THE PROLIFERATION AND FUSION OF MYOBLASTS IN VIVO

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In this paper the term myoblasts is a general term for all muscle precursor cells. The aim of myoblast therapy is to introduce normal myoblasts into dystrophic (dy) hosts, and to fuse the maximum number of normal donor myoblasts with dy host muscle cells. Some of the questions that need to be discussed are: How many host muscle precursors or areas of regenerating muscle fibres are normally available at any one time to fuse with the implanted myoblasts, and therefore what proportion of the dy muscle fibres will actually incorporate donor muscle nuclei after a single injection of myoblasts? We also need to know whether the introduced myoblasts should be encouraged to proliferate locally or should they just fuse after implantation? For how long can these implanted myoblasts persist without fusing? (This may have immunological implications). Finally, what are the conditions in vivo that regulate or enhance the proliferation or the fusion of myoblasts?

At all times we should be aware that we will be dealing with dy host myoblasts which will be derived from postnatal muscle of juveniles (presumably from satellite cells), we are not likely to be talking about embryonic host muscle precursors. However, the potential source of implanted donor myoblasts may be postnatal adult or juvenile muscle, or embryonic tissue, or perhaps cell lines. There may be significant differences between postnatal myoblasts and those from embryos or cell lines.

I shall briefly discuss 3 topics.

1. Proliferation of host (dy) myoblasts.
2. Factors controlling the proliferation and fusion of myoblasts in vivo.
   Models of myogenesis
   Growth factors
3. Genetic influences in regeneration

Proliferation of host (dy) myoblasts

It has been shown that introduced normal myoblasts will fuse with regenerating dy host muscle cells (Partridge et al., 1989); but it is not known whether the donor myoblasts fuse directly with new membranes formed around damaged areas of the dy muscle fibres, or with host muscle precursors to form myotubes. Are enough dy muscle fibres regenerating at any one time, to take full advantage of the myoblast transfer? If not, then should the number of host muscle cells available for fusion be increased? One way to achieve this is to induce regeneration in dy host muscles. This would result in large numbers of host (dy-) myoblasts being available for fusion.
with implanted (dy+) myoblasts and the formation of more mosaic muscle fibres. If we are prepared to induce regeneration, then a local anaesthetic like Marcaine which is known to be highly myotoxic (Foster and Carlson, 1980) might be the agent of choice in dy human subjects.

Most of the results that I shall refer to are from autoradiographic studies on muscle regeneration in vivo carried out in collaboration with Dr. John McGeachie from the University of W.A. Essentially what we do in these experiments is to injure or transplant muscles in mice, inject ³H-Tdr at various times after injury to label cells synthesising DNA at that time, leave the animals for 10 days until the labelled muscle precursors have fused to form myotubes, and then retrospectively determine the pattern of muscle precursor replication (McGeachie and Grounds, 1989). Autoradiographic results after muscle injury in BALB/c mice demonstrate the onset, peak and duration of muscle precursor cell replication (McGeachie and Grounds, 1987), and show that there is essentially no DNA synthesis in muscle precursors before 30 hours and little after 5 days. The peak of precursor replication is around 2 to 3 days, depending on the type of injury. We know that many of these precursors fuse after only 2 cell divisions (Grounds and McGeachie, 1987) and this is confirmed by the appearance of myotubes from about 3 days.

Similarly, in dy muscles injured with Marcaine, we would expect that many host myoblasts would be proliferating after 2 to 3 days. So if we injured dy host muscles with Marcaine and then around 3 days later injected the donor myoblasts, maximum fusion should be possible between host and donor muscle cells. Although it seems radical, such a procedure might be recommended in humans to maximise the benefit of injecting myoblasts.

Factors controlling the proliferation and fusion of myoblasts

Factors controlling muscle precursor cell behaviour in vivo are largely of relevance to satellite cells, but also to the implanted myoblasts whatever their actual source.

Two different models of myogenesis have emerged from tissue culture studies. The cell lineage model says that stem cells give rise to committed cells and that these must undergo a series of obligate cell divisions before they are terminally differentiated and their only option is to fuse. This relatively inflexible model has important repercussions in vivo as committed precursors cannot be made to fuse prematurely, and they cannot be made to proliferate beyond the quantal cell cycle. The alternative permissive or opportunistic model says that there is no strict lineage and that, depending on the particular set of conditions, the muscle precursors can continue to proliferate or alternatively can undergo fusion. We have tested the cell lineage model of myogenesis as defined by Quinn and her colleagues (Quinn et al., 1985) in regenerating mature muscle of mice and chickens in vivo, and our autoradiographic results do not support its predictions (Grounds and McGeachie, 1987; 1989a). This issue needs to be resolved, as these models have relevance to the potential manipulation of muscle precursor behaviour in vivo.

Tissue culture studies also show that various hormones (Florini, 1987; Cossu et al., 1989), growth factors (Florini and Magri, 1989) and components of extracellular matrix (Sanes, 1986; Grounds, 1989) affect the proliferation and fusion of muscle precursors: some of these studies have been carried out on satellite cells from mature muscle (Allen and Boxhorn, 1989; Cossu et al., 1989).

The main effects of some hormones and growth factors (GF) are shown below: their action may depend on the dose used and the combination with other growth factors (Florini and Magri, 1989). It will be very interesting to find out how these substances operate in vivo, and whether they can be used therapeutically to improve muscle regeneration, or to specifically enhance proliferation or fusion of the host or donor myoblasts in the proposed therapy.
FACTORS AFFECTING MYOBLAST BEHAVIOUR IN VITRO

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Genetic factors

In myoblast therapy in humans, donor myoblasts will be fused with host muscle cells of a different genotype. The genetic differences between donor and host may be quite pronounced. There clearly may be problems of immunological rejection. In addition, it has already been recognised that there are problems associated with efficiently fusing together myoblasts from combinations of different genetic mouse strains, and that this also depends on the immunological status of the host (Watt, 1982; Partridge, 1982; Partridge et al., 1989).

Two genetic mouse models of muscle regeneration that we have described may enable us to identify some of the important factors controlling regeneration (Grounds and McGeachie, 1989b). In Swiss mice muscle regeneration is excellent, whereas in BALBc mice it is poor. At 10 days after severe crush injury large numbers of myotubes are present and there is very little fibrosis in lesions of adult Swiss mice, however, in BALBc mice myotube formation is poor and large areas of fibrous and cellular connective tissue are present in the centre of the lesions. It would be useful if conditions in BALBc mice could be manipulated to improve their muscle regeneration. Why is muscle regeneration so different in the two strains? What are the genes involved? It is desirable to know to what extent the general host environment is important in controlling the effectiveness of muscle regeneration, e.g. the capacity to revascularise, macrophage function, the extracellular matrix and circulating factors: as compared with the importance of genetic factors inherent in the muscle itself, such as the capacity to form new membranes to seal the ends of the damaged fibres, the production of specific growth factors (Bischoff, 1986), production of growth factor receptors and responsiveness to growth factors.

An understanding of the genetic factors controlling different aspects of muscle regeneration has direct relevance to myoblast transfer therapy, as the success of this procedure may be determined by the genotype of the host environment and host myoblasts, in combination with the genotype of donor myoblasts.

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REFERENCES


DISCUSSION

Partridge: First of all could you talk about the idea of inducing a crisis as a means of enhancing fusion. You're suggesting that one might put Marcaine into dystrophic muscle: could you expand upon the timing if you were led to do so grave a thing?

Grounds: One really needs to know to what extent the donor myoblasts want to fuse when they're introduced, or to what extent they're prepared to stick around and proliferate. But if one assumes that when you implant them they want to fuse, then I think that you have to injure the host muscle prior to implanting the donor myoblasts such that there are large numbers of host muscle precursors available and ready to fuse with the donor myoblasts in order to produce the mosaic fibers.

There is really very little information on the number of host muscle precursors in dystrophic muscle that are available for fusion at any one time. There has been only one study that I know of which provides some information on this subject. Anderson and colleagues (1988) injected tritium thymidine into mdx mice aged four, or 32 weeks of age, took the samples out one hour later and looked at (premitotic) subsarcolemmal nuclei, which are considered to be satellite cells. They found that two to three percent of the subsarcolemnuclei were labelled. This represents a 200
fold increase over satellite cell turnover (0.014%) in normal adult mouse muscle (McGeachie and Grounds, 1989). Tritiated thymidine is only available for about an hour after injection, so only cells which are actually synthesizing DNA at that time are labelled. The DNA synthesis part of the cell cycle is perhaps roughly a third of the entire cell cycle, so the two to three percent might represent as much as nine percent of satellite cells which were moving through the cell cycle and proliferating at this time: not necessarily synthesizing DNA, but in a proliferative phase. Now, nine percent is a very high proportion because satellite cells are normally considered to represent only about one to five percent of all muscle nuclei in adult host muscle fibres (Allbrook et al., 1971). There may be very large populations of satellite cells available in dystrophic muscle, and I think that it is important to obtain more precise information on this subject.

Another option that exists if you do have quite a large population of activated proliferating dystrophic host muscle precursors, is to directly enhance the proliferation of these cells (not by causing degeneration and regeneration with Marcaine) by putting in a mitogenic growth factor like fibroblast growth factor, which will increase the proliferative capacity of the host muscle precursors, before you put in the donor myoblasts.

I think that if you're going to go through the trauma of injecting the donor myoblasts, then you want to ensure that you get the maximum efficiency out of the procedure. Although it is slightly radical to consider interfering with the dystrophic subject in this way I think that this approach should be seriously evaluated.

**Partridge:** As Marcaine is a local anaesthetic, at least it would be painless!

**Grounds:** It is certainly preferable to the use of myotoxic snake venoms.

**Partridge:** You put forward two extreme versions of the developmental basis of myogenesis. If one of these were to be confirmed to the exclusion of the other in mature muscle *in vivo*, then what sort of changes in strategy would that lead you to adopt with respect to myoblast implantation therapy?

**Grounds:** Well there are two aspects to this: One is that the donor cell which you are going to implant will be relatively synchronized, as the muscle precursor cells will have been cultured for a particular length of time and will presumably have passed through several cell cycles. If you assume that the cell lineage model applies - with its obligate cell divisions and a terminal cell cycle - then your donor myoblasts might be fairly close to the stage of terminal differentiation. Whereas your dystrophic host myoblasts, if you haven't modulated them in any way, would presumably be a very heterogeneous population of cells - some would be stem cells, some early committed precursors, and some might be almost terminally differentiated. So when you put these two groups of muscle precursor cells together, there might be difficulty in actually fusing the donor myoblasts (many of which may be essentially terminally differentiated), with the host muscle precursors (which are at various stages of differentiation, many not able to undergo fusion). This simplistic scenario results from the strict cell lineage model, which clearly has implications for the efficiency of fusion between the donor and host muscle precursors. In contrast, the opportunistic idea says that muscle precursors if they find themselves in the right environment, will quite readily fuse together: there is no barrier to fusion.

The second aspect is relevant if you want to entertain the idea of playing around with enhancing the proliferation of either the host or the donor myoblasts, or perhaps delaying of enhancing the fusion between them. This is much more difficult if the cell lineage model is the situation that applies *in vivo*, because you just don't have the flexibility. In contrast the opportunistic model says that if you change the environment around the muscle precursors, then they will respond accordingly.

**Partridge:** What about growth factors? Could you use them systemically, or would you have to apply them locally?
Grounds: Well, again, not many people have worked with, or explored these avenues in vivo. The situation depends upon what questions you’re asking.

You may want to use a mitogen like fibroblast growth factor (FGF) or perhaps the Bischoff muscle growth factor. If it is a competence factor like FGF, then it probably only has to be available for a very short period of time, in order to move the cells from G0 into G1. So if the aim is to promote host muscle precursor cell proliferation by putting in a competence factor, the factor only has to be available transiently and could be injected. A local intramuscular injection would seem to be much more efficient than systemic injection which would consume a large amount of expensive growth factor. In dystrophic humans an intramuscular injection like this is feasible.

Another method of administering growth factors is the incorporation into substances like Agarose gel which are biodegradable, would disperse throughout the tissue, and would have a slow release effect (Hayek et al., 1987). This approach is more important when the growth factor needs to be available for a longer period of time, such as with progression factors, or with growth inhibitors like transforming growth factor beta.

A third option which lends itself more to experimental work in mice, is to incorporate the growth factors into plastics such as Hydron (hydroxy methyl methacrylate : Langer and Folkman, 1976) or L-vax 40 (ethylene vinyl acetate copolymer : Rhine et al., 1980). These plastics can be made up into little buttons, pellets or sheets, and implanted under the skin where they are non-inflammatory and slowly release the growth factor. You can devise scenarios whereby pellets are implanted containing combinations of several growth factors which would enhance proliferation but restrict differentiation and fusion (Allen and Boxhorn, 1989): the pellet could later be removed and the large numbers of muscle precursor cells that had been generated could fuse to form myotubes. The use of pellets in this way is attractive for experimental purposes in animals, but is less applicable to humans.

REFERENCES