

Histochemical studies of developing and diseased muscle. From bench to bedside and back. A tale of two interests

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Abstract

My first exposure to Duchenne muscular dystrophy during a residency post in London in 1957 led to a life-time commitment to neuromuscular disorders. In addition to the clinical aspects, I was also keen on basic research, which channelled me into histochemistry, under the guidance of Everson Pearse at the Postgraduate Medical School, a pioneer in the field. Initial studies of dystrophic muscle were followed by normal muscle in human and various animals, the definition of fibre types in muscle on histochemical grounds, and subsequent application to developing human and animal muscle and the effects of denervation, reinnervation and crossed-innervation in muscle. But in the long term my first love has been for clinical medicine and a return to the diagnosis and management of children with neuromuscular disorders in parallel with continuing multidisciplinary research.

Key words: histochemistry, Duchenne muscular dystrophy, human and animal muscle, development, denervation, reinnervation, crossed-innervation

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From the patient to the laboratory

My life-long interest in muscle and its diseases was sparked by a chance three week temporary resident's post in Queen Mary's Hospital for Children, south of London, in 1957. Apart from my general paediatric responsibilities, I might also be called to one of the two long stay muscular dystrophy wards if a child needed attention for pneumonia or other problems. Having never heard of muscular dystrophy, let alone seen a case, my curiosity was aroused and took me to the wards the next day and I have been hooked ever since. In addition to acquainting myself with the clinical and pathological features of the disease, I was also keen to do some basic research and advised to consult professor Everson Pearse, one of the founding fathers of enzyme histochemistry, at the Royal Postgraduate Medical School. He welcomed me to his lab despite my complete ignorance of the field.

My initial studies of normal and dystrophic muscle with a wide range of enzyme histochemical techniques led to the discovery of a reciprocal activity of oxidative and glycolytic enzymes in individual fibres and the definition of type 1 and type 2 fibres in human muscle on histochemical grounds. I then extended the studies to various animals including mouse, rat and guinea pig, as well as pigeon breast muscle, fish muscle and the fast and slow twitch muscles of the chicken, and also a study of developing muscle in the human fetus and the newborn mouse, rat and guinea pig.

Mouse and rat muscle were undifferentiated at birth and comparable to a 20 week human fetus, whereas guinea pig muscle had a fully mature differentiated pattern at birth.

An opportunity of applying these histochemical techniques to one of the unusual congenital myopathies, central core disease, confirmed the potential value of enzyme histochemical techniques in the study of diseased muscle. It demonstrated two unusual features. Firstly, all the fibres were uniformly high oxidative type 1 fibres, with loss of the normal checkerboard pattern, and secondly the cores, previously shown with trichrome staining to be a more compact aggregation of myofibrils, were shown to be devoid of oxidative and glycolytic enzymes and also glycogen, and presumably non-functional.

The same year, 1960, also saw the publication of an interesting experiment in Eccles' lab in Australia. Crossing the nerves of a slow twitch muscle (soleus) and a fast twitch muscle (flexor hallucis) in the cat resulted in the switch of the fast or slow twitch characteristics to conform to the corresponding nerve. This looked like an interesting model to study to potential change in histochemical pattern of the respective muscles after cross innervation. But at that stage I decided to return to clinical medicine and obtained a lecturer post in the Department of Child Health in Sheffield. It was not until 1965 that I had the opportunity of pursuing this further along two separate

Heterogeneity of human fibres

Basic Applied Myology 17 (3&4): 147-149, 2007

channels, when I arranged a sabbatical year at the Institute of Muscle Disease in New York in parallel with a clinical attachment in the paediatric department of the Cornell Medical College nearby.

Cross innervation of fast and slow muscles in the mature cat produced a remarkable change in histochemical pattern in parallel with the change in twitch characteristics. A further series of studies were undertaken in mature and newborn rabbits. I was also struck by the bizarre structural changes in the muscle during the phase of regeneration in a series of rabbits I studied soon after the cross innervation, near the end of my time there. These bore some superficial resemblance to the structural changes seen in dystrophic muscle and raised the question of a neural influence in the pathogenesis of muscular dystrophy. This was one of the theories current at the time in addition to the more popular theory of a defect in the muscle membrane.

After my return to Sheffield at the end of 1966, I started a laboratory research program in parallel with my general clinical responsibilities and the additional specialised muscle clinic I had started for diagnosis and management of muscle diseases in children.

This research evolved along two main channels. Firstly we applied the technique for transplantation of minced muscle, recently published by Bruce Carlson, based on the pioneering work of the Russian biologist Studitsky. In this way we could track the regenerative ability of normal and dystrophic muscle either reinserted into the same animal or into a normal or dystrophic littermate.

We studied two separate animal models of muscular dystrophy, the *dy/dy* mouse and the BIO 14.6 rabbit. We also started a program of tissue culture of normal and dystrophic human muscle explants, which matured to the point of myotubes with cross striations and occasional spontaneous twitches. This was followed by a series of experiments of co-culture of explanted human muscle with isolated slices of mouse embryonic spinal cord, which innervated the muscle and led to further maturation and rhythmical twitching of the muscle. This technique was pioneered by Edith Peterson at the Albert Einstein College of Medicine in New York, whom my research fellow Belinda Gallup visited. We later used this technique to study various forms of diseased human muscle including structural congenital myopathies in order to see whether one might be able to reproduce the structural changes in long-term cultured muscle preparations.

It was in the course of this ongoing program of experiments initiated in Sheffield in the late 1960s and subsequently continued in my muscle unit at the Royal Postgraduate Medical School in London after I took up the Chair of Paediatrics and Neonatal Medicine in 1973, that I had the pleasure and privilege of meeting Ernest Gutmann on a number of occasions, including also a visit to his laboratory in Prague when I attended a paediatric neurology conference. I vividly recall his enquiring mind and how he was always keen to delve in depth into a particular topic and also to intermittently take his constant companion notebook out of his pocket to jot down a few notes in it.

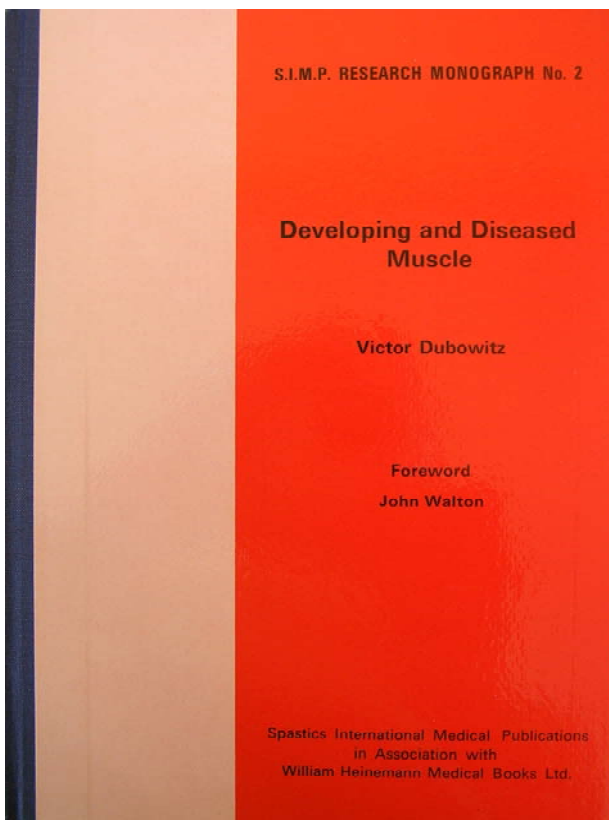
Back to the bedside

The 1980s saw a totally new direction of research in relation to neuromuscular disorders, with the molecular genetic explosion and the development of powerful techniques for the location, isolation and cloning of genes in relation to previously unknown diseases, such as muscular dystrophy, and then to identify the protein involved. This in turn opened a completely new potential for therapy of these previously enigmatic diseases.

We now find ourselves in exciting times and seemingly on the verge of a major breakthrough in the potential cure of these diseases by replacement, or manipulation, of the defective genes.

Unfortunately, we have now been on the verge for some 10 years or more and the recurrent promises of the basic scientists that the cure was just round the corner, have not really brought the corner any nearer. Instead of the orderly and rather slow progress one often experiences in scientific studies, the advances in this field have been punctuated by wild swings to the crest of the wave, only to be followed by descent into the trough again.

The main reason for this is that the laboratory scientists doing the delicate genetic manipulative work on animal models of human muscular dystrophy, have



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published short term acute experiments in prominent scientific journals, such as "Nature" and "Science", with a fanfare of publicity in the media. This inevitably was followed by a parallel crescendo of hope on the part of the sufferers and their family. In addition, these scientists routinely recommend going straight from uncontrolled, relatively short term observations in animals directly to clinical trials in human patients. What is sorely lacking is a cohort of animal clinicians doing clinical trials on the dystrophin deficient mdx mouse, which has a much milder phenotype than the human disease, or on the dystrophin deficient golden retriever dog, which has a disease of comparable severity to the human. A recent example of this recurrent hype was the worldwide publicity through the BBC World Service, following a publication in Nature in November 2006, of a series of uncontrolled studies of stem cell treatment in dystrophic dogs. The stem cells used were a novel mesangioblastic stem cell line capable of ready conversion to myoblasts, obtained originally from the embryonic aorta and more recently from the peripheral vasculature of mature animals. The therapeutic trial was compounded by all the animals having prednisone and those given heterologous, rather than autologous stem cells, also having a variety of additional immunosuppressive drugs. As prednisone has been well documented over the past 15 years to have an unequivocal therapeutic benefit in Duchenne dystrophy, one would need to do controlled studies on the dog with and without prednisone and in addition also control studies for the immunosuppressive drugs. So the jury is still out on the stem cells and a lot more carefully controlled studies need to be done on the dystrophic dogs. One way round the usual counterargument of the researchers that dogs are expensive and in short supply, would be to treat the same dog sequentially with add-on regimes, such as a period on prednisone, followed by a period on other immunosuppressive drugs, followed by the actual stem cell therapy, and assessing the change with each variable, rather than a blunderbuss approach with multiple agents simultaneously in the dog.

Another exciting area of potential therapeutic benefit, is gene repair, with antisense oligonucleotides. As approximately 70% of Duchenne cases are due to out-of-frame deletions of exons in the gene, it has been shown that knocking out further selected exons may put the residual exons back in frame, and thus potentially produce a milder disease. This programme has already passed through several carefully controlled and documented stages, from cultured myoblasts, from mdx mice, to in vivo studies in mice and subsequently,

manipulation in culture of various known deletions in muscle from cases of Duchenne dystrophy and putting the mutation back in frame and producing dystrophin in the cultured muscle. This program is now at the phase of clinical trials and the two main collaborative studies are those of van Deutekom in Leiden and Muntoni in London. Moreover, van Deutekom has shown that with the use of a limited number of antisense oligonucleotides, aimed at about 15 different exons, one should be able to put back into frame about 70% of the common deletions, recorded in the cumulative Leiden database of Duchenne muscular dystrophy.

The future for the treatment of Duchenne dystrophy certainly looks rosy but we have not yet reached the light at the end of the tunnel. Meanwhile, the advances in the basic care and management of cases of Duchenne dystrophy over the past 25 years, have had a remarkable impact on the disease. Thus the median survival in Duchenne dystrophy is now around 25 years, compared to about 16 years in the 1960s. Provision of lightweight, polypropylene orthoses at the time of loss of ambulation, can achieve a further 2 to 3 years of active, independent ambulation and also forestall the development of scoliosis which occurs after loss of ambulation. Cumulative experience with the use of low dosage corticosteroids over the past 20 years have also shown considerable benefits in the short and longer term, both on the physical function and prologation of ambulation, but also on respiratory function. The wider use of non-invasive mask ventilation at night once these boys go into respiratory failure in their late teens, has improved not only the survival, but also the quality of life and well-being day to day. More sophisticated monitoring of cardiac function in Duchenne boys with echocardiography from an early age, has also lead to the early detection of left ventricular dysfunction and potential therapeutic intervention with ACE inhibitors and also pharmacological approaches which may further improve survival. With the much more widespread positive attitude amongst physicians and orthopaedic surgeons to the management of boys with muscular dystrophy, the scene is set for maintaining these boys with their progressive disease in optimal condition pending the advent of the final cure.

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