Structural organisation of connective tissue

D.A. Brüggemann[1], A.P. Harrison[2] , Jens Risbo[1] & Anders H. Karlson[1]

[1] Department of Food Science, Faculty of Life Science, University of Copenhagen, Rolighedvej 30, 1958 Frederiksberg C, Denmark , E-mail: dab@life.ku.dk; ahk@life.ku.dk

[2] Department of Animal and Veterinary Basic Sciences, Faculty of Life Science, Grønnegårdsvej 7, 1870 Frederiksberg C, Denmark, E-mail: adh@life.ku.dk

Abstract

The mammalian skeletal muscle cell is very well investigated and the spatial arrangement of the molecules organizing it into sarcomeres, myofilaments, myofibril and myofibres can be considered as completely understood. It is also known that the structural integrity of muscle fibres is maintained by three layers of intramuscular connective tissue: the endomysium that surrounds the individual muscle fibre, the perimysium that bundles a group of muscle fibres and the epimysium that enfolds the whole muscle. On the other hand, much less is known about the hierarchical organisation of the structural components within these intercellular tissues. To make matters even more complicated, in these intercellular spaces, muscle tissue is also mixed with vascular and nervous tissues. Using chemical methods, the molecular components of these tissues are not distinguishable from the muscle tissue. Scanning electron microscopy (SEM) can be used to get an overall view of the localisation and distribution of the vascular and nervous tissue. The further use of confocal laser scanning microscopy (CLSM) in combination with immuno - histochemistry allows for identification and localisation studies of molecular components**.**

Introduction

If one considers the contractile machinery of muscle in the context of interrelated factors, then suddenly one is faced with a very complex area of study. At the one level it is essential that one can combine contractile units in such a way as to augment and optimize force production, but at another level one needs also to provide space for an ancillary network to facilitate the supply of nutrients and the removal of waste compounds. It is this interconnection both between the anatomical and physiological parameters that comprise muscle and between the fundamental requirements of contraction *versus* the continued supply and removal of compounds that makes muscle a fascinating subject to research.

The traditional way of looking at connective tissue in skeletal muscle focuses primarily on the structural integrity of muscle, which is thought to be maintained by three layers of intramuscular connective tissue: the endomysium that surrounds the individual muscle fibre, the perimysium that bundles a group of muscle fibres and the epimysium that enfolds the whole muscle. Moreover, since the most plentiful and widespread protein component of the connective tissue is collagen, there has been a great deal of focus on the identification and localization of collagenous structures. However, the connective tissues are also the place where vascular and nervous structures are embedded. Vascular structures in particular, are highly abundant in skeletal muscle and could therefore considerably contribute to the quality of meat and meat products. There is a lack of knowledge concerning these structures, which is grounded in a lack of methodology. However, with the development of confocal laser scanning microscopy it is possible to study the molecular composition and behaviour of such structures.

Material and methods

Muscle samples from *M. biceps femoris* were collected at 24 h *post-mortem* and consequently snap frozen in liquid nitrogen. Antibodies against Desmin DE-R-11, LYVE 1 and Collagen I were purchased at Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The antibody against laminin came from the Developmental Studies Hybridoma Bank (University of Iowa, Dept. of Biological Sciences, Iowa City, IA 52242 U.S.A.). The phallotoxin Atto 647N-Phalloidin and fluorescently labelled wheat germ agglutinin (WGA) from Fluka was purchased via Sigma-Aldrich Inc. (St. Louis, MI, U.S.A.). Secondary antibodies conjugated to Alexa 488, 555 and 647 and the lipid stain Bodipy were acquired from Invitrogen, Molecular Probes (Eugene, OR, U.S.A.). Immuno-histochemistry was performed as previously described in Brüggemann and Lawson (2005). A CLSM (SP2, Leica Laser Technik GmbH, Heidelberg, Germany) was used for imaging. In order to avoid false positives by collecting fluorescence emission from adjacent fluorophore channels, a sequential confocal imaging technique was used. The images shown are the result of a projection from the stack of images in the vertical direction using the maximum intensity mode. The

resultant grey-value for each pixel was the highest value present in the column of pixels in the stack with the same (x,y)- position. For scanning electron microscopy samples of rabbit *M. biceps femoris* were studied using a FEI scanning electron microscope.

Results and discussion

Pork and rabbit *M. biceps femoris* comprise an extensive vascular system of different hierarchies. Arteries, arterioles, capillaries, venules and veins as well as lymphatic vessels can be identified by their morphological features and molecular wall composition. All of these vessels consist of single or multiple layers of endothelium and consequently a basement membrane. The basement membrane of the vasculature can be visualized using anti- Laminin (Fig. 1 b,c). Smooth muscle cells help the vascular vessel to dilate and contract and their arrangement can be studied by labelling for Desmin (Fig. 1a). Further wall components' are elastin and collagen to different extents and in different arrangements depending on the type of vessel. Arteries, venes, arterioles and venules are accompanied by a meshwork of nerves within their walls, which serves to ensure the maintenance of an appropriate blood pressure.

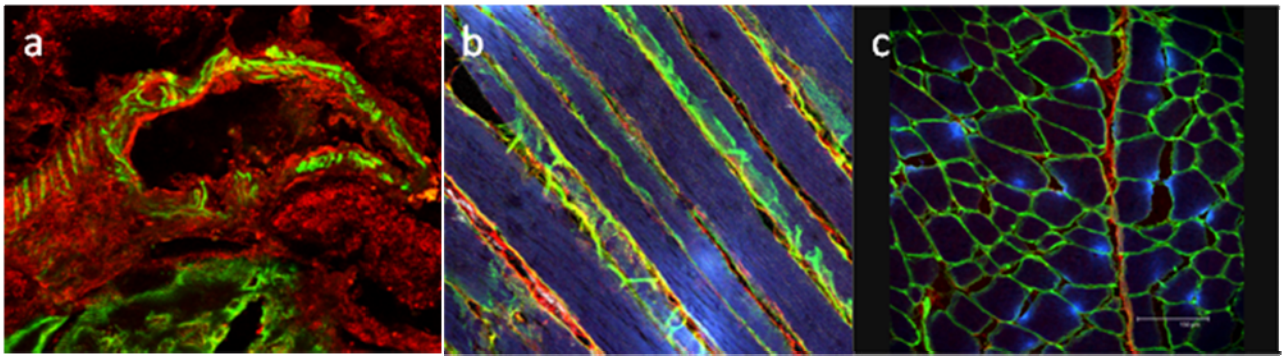

Figure 1. Molecular markers for the vascular system (a) smooth muscle cells in the vascular wall are shown using anti-Desmin (green) and anti-Decorin (red), bar equals 5µm. Branching vascular vessels of different hierarchies are visualised using anti Laminin (green), Phalloidin (blue) and WGA (red). The same labelling is used in the longitudinal (b,) and in the cross section of *M. biceps femoris*. Bar = 60µm.

However, vascular vessels do not only run straight, or parallel with the muscle fibres. In fact they are corrugated, branching structures and the degree of corrugation is dependent on the ultimate sarcomere length of the muscle fibres. This make capillary counts based on transverse sections questionable in terms of their accuracy, as shown by Mathieu-Costello et. al. (1988). New approaches include stereological measurements based on 3-D constructions of optical sections based on immuno labelling and CLSM (Kubinova et al. 2001).

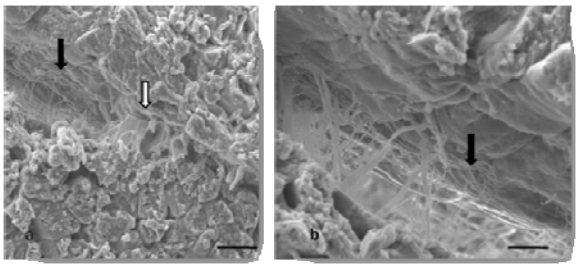

Figure 2. Vascular wall material degraded during storage of meat. SEM images showing a cross section of rabbit *M. biceps femoris*. In the endomysium between two neighbouring skeletal muscle cells vascular vessels can be seen. At the level of the white arrow the vascular wall is still intact, while at the level of the black arrow, only the collagenous and elastic framework is left. Bars = 50µm (left)/ 10µm (right).

Using the lymphatic marker LYVE 1, an extensive lymphatic network has been detected in *M. biceps femoris* perimysium and endomysium (Fig 3a) . In contrast to the vascular system, the lymphatic system does not form a circulatory circuit, instead, the lymph is transported unidirectionally from tissues back to the systemic circulation. The largest lymphatic vessels run in the perimysium (Fig.3b) as well as big lymph nodes (Fig. 3d). Finer vessels, blind ending sacs (3c) and small lymph nodes can be found in the endomysium. The smaller absorbing lymphatic capillaries are different from blood capillaries in that they lack fenestrations, a continuous basal lamina and pericytes. The larger lymphatic vessels contain a

continuous muscular layer, an adventitial layer which comprises collagen and elastin, a basement membrane, and valves to prevent retrograde flow. They run in close proximity to the venous system, which they enter, so that the excess interstitional fluid and protein can return into the vascular system.

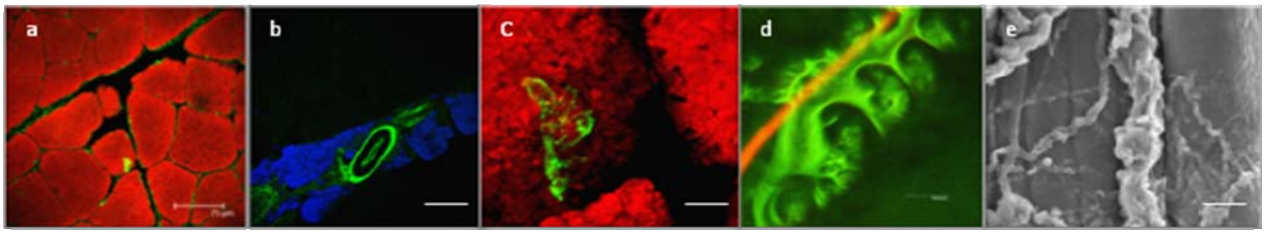

Figure 3. Skeletal muscle contains an extensive lymphatic vasculature. Images visualizing the lymphatic system in pork and rabbit M.biceps femoris (a) cross section labelled with the vascular endothelium marker LYVE 1 (green) and muscle fibres (red), bar = 75µm. A large lymphatic vessel (green) surrounded by intramuscular fat (blue) is shown in the perimysium (b), bar = 300 µm. Lymphatic vessels can end in blind sacs (c), bar = 5 µm. A large lymph node could be visualised by 2P autofluorescence (d) and an SEM image showing lymphatic vessels of different hierarchies on the surface of muscle fibres (e), bar = 10µm.

References

Brüggemann D. A. & Lawson, M.A. (2005). The extracellular matrix of *Gadus morhua* muscle contains types III, V, VI and IV collagens in addition to type I **.** J. Fish Biology 66 (3) , 810–821

Kubinova, L., Janacek, J., Ribaric, S., Cebasek, V & Erzen, I. (2001). Three-dimensional study of the capillary supply of skeletal muscle fibres using confocal microscopy. Journal of Muscle Research and Cell Mortality 22, 217-227.

Mathieu-Costello, O, Potter, R.F, Ellis, C.G. & Groom, A.C.(1988). Capillary configuration and fiber shortening in muscles of the rat hindlimb: correlation between corrosions casts and stereological measurements. Microvascular Research 36, 40-55

Taylor, R. & Koohmaraie (1998). Effects of Postmortem Storage on the Ultrastructure of the Endomysium and Myofibrils in Normal and Callipyge Longissimus. J. Anim. Sci. 76, 2811–2817