Expression of specific white adipose tissue genes in denervation-induced skeletal muscle fatty degeneration

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Abstract Denervation of skeletal muscle results in rapid atrophy with loss of contractile mass and/or progressive degeneration of muscle fibers which are replaced to a greater or lesser degree by connective and fatty tissues. In this study, we show that denervated rabbit muscles are transformed into a white adipose tissue, depending on their fiber types. This tissue does express LPL, G3PDH and particularly the *ob* gene, a white adipose tissue-specific marker, and does not express the brown adipose tissue molecular marker UCP1 mRNA.

Key words: Muscle degeneration; Denervation; Skeletal muscle; White adipose tissue; *ob* gene; Rabbit

1. Introduction

A muscular degenerative process including fat accumulation occurs in some X-chromosome-linked recessive muscular disorders, such as Duchenne muscular dystrophy (DMD) [1] and Emery-Dreifuss muscular dystrophy in which myocardium is concerned [2]. Similarly, denervation of skeletal muscle results in rapid atrophy with loss of contractile mass and/or progressive degeneration of muscle fibers which are replaced to a greater or lesser degree by connective and fatty tissues depending on animal species and muscles. For example, denervated human muscles are transformed into a fibrotic containing fat tissue several years after trauma. In contrast, it has been known for a long time that denervation induces in rabbit muscles a striking and almost complete fatty degeneration [3,4]. These observations have been completed recently by in vitro experiments showing that co-expression of the peroxisome proliferator-activated receptor-gamma (PPARγ) and the CCAAT/enhancer-binding protein (C/EBPα), two adipogenic transcription factors, induced transdifferentiation of myogenic cells into adipose-like cells [5]. Similarly, agonists of PPARγ such as thiazolidinediones or long chain fatty acids convert non-terminally differentiated myoblasts into adipoblasts [6].

In this study, we wanted to determine the nature of the fatty tissue which develops in muscles deprived of innervation. For this purpose, we took advantage of two denervated rabbit muscles, the slow-twitch semimembranosus proprius (SMp; 100% type I fibers) and the fast-twitch semimembranosus accessorius (SMa; nearly 100% type II fibers). We investigated the nature and the kinetics of development of the invading fatty tissue by studying the expression of genes which are known to characterize adipose tissue and myogenesis or muscle regeneration. The former were *ob* [7,8], α2 chain of type VI collagen (A2COL6) [9,10], lipoprotein lipase (LPL) and glycerol 3-phosphate dehydrogenase (G3PDH) [11]. The latter was myogenin, as a marker of fusion during muscle differentiation and degeneration/regeneration [12,13].

The results reported in this study show that denervated rabbit muscles are transformed into a white type adipose tissue characterized by the expression of specific genes, such as *ob*. In addition, the kinetics of fatty degeneration is different in fast-twitch and slow-twitch muscles, the former being transformed faster and more completely than the latter.

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Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue; A2COL6, α2 chain of type VI collagen; LPL, lipoprotein lipase; G3PDH, glycerol 3-phosphate dehydrogenase; SMa, fast-twitch semimembranosus accessorius muscle; SMp, slow-twitch semimembranosus proprius muscle; SMm, semimembranosus muscle, i.e. fast-twitch semimembranosus accessorius plus slow-twitch semimembranosus proprius muscles

2. Materials and methods

2.1. Animals and surgery procedures

This study was carried out with New Zealand White rabbits of our breeding. All surgical experiments were performed under aseptic conditions. Adult rabbits (2.5 kg) were anesthetized with sodium pentobarbital (Nembutal, 30 mg/kg i.v.) and ketamine (Ketalar 50 or Imalgène 500, 15–20 mg/kg i.v.). Rabbit SMa and SMp muscles were specifically denervated bilaterally under a binocular lens according to the surgical procedure previously described [4]. Briefly, a longitudinal section of the gracilis muscle gives access to the SMm and its innervation. The branches innervating the SMa and SMp muscles were sectioned, ligated twice, and their proximal parts reflected carefully backwards to prevent reinnervation.

2.2. Histological procedures

Samples were fixed in 4% formaldehyde, embedded in paraffin and sectioned at 16 μm. Dewaxed transverse or longitudinal sections were stained by the Masson trichrome technique as described by Bradbury and Rae [14]. The celestin-hemalum sequence was used and light green was substituted for methyl blue.

For immunohistochemical observations, unfixed muscles were frozen in CO_2-cooled isopentane. Transverse sections 8 μm thick were cut in a cryostat. The expression of MyHC isoforms was performed with a monoclonal antibody reacting with all MyHC isoforms (1F11, kindly provided by F. Pons, U300 INSERM, Montpellier, France). Sections were incubated first with the antibody, followed by fluorescein-conjugated goat anti-mouse antibody for 30 min at 37°C. Stained sections were mounted in glycerol containing 1 mg/ml paraphenylenediamine and viewed with fluorescein optics.

2.3. Northern blot analyses

Total RNA were prepared by the guanidinium isothiocyanate extraction method [15] from 2–3 different SMa and SMp muscle sample per stage, adult perirenal white adipose tissue (WAT) and neck brown adipose tissue (BAT) from newborn rabbit. For Northern blot anal-

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yses, total RNAs were electrophoresed in a 1% agarose gel and transferred to nylon membranes (Hybond-N, Amersham UK). RNA loading and transfer efficiency were ascertained by human 36B4 cDNA hybridization [16]. cDNA probes for A2COL6 [10], LPL [17], G3PDH [18], rabbit *ob*, rabbit UCP1 [19] and myogenin [20] were labeled by random priming with [α-^{32}P]dCTP (Rediprime Kit, Amersham, UK). The same RNA blots were hybridized overnight at 65°C with the different probes, with successive cycles of hybridization, washing and stripping. Blots were exposed to autoradiography (Kodak X-Omat films) at −80°C. Autoradiographs were scanned with the Adobe Photoshop/VistaScan system, quantified with an NIH Image program using an Apple Macintosh computer to determine the intensity of the bands and corrected for slight variations in the amount of RNA loaded on each track using 36B4 cDNA.

3. Results and discussion

As previously reported [4,21], the SMa (nearly 70% type IIb and 30% type IId/IIx fibers) and SMp (100% type I fibers) muscles undergo general atrophy after denervation, followed by loss of fibers and fatty degeneration. However, their kinetics of degeneration differ. One month after denervation, the SMa shows large patches of fatty infiltrations and at 2 months it is almost completely constituted of adipose tissue (Fig. 1). This contrasts with the slower and incomplete transformation of the slow-twitch SMp. In fact, the SMp still contains numerous muscles fibers surrounded by adipose cells 1 year after denervation [22]. Thus, muscles constituted by fast muscle fibers are the most affected by denervation. They differ from muscles composed of slow fibers which are much less sensitive to denervation and subsequent degeneration. As in the rabbit, it should be noted that in DMD patients muscle fast type IIb fibers are more affected than slow ones [23].

Fig. 2 shows the expression of adipose tissue and myogenic marker genes in 2-month denervated SMa and SMp. It is noteworthy that at this stage denervated SMa have a high level of expression of the genes characterizing the control WAT, i.e. LPL, G3PDH, A2COL6, and particularly *ob*. Denervated SMp expresses most of these genes at this stage but at a lower level, excepted for A2COL6. As in WAT, both denervated muscles do not express UCP1 which is detected only in BAT. Thus, we can conclude that denervation induces the transformation of rabbit muscles into white adipose tissue.

Myogenin mRNAs are highly expressed in both denervated muscles. This suggests that muscle transformation is accompanied by muscle regeneration, as illustrated in Fig. 3 after immunostaining of SMa and SMp with the myosin antibody. However, these microphotographs from 2-month denervated muscles show that the structure of SMp is well retained, in contrast to that of SMa in which only islets of myotubes surrounded by adipose tissues are observed.

Fig. 4 illustrates the expression of adipose and myogenic molecular gene markers during the kinetics of denervated

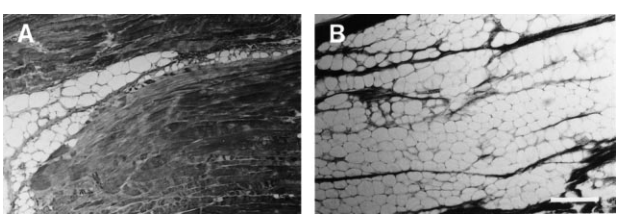

Fig. 1. Longitudinal sections of the fast-twitch semimembranosus accessorius rabbit muscle after 1 month (A) and 2 months (B) of denervation. Bar in B = 50 μm.

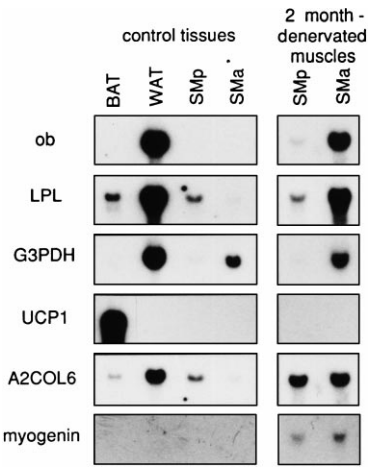

Fig. 2. Expression of *ob*, LPL, G3PDH, UCP1, A2COL6 and myogenin mRNAs in 2-month denervated slow-twitch semimembranosus proprius (SMp) and fast-twitch semimembranosus accessorius (SMa) rabbit muscles. RNAs from cervical brown adipose tissue (BAT) of newborn rabbit, adult perirenal white adipose tissue (WAT), and innervated SMp and SMa muscles were taken as controls. Northern blot loading: 20 μg RNA per lane, except for BAT (5 μg).

muscle transformation. In the SMa, all genes are expressed as soon as 15 days after denervation. Thereafter, the LPL, G3PDH and *ob* gene expression increases linearly to 2 months and is maintained at a high level later on for *ob*, and decreases regularly to 6 months onwards for LPL and G3PDH mRNA. Myogenin is expressed at a constant level and A2COL6 is mostly expressed at 2 and 4 months after denervation. This differs from the denervated SMp in which expression of *ob*, LPL and A2COL6 genes appears to be stage-dependent. This contrasts with the myogenin gene which is expressed at a low and constant level during the experimental period. G3PDH mRNA is not detected under our conditions.

Denervation of SM muscles induced a high expression of the myogenin gene transcript. As previously reported, mRNA levels of myogenic factors increase transiently in denervated skeletal muscle [24,25]. However, we did not observe a transient increase of these mRNAs within a few days after nerve resection, as in rat and mouse, but only a quite stable level of accumulation. Thus, denervation is capable of reactivating the expression of myogenic regulatory factors which are related to muscle regeneration, in spite of the striking fatty transformation of a muscle such as the SMa.

The most striking result of these observations is the expression of the *ob* gene in denervated muscle, as *ob* gene expression is known to be adipose tissue-specific in different animal species, including man [7,8,26]. The increased expression of *ob*

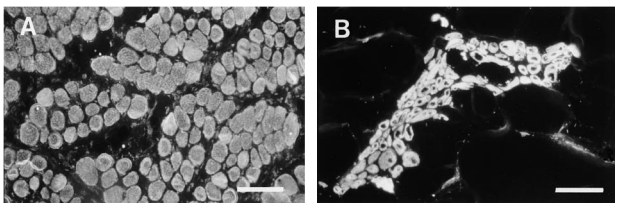

Fig. 3. Immunohistochemical staining with myosin heavy chain 1F11 antibody of sections from 2-month denervated slow-twitch semimembranosus proprius (A) and fast-twitch semimembranosus accessorius (B) rabbit muscles. Bar in A = 100 μm; bar in B = 200 μm.

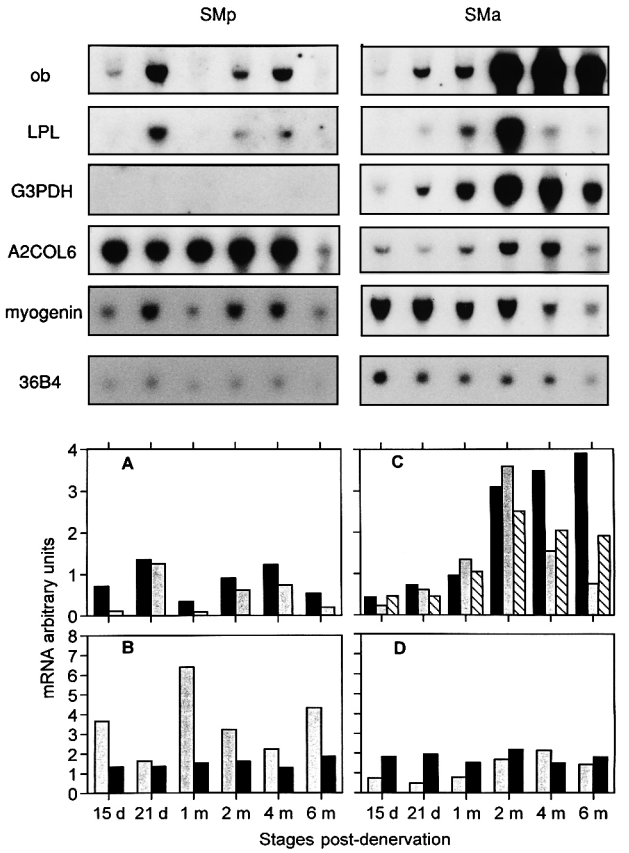

Fig. 4. Upper panel: Expression of *ob*, LPL, G3PDH, A2COL6 and myogenin mRNAs as shown by Northern blot analysis. For each probe, the homogeneity of RNA loading is shown by hybridization of the 36B4 cDNA probe. RNAs were extracted from slow-twitch semimembranosus proprius (SMp) and fast-twitch semimembranosus accessorius (SMa) rabbit muscles at different stages post denervation (pooled RNA from 2–3 different SMp and SMa denervated muscle samples per stage). Loading: 20 μg RNA per lane. Lower panel: Graphic representation (arbitrary units) for SMp (A, B) and SMa (C, D) of scanned molecular marker levels as deduced from the two Northern blots, corrected for variations in the amount of RNA loaded on each track using 36B4 cDNA. A and C: *ob* (black column), LPL (dotted column) and G3PDH (hatched column); B and D: myogenin (black column) and A2COL6 (dotted column).

mRNA, as well as mRNAs of lipogenic enzymes LPL and G3PDH, suggests that denervation induces progressive muscular transformations towards the development of a white adipose tissue. A2COL6 gene expression is also markedly increased in both muscles. A2COL6 mRNA, which is expressed in growing tissues, has been identified as a marker of preadipose and myoblast states [10]. Its increase in denervated muscle could be related either to satellite cell proliferation, relevant to muscle regenerative process occurring after muscle injury, or to the proliferation of quiescent preadipocytes preexisting in SMm [11]. Thus, the expression of both adipogenic and myogenic markers raises the question of the origin of adipose cells which appear in denervated muscle. Two hypotheses could be suggested, either an adipoblastic or a myoblastic origin.

According to the first hypothesis, it is well known that in muscles connective tissue that ensheaths muscle fibers contains fibroblasts and/or preadipocytes. It has been shown in vitro that human and rabbit preadipocytes need glucocorticoids and insulin to differentiate [27,28]. Glucocorticoid hormones, which are involved in inflammatory processes, could act by themselves or by stimulating the activity of phospholipase A2 and the production of prostaglandins such as PGE2 and/or PGI2 by macrophages. Increased levels of phospholipase A2 and PGE2 have been shown to be associated with atrophy of denervated rat soleus muscle [29,30] and dystrophynopathies [31]. Recruitment of adipose cell precursors could also result from nerve resection itself, as it has been shown that sympathetic denervation of the retroperitoneal white adipose tissue induces an increase in both A2COL6 mRNA level and DNA content, showing an increase in the number of small adipocytes derived from quiescent preadipocytes [32].

In the second hypothesis, denervated skeletal muscle of most animal species shows a dramatic DNA synthesis increase as many classes of cells – satellite cells, macrophages, mast cells, etc. – proliferate [33,34]. Most of these cells secrete mediators, such as pro-inflammatory cytokines (interleukins, TNFα), growth factors (TGF, FGF, PDGF, IGF1, etc.) and prostaglandins or derivatives (E2, F2α, D2, 15-d-PGJ2). bFGF, TGFβ1 and IGF1 have been shown to stimulate macrophages, mast cells, fibroblasts, preadipocytes, and muscle precursor cells via myogenic regulatory factors [35,36] to promote their proliferation and differentiation leading to muscle repair or adipose degeneration. In this biochemically modified environment, satellite cells might transdifferentiate into preadipocytes as recently shown by in vitro studies [5,6]. In fact, co-expression of the two adipogenic transcription factors PPARγ and C/EBPα by transfection of G8 myoblasts inhibits normal myogenesis and synergizes to convert myogenic cells into adipocytes [5]. Similarly, myogenic conversion into adipocytes has also been obtained with exposure of C2C12N myoblast cells or satellite cells with potent antidiabetic drugs such as thiazolidinediones or polyunsaturated long chain fatty acids and arachidonate leukotrienes such as 5,8,11,14-eicosatetraynoic acid [6].

In conclusion, our studies show that a white adipose tissue develops in denervated rabbit fast- and, to a lesser extent, slow-twitch muscles. However, the origin of adipose cells still remains unknown. Denervated rabbit muscles could represent an interesting model for studying human myodystrophic and myohypotonic diseases, where a similar pattern of fatty degeneration has been observed.

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