LOCALIZATION OF PREADIPOCYTES IN BOVINE MUSCLE TISSUE

Jochen Wegner, Elke Albrecht, Phillip Huff and Klaus Ender

Research Institute for the Biology of Farm Animals, D-18196 Dummerstorf, Germany

Background:
Adipocytes have multiple roles and specialized functions in animal systems. These roles include protection from physical injury, accumulation of triacylglycerol as a form of stored energy, regulation of energy homeostasis, and increasing the palatability of meat from farm animals. These central roles, in addition to the increased public concerns and health consequences of obesity, has increased the attention given to the process of adipocyte differentiation. This report describes our preliminary study to immunohistochemically localize preadipocytes in bovine skeletal muscle tissue using monoclonal antibodies (MAbs) specific to pref-1 and polyclonal antibodies (PAbs) specific to PPARγ2. Pref-1 is a novel transmembrane protein of 45-60 kDa with six tandem EGF-like repeats in the putative extracellular domain. Pref-1 is highly expressed in preadipocytes, is absent in adipocytes, and appears to play a role in maintaining preadipocytes in an undifferentiated state (Smas and Sul, 1993). Pref-1´s biological role in adipose tissue has been elucidated to be an inhibitor of adipocyte differentiation as well as responsible for blocking the differentiation of preadipocytes to adipocytes. PPARs are members of the nuclear hormone receptor superfamily. PPARγ has been shown to be an integral regulator of adipocyte differentiation and has been shown to be able to convert fibroblastic cells with little or no inherent adipogenic potential into preadipocytes (Tontonoz et al., 1994). One isoform, PPARγ2, is mainly expressed in adipose tissue thus suggesting its relative importance in the adipocyte differentiation process (Spiegelman and Flier, 1996). In previous work, Cinti et al. (1989) localized preadipocytes via biochemical and immunocytochemical methods using the S-100 protein, and Hausman and Richardson (1998) developed the AD-3 antibodies which localize both preadipocytes and adipocytes in their study of ECM expression by preadipocytes. There currently is limited information reported concerning the use of immunohistochemistry in the localization of preadipocytes in muscle tissue. Current methods of determining fat cell number and size is only possible for lipid-filled cells.

Objectives:
The objective of this study was to develop an immunohistochemical test to be used in estimating of the developmental potential of fat deposition in muscle tissue.

Material and Methods:
Muscle samples were obtained immediately post mortem from the central surfaces of longissimus dorsi muscles of German Holstein bulls (18 months old). These samples were immediately frozen in liquid nitrogen and stored at -80°C. Ten micron thick transverse sections were cut using a Cryostat 2800N Frigocut (Reichert-Jung, Leica, Bensheim, Germany) set at -21°C, mounted to SuperFrost*/Plus slides (Menzel-Gläser, Karlsruhe, Germany), and fixed with 4% formaldehyde. After fixing, all sections to be stained were washed twice with phosphate buffered saline (PBS, 0.0005 M KH_2PO_4, 0.005 M Na_2HPO_4, 0.0685 M NaCl, 0.00135 M KCl, pH 7.4), once with PBS containing 0.1% Triton X-100, and pre-treated for 15 minutes with 3% normal goat serum diluted in PBS. Sections were incubated at room temperature for 1 h with pref-1 antiserum (1:300) or at 37°C for 20 minutes with PPARγ2 (Affinity Biogeants Inc., Golden, CO) (1:300). Both antibodies were diluted in PBS containing 0.1% Triton X-100 and 1% BSA (Boehringer Mannheim GmbH, Germany). The antiserum against pref-1 was preincubated in a bovine liver homogenate for 16 hours at 4°C. Control sections were incubated without the addition of any antibody. Sections were washed once with PBS containing 0.1% Triton X-100, twice with PBS, and subsequently incubated at room temperature for 45 minutes with Rhodol Green™ goat anti-rabbit IgG (H + L) conjugate (Molecular Biologische Technologie, Göttingen, Germany) diluted 1:400 and 800 for PPARγ2 and pref-1, respectively. Sections were washed once with PBS containing 0.1% Triton X-100, twice with PBS, incubated at room temperature for 10 minutes with bis-benzimide (Sigma Chemical Co., St. Louis, MO) for nucleic acid, and subsequently washed once with PBS and once with d_2H_20. Sections were stained with oil red-O for lipid and visualized using an image analysis system (Quantimet 570, Leica, Bensheim, Germany) equipped with 3-CCD color camera (Sony, Japan) and a Microphot-SA fluorescence microscope (Nikon, Europe B.V., The Netherlands).

Results and Discussions:
The results of immunohistochemical stainings of bovine skeletal muscle tissue presented as stains with anti-pref-1 and anti-PPARγ2 are shown in Figure 1 (left). Each image is provided with the corresponding stain for the cell nuclei (right). Since the MAb against pref-1 protein recognizes the C-terminal peptide fragment it is reasonable to state that the soluble rather than the membrane bound form of pref-1 was localized. As well, immunohistochemistry showed that the level of immunostain was highest near the cell´s nucleus as demonstrated by the strong correlation between nuclei stain and anti-pref-1 immunostain (Fig. 1a and b). However, it should be mentioned that Halder et al. (1998) observed a similar immunostaining pattern for another anti-pref-1 MAb, raised against an extracellular domain of pref-1, as for the pref-1 MAb used in this study. The regions within the connective tissue around adipocytes exhibited strong immunostaining with the anti-pref-1 Ab (Fig. 1a). Adipocyte depots develop within the connective tissue between large muscle fiber bundles. The pref-1 protein immunolocated in this region is believed to correspond to preadipocyte cells. Pref-1 protein was also localized within nerve fibers (Fig 1b). Immunostaining occured between axons directly aligned with a cell´s nuclei. This study did not investigate the identification of the cell type. The antibody against pref-1 also stained an unknown protein within the wall of the blood vessels within muscle tissue (results not shown).

Positive immunostainings for PPARγ2 were observed within the connective tissues, within the blood vessel walls, between muscle fibers, and adipocytes (Fig 1c and d). PPARγ2 is expressed at high levels in adipose tissue as well as being present at higher levels in preadipocytes than other fibroblastic cells (Spiegelman and Flier, 1996). The PAb against PPARγ2 recognizes a 16 amino acid residue whose sequence was derived from mouse PPARγ2 and is absent in PPARγ1. Our immunolocalization of PPARγ2 in adipocytes and connective tissue confirms these results obtained in cell culture. PPARγ has been recently immunolocalized within skeletal muscle (Zierath et al., 1998). The techniques employed in this study allow a visual comparison of PPARγ expression between adipocytes, connective tissue, and muscle fibres (Fig 1c). Immunostaining also revealed PPARγ2 to be expressed in blood vessels (Fig 1d). The role of PPARγ2 in blood vessels is unknown but it has recently been described to have a gene regulation role in endothelial cells (Marx et al., 1999). PPARγ2 may have a role in the regulation of plasminogen activator inhibitor type-1 (PAI-1) whose plasma levels have been correlated with both mycardial infarction and venous thrombosis. PPARγ2 may therefore have an important implication between obesity and artherosclerosis. No immunostaining was observed in the muscle fibers, adipocytes, nerve bundles, within connective tissue, and within blood vessels, when primary antibodies were not included (results not shown).

Conclusions:
Through the use of immunohistochemical techniques, potential fat depots and associated systems can be localized and may aid in the study of the process of adipogenesis. Our preliminary study suggests that the pref-1 Ab utilized in the study and PPARγ2 are not specific enough in determining the developmental potential of marbling. An antibody with greater specificity could be created and utilized in a future attempt.

We thank Dr. Mitsuhiro Okamoto from the Osaka University Medical School for providing us with the anti-pref-1 antiserum.

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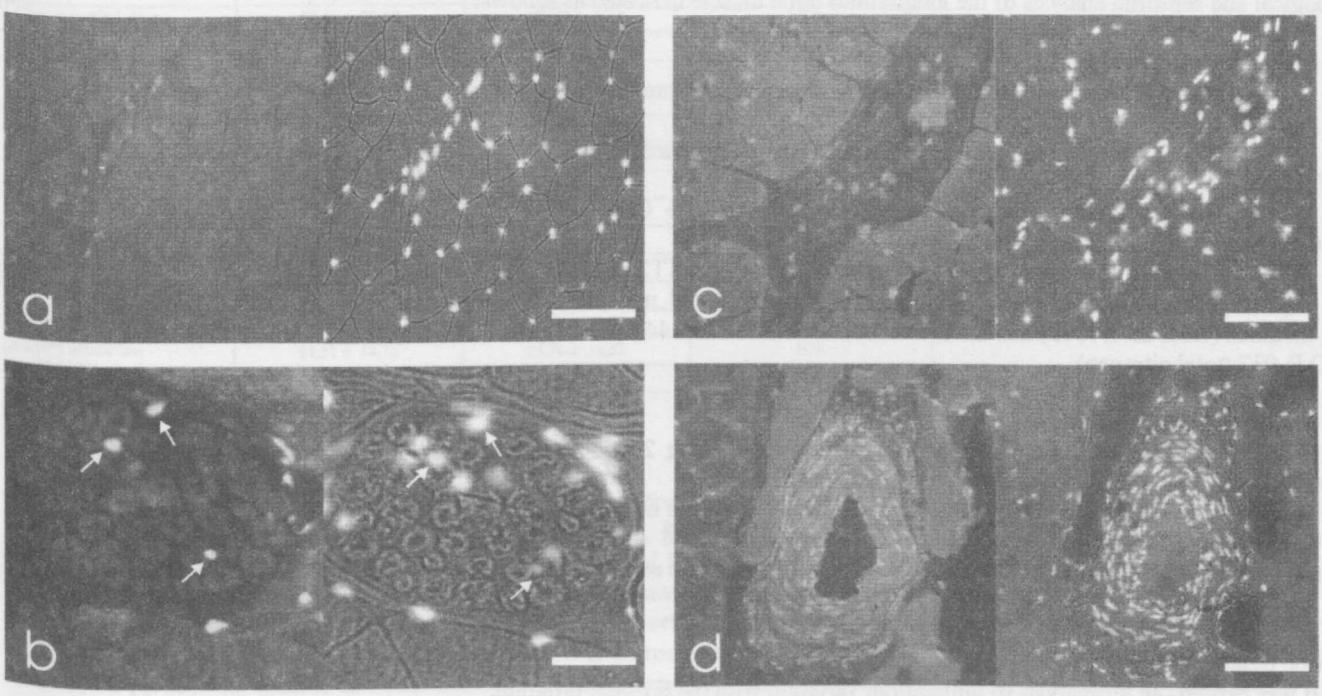

Figure 1. Immunohistochemical staining of preadipocytes in bovine longissimus dorsi muscle tissue cross sections with anti-preadipocyte-1 antiserum (a) and (b) and anti-PPARγ2 antibody (c) and (d). The left column of images depicts staining with antibody and the right column of images depicts the corresponding nuclei. Positive immunostainings were observed within the connective tissue between the muscle fiber bundles and between the muscle fibers (a and c).The arrows (in b) indicate bound pref-1 and the corresponding nuclei in a nerve bundle. The image in (d) depicts a blood vessel. Scale bar for (a), (c), and (d) indicates 100 μm while the scale bar for (b) is 30 μm.