techniques), the ethical issues surrounding their use are self-evident (see the following websites for further useful information: www.nuffieldfoundation.org/bioethics; www.dti.gov.uk; www.royalsoc.ac.uk; www.doh.gov.uk and www.mrc.ac.uk).

Despite current hurdles, potential clinical applications for stem cell technologies include: the production of cardiomyocytes to replace damaged heart tissue, the manufacture of insulin-producing pancreatic cells for patients with diabetes and the generation of neurones for the treatment of patients with Parkinson's disease. Indeed, the use of bone marrow transplants for patients with leukaemia, is an example of stem cell therapy already in practice. Although ES cells may initially appear the more versatile when required for the regeneration or repair of specific tissues, these cells can only be used in the form of allografts unless therapeutic cloning techniques are used to generate autologous ES cells. Furthermore, recent studies have highlighted the potential of ES cells to become cancerous with age, adding additional complexity to these cells[2]. Post-natal stem cells may not develop this problem, and can be used for autologous transplants. Progress is being made in the potential use of these adult cells, particularly for the treatment of muscle and connective tissue diseases and damage.

Skeletal muscle stem cells

Muscle wasting is frequently linked with increased protein catabolism, which is associated with altered cytokine profiles, in particular with increased levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF) and interleukin-6 (IL-6). This catabolism is characterized by progressive muscle protein loss and negative nitrogen balance, both of which may culminate in high levels of morbidity and mortality[3]. While limited protein mobilization is beneficial in acute catabolic disease, that associated with chronic catabolic conditions has severe implications for the patient, including: impaired respiration, mobility and efficacy of potential treatment. In addition to the disease state, healthy skeletal muscle is also subject to continuous injury, as a consequence of exercise, weight-bearing and trauma. In either situation (catabolism or injury), adult skeletal muscle exhibits a remarkable capacity to regenerate following myotrauma, which is dependent upon an available, local, renewable source of cells to repair the loss or damage. Because adult myofibres are terminally differentiated and are therefore incapable of replication, the regeneration of skeletal muscle is largely dependent on a small population of resident cells, termed muscle satellite cells[4,5]. Despite their identification 40 years ago, little is known regarding their

molecular phenotype, regulation or responses to physiological and pathological stimuli. While the potential of these cells remains ill-defined, the phenomenon of regeneration biology is of growing medical interest, therefore muscle regeneration research is essential.

Skeletal muscle satellite cells were first identified in frog leg muscles by electron microscopy, and have subsequently been identified in all higher vertebrates, including humans. They reside dormant beneath the basal lamina of skeletal muscle fibres, thus being ideally located for timely repair of damage or loss. These quiescent cells are activated to proliferate following damage, an essential first step towards generating sufficient numbers necessary for differentiation, fibre production and self-renewal. In humans and mice, these nonfibrillar stem cells are plentiful at birth (approximately 35%), but numbers decline with age to 1-5% in adults[6,7]. Following initial identification of satellite cells, further stem cell populations resident in skeletal muscle have been identified. These have not only added complexity, but also potential to the process of skeletal muscle regeneration[7,8].

In addition to regenerating muscle, these cells also display a large degree of plasticity, differentiating into non-muscle cells. Studies describing the plasticity of adult somatic stem cells have become a focus of interest because of potential clinical applications in the treatment of age or disease related degeneration. If exposed to strong osteogenic (e.g., bone morphogenetic protein 4(BMP-4)) or adipogenic (e.g., thiazolidinediones) inducers, or if they are cultured on soluble basement membrane matrix (matirgel), muscle stem cells spontaneously form osteocytes or adipocytes[9,10]. Indeed, early studies into the possible link between muscle loss and fat gain in the elderly suggest that skeletal muscle stem cells become more adipocyte-like with age, leaving the elderly individual less able to recover from muscle injury[11]. Furthermore, upon stimulation with vascular endothelial growth factor (VEGF) or nerve growth factor (NGF), skeletal muscle stem cells can be induced to express cellular markers of endothelial or neuronal cells, respectively[12]. The potential for and heterogeneity of muscle stem cells is underscored by the observation that muscle side population cells, positive for the haemopoietic marker CD45 are capable of reconstituting bone marrow in lethally irradiated mice, and also of contributing to neo-vascularization of regenerating muscle[7]. Finally, initial attempts to replace infarcted myocardium began with the direct injection of skeletal myoblasts into the infracted myocardium[13]. Cells successfully engrafted into the normal and infracted tissue, and cardiac function was improved, relative to controls at two months. External stimuli, were, however, required to stimulate contraction and relaxation of the cells and no meaningful gap junctions were formed[14].

While these initial findings are enormously exciting, with regard to the potential of adult skeletal muscle stem cells in regeneration and repair, little is known about the *in vivo* signals, which orchestrate these events. Detailed knowledge about the physiological, biochemical and molecular process-

es, which steer the progression of regulated differentiation and renewal are critical for translational research, if we plan to exploit the clinical use of stem cells for replacement therapies. Indeed, the potential for these adult cells in treating wasting disorders, many of which are associated with the aging process, has already triggered the advancement of research into the concepts of reprogramming cells for transplantation. Tissue engineering involving the reprogramming or rejuvenation of stem cells is emerging as a promising strategy. It is feasible to imagine a situation where a patient's cells are removed and expanded *ex vivo*, engineered to enhance specific properties, and reintroduced into the patient to create or repair specific tissues. This clinically relevant strategy of combining genetic and tissue engineering processes could be critical for treating among others, the elderly patients with wasting disorders. Cells, which are more plentiful than myoblasts (e.g., fibroblasts), could be harvested, expanded and reprogrammed into myogenic cells via the introduction of the muscle specific transcription factor MyoD. Senescence of these cells, as is observed with age, could be overcome by the expression of the catalytic protein of telomerase (hTERT), which would immortalize the cells. These cells could then be reintroduced into the patient, to repair damage when needed[15]. These processes are by no means pipe dreams of science fiction writers, but are fast becoming reality. However, certain caveats remain about the safety of these latter techniques over the long term, particularly with regards to oncogenesis. Efficiency and safety therefore need to be improved before engineered cells can be routinely used for transplantation.

Sarcopenia and cachexia

While huge potential appears to exist for skeletal muscle stem cells, perhaps the most relevant use for these cells is in the regeneration of skeletal muscle lost with aging or cancer. A progressive decline in muscle mass together with a parallel increase in fat mass are considered hallmark features of the aging process[16]. These losses of muscle mass and ultimately of strength, which are associated with age (sarcopenia), and with cancer (cachexia) are altered by factors that manipulate the balance between anabolism and catabolism of muscle. What these factors are, and how they function is of fundamental medical interest, because people die when they lose approximately 40% of their lean body mass[17]. Poor muscle strength as a result of muscle loss is a strong predictor of mortality. There is an approximate 30-40% loss of muscle strength between the second and seventh decades of life[18], and by the time they are in their 80s, most successfully aging people have sarcopenia. Extrapolated on a population basis, these data suggest that approximately 9 million people in the US have sarcopenia[17], making it a major health care issue. Cross-sectional studies suggest that the main source of muscle loss is in type II fibres. Importantly, these fibres are the fibres that have the highest number of associated satellite cells[7,17]. The natural progressive loss of muscle fibres and of

stem cells with age, together with a reduced capacity of myoblasts to proliferate, and a propensity to accumulate fat, may all contribute to an inability to regenerate muscle with age. Gaining a better understanding into the anabolic and catabolic agents contributing to muscle loss, may enable the development of therapeutic interventions.

Anabolic and catabolic agents in muscle

We believe that the insulin-like growth factor (IGF) system positively impacts on muscle anabolism, while the TNF system acts to trigger catabolism. We hypothesise that growth factor/cytokine interactions are fundamental to a normal muscle phenotype, and that a cytokine environment will culminate in destruction. We believe the IGF system will allow protection, survival, cytoskeletal integrity and differentiation, and that these positive effects will be reduced by TNF, decreasing muscle maintenance and repair.

Many growth factors stimulate myoblast replication *in vitro*, but the IGFs uniquely induce terminal differentiation[19,20], potentially via the induction of myogenin[21]. IGF effects are altered by IGF binding proteins (IGFBPs), with IGFBP-5 overexpression prolonging myoblast survival, and inhibiting differentiation[22]. In conjunction with its role as a positive differentiation factor, IGF-II minimizes cell death during the transition from proliferating to differentiating myoblasts. We have shown that IGF-II antisense skeletal muscle cell clones undergo rapid apoptosis when incubated in low serum. Death was blocked, and differentiation promoted by exogenous IGF[23]. Similar anti-apoptotic effects of IGF-II are evident in myoblasts from dystrophic animals[24]. Murine gene knock out studies ablating the IGF system result in growth impairment accompanied by severe skeletal muscle hypoplasia[25,26]. Conversely, overexpression of IGF-I in skeletal muscle of mice stimulates myofibre hypertrophy[27]. We have shown that mice exposed to excess IGF-II through IGF-IIR ablation, exhibit significant overgrowth, most marked in cardiac and skeletal muscle[28]. IGF-I mRNA and protein are detected in newly replicating rat skeletal myoblasts after injury[29] and IGF gene expression is seen as an early event during work-induced muscle hypertrophy[30], muscle overload or eccentric exercise[31]. These data clearly indicate a role for both IGF-I and -II in muscle growth, regeneration and in response to injury.

The circulating and intramuscular concentration of IGF-I, and the reponsiveness of muscle to IGF-I are reduced in most medical conditions where circulating cytokines are elevated and muscle wasting is evident. Studies conducted in humans show that concentrations of pro-inflammatory cytokines, at concentrations often found in the elderly population, interfere with IGF-I gene and protein expression, and the capacity of IGF-I to interact with its receptor[16]. TNF is believed to be important in the pathogenesis of muscle wasting, with acute administration causing severe, transient weight loss in rodents[32], and prolonged exposure resulting in profound wasting[32,33]. Site-specific TNF production also alters the pat-

tern of tissue wasting[33], suggesting that local production may be sufficient to induce degeneration. Neutralizing TNF antibodies administered to tumour-bearing mice reduced protein and fat losses, slowed tumour growth, and prevented TNF-induced cytokine cascades[33,34]. Furthermore, intravenous infusion of TNF into rats caused a significant reduction in circulating levels of IGF-I and IGFBP-3, and increased IGFBP-1. Furthermore, high circulating levels of TNF, IL-6 and low circulating levels of IGF-I are synergistic risk factors for poor muscle strength, and are independent risk factors for mortality[35]. TNF is overexpressed in adipose tissue of obese rodents and humans, and is associated with insulin resistance[36]. TNF message is detectable in skeletal muscle, and cultured skeletal myoblasts secrete TNF, with higher detectable levels in muscle from subjects with insulin resistance[37], suggesting a local autocrine function for TNF in skeletal muscle resistance to the anabolic effects of insulin. Furthermore, TNF blocks morphological and biochemical differentiation, and induces significant apoptosis of murine skeletal myoblasts, when compared with controls[38]. Concomitant with a block in differentiation and the induction of death, TNF suppresses the secretion of both IGF-II and IGFBP-5 by these cells[38].

These important interactions between the IGFs and TNF/IL-6, regarding muscle maintenance in aging or disease, warrant further investigation. Since sarcopenia is a relatively slow process, longitudinal studies would need to be performed to monitor these questions. Due to underlying similarities between sarcopenia and cachexia (muscle loss with increased cytokines and decreased responsiveness to IGFs), we chose to investigate the impact of disease and age on muscle stem cell retrieval, as well as the responsiveness of these cells to growth factors and cytokines. Following ethical approval and informed consent, perioperative muscle biopsies were taken from the anterior abdominal wall of patients undergoing elective surgery for benign or upper gastrointestinal malignant conditions. All patients had comparable BMIs fat and lean body weights. Primary skeletal muscle cultures were derived from all biopsies, irrespective of age, sex and disease state[39,40]. All cultures could be induced to differentiate, with a significant correlation existing between initial myoblast number and biochemical markers of differentiation[40]. As would be expected, in the benign patients there was a negative correlation, which tended to significance between patient age and myoblast number (r=-0.442, p=0.066, n=18). In contrast to what we anticipated, rather than seeing an accelerated decline in myoblast number and differentiation capacity, the age relationship was lost in malignancy. In benign cultures, the percentage myoblasts decreased significantly in patients over 50 years of age (p<0.05), whereas in the malignant cultures, numbers remained constant[40]. The potential of these findings is enormous. If we can ascertain what is occurring in the disease state, prior to the onset of cachexia, perhaps we can apply these findings not only to the issues of chronic catabolic conditions, but also to the issues of stem cell depletion in sarcopenia in an attempt to reverse or stem the process of muscle loss.

Conclusion

Myofibre degeneration, with lack of regeneration, may be attributable to more than simply deregulated protein synthesis/degradation, with altered specification, migration, proliferation, survival, and differentiation playing contributing roles. These are complex processes involving intrinsic and extrinsic cues, of which very little is known. Although progress is being made, muscle satellite cell biology is still an emerging field, such that informative answers to some of the most fundamental biomedical questions remain to be answered.

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