Skeletal muscle satellite stem cells at different postmortem intervals

Mihai Ceausu1, Sorin Hostiuc2*, Dan Dermengiu2

Abstract: Skeletal muscle satellite stem cells are difficult to isolate, have a low proliferative capacity and lose in vivo regenerative potential upon ex vivo expansion. A recent study by Latil et al. suggested that viable and functional skeletal myogenic cells from humans could be obtained up to 17 days postmortem. The purpose of this study is to see up to which day these SCs can be identified in human autopsy samples from uncontrolled environments, and to better characterize these cells immunohistochemically at different postmortem intervals. Materials and methods: histologically normal samples of skeletal striated muscle were obtained from 4 cadavers after autopsy; the tissue specimens were fixed in formalin at different intervals post-mortem (1 day, 2 days, 6 days and 8 days). We used various histochemistry stains (PTAH, PAS, and May Grunwald Giemsa) and immunohistochemistry markers (CD56 / N-CAM, CD117 / c-kit and CD34). Results. Satellite stem cells were identified up to six days post-mortem; their characteristics varied depending on the post-mortem interval. In conclusion, our study suggests that satellite stem cells can be identified up to six days after the estimated moment of death in uncontrolled environments. A positive reaction for CD117/c-kit is suggestive for SCs, contrary to recent studies that considered them to be negative for this marker.

Key Words: satellite cell, N-CAM, regeneration, skeletal muscle.
were found to be enriched in post-mortem tissue, most likely due to a selective survival advantage compared to other muscle cells [7]. Their study suggested that SCs, in the unique postmortem microenvironment, characterized by the lack of oxygen and nutrients, and extensive necrosis, may trigger a cellular response in these SCs, causing them to adopt a deeper state of quiescence [7]. The purpose of this study is to see up to which day these SCs can be identified in human autopsy samples from uncontrolled environments, and to better characterize these cells immunohistochemically at different postmortem intervals.

MATERIAL AND METHODS

Case selection for the human tissue specimens
Histologically normal samples of skeletal striated muscle were obtained from 4 cadavers after autopsy: three men (of 41, 46, and 48 years old) and one woman (76 years old), individuals who had no muscular autoimmune disorders or other significant diseases (inflammation, tumors etc.). The tissue specimens were fixed in formalin at different intervals post-mortem (1 day, 2 days, 6 days and 8 days). Samples were harvested in late Spring/Summer, with the mean outside temperature between 16 and 28 degrees. The bodies were refrigerated at 2 degrees Celsius between 16 and 25 hours, until the autopsy was performed.

Ethical issues. The tissue specimens for microscopy analysis were collected according to the national legislation, in accordance to generally accepted international practice (Declaration of Helsinki).

Tissue sampling and stains
The fragments were obtained from different areas of the psoas, deltoid and diaphragm muscles. Tissue samples have been divided into appropriate-sized slices for conventional microscopy and immunohistochemistry. The selected tissue samples were fixed in formalin at different intervals post-mortem (1 day, 2 days, 6 days and 8 days). Samples were harvested in late Spring/Summer, with the mean outside temperature between 16 and 28 degrees. The bodies were refrigerated at 2 degrees Celsius between 16 and 25 hours, until the autopsy was performed.

Immunohistochemistry
Immunohistochemical analysis (IHC) was done using sections displayed on slides treated first with poly-L-lysine. IHC was performed on 3 μm thick sections from formalin-fixed paraffin-embedded specimens.

The method used was an indirect tristadial Avidin-Biotin-Complex technique, with a NovoLink Polymer detection system which utilizes a novel control polymerization technology to prepare polymeric HRP-linker antibody conjugates, according to the manufacturer’s specifications (Novocastra, UK).

Briefly, the procedure comprised: deparaffinization in toluene and rehydration in alcohol series, washing in phosphate buffer saline (PBS), blocking with normal serum, for 5 min., incubation with primary antibody 60 min., incubation with post-primary block 30 min., then with NovoLink Polymer 30 min. Sections are further incubated with the substrate/ chromogen 3,3’-DAB and counterstained with Meyers’ hematoxylin.

The antibodies used for IHC were: CD56/ N-CAM (clone: 1 B6, RTU, Novocastra), CD117/ c-kit (T595, RTU, Novocastra) and CD34 (QBend/10, RTU, Novocastra).

Antigen retrieval techniques (thermal or enzymatic pretreatment) were done, according to the producer’s specifications. Both positive and negative controls were used.

Negative control was made by using a primary irrelevant antibody or by replacing the secondary antibody with phosphate buffered-saline (PBS). Positive control was made comparatively with the expression of antibody investigated in specific cells or tissue structures (positive intern control on slides).

To ensure the reliability of the experimental study, internal quality control of special stains and IHC techniques were performed as a part of an implemented and certified quality assurance system (ISO 9001/2008).

All slides were examined and photographed on a Zeiss Axio Imager microscope (Göttingen, Germany). Digital images acquired with Zeiss Axio Vision program have been processed and analyzed with ACDSee Pro Photo Manager (Washington DC), running under Windows Vista.

IHC assessment and statistics
The distribution of markers-positivity has been assessed using the modified Quick score method [8], which takes into account the intensity and distribution of the IHC reaction: negative (no staining) = 0; weak (only visible at high magnification)= 1; moderate (readily visible at low magnification)= 2; strong (strikingly positive at low magnification)= 3.

Descriptive statistics was used for uniform distributed data using mean, median and standard error. Statistical analysis was performed in SPSS–13, running under Windows Vista.
RESULTS

From the morphology standpoint, the skeletal muscle satellite stem cells (SSC) or myosatellite cells are small, plump, ovoid to elongated, mononuclear cells, with distinct borders, scant cytoplasm, centrally placed nuclei (sometimes hardly noticeable, depending upon the incidence of the cut section) and a high nuclear to cytoplasm volume ratio.

Citometry: SCs, measured at 400x magnification had a minimum diameter of 6.08 μm (min.) and a maximum diameter of 16.66 μm. (max.) [m= 13.47 μm, M= 14.16 μm, SE= +/- 3 μm].

Satellite stem cells were located interstitial and perifibrillar, adjacent to the sarcolema of muscle fibers. Their distribution along the muscular fiber appeared to be random, with various positions; they either were oriented parallel or perpendicular to the longitudinal axis of the muscle fiber.

The aspect of the cells was also influenced by the surrounding micro-environment, such as vascularization, other stromal cells etc. In context of hypoxia and anoxia, they tended to shrink, diminish their dimensions and became more globular or spherular, had a more faded staining with the used IHC markers, and were harder to distinguish (for the evolution of those changes see Figs 1-3).

Satellite stem cells stained positive for CD56/N-CAM (positive intern control in neural fibers), CD117/c-kit (positive intern control in mast cells) and CD34 (positive intern control in capillary vessels).

We identified these cells in skeletal striated muscle after 1 day, 2 days and 6 days after death (Figs 1-3); we were not able to identify these cells 8 days after death.

The micro vascular density in post-mortem skeletal muscle assessed by CD34 (Fig.4) showed that the capillary network decreased significantly after 24h and subsequently diminished the number and dimensions of satellite cells, making them more difficult to identify.
In normal healthy muscle, most satellite cells are quiescent [9]; in response to tissue damage, satellite cells become activated, proliferate initially as skeletal myoblasts and then undergo myogenic differentiation [10]. Stem cell viability was initially thought to decline significantly about 2 days after death [7].

In cultures, survival of these cells is prolonged and they can be used for experimental procedures. Recently was proposed that the lack of oxygen and nutrients or the presence of extensive necrosis may lead to adaptation of these cells to harsh conditions, adopting a deeper state of dormancy and retaining regenerative capacity [7].

In homeostatic conditions, satellite cells can be activated under certain stimuli, such as physical trauma or growth signals. After activation, satellite stem cells undergo symmetric divisions to expand their number or asymmetric divisions to give rise to committed satellite cells. Myogenic progenitors proliferate, sometimes differentiate and fusion with each other in order to reconstitute fiber integrity and function [11]. In pathologic conditions, signals released from the inflammatory cells and from mesenchymal interstitial cells, also known as fibro-adipogenic progenitors appear to instruct satellite stem cells to break quiescence, proliferate and differentiate [12]. Satellite cells are also involved in the endothelial activation of key angiogenic signalling molecules, including VEGF, Ang-2 and VEGFR-2 [13].

We showed here that SCs could be easily identified up to six days after the estimated moment of death; at eight days these cells could not longer be found. An increase in the number of cases might have shown a positive identification after six days in some cases, but our opinion is that their identification and culture after this post-mortem interval isn’t feasible in practical conditions. Moreover, as it is seen in Figs 3a and 3b the SCs at six days have a clearly altered cellular architecture, making improbable their “revival” to an optimal status in culture conditions. This result partially confirms the one of Latil et al., who found that at day 8 the clonogenity of SCs dropped at 1.8%. We do not know the particular conditions in which the human samples were obtained in that study as it was not specified [7], but if they were properly refrigerated before sampling, it is possible that a positive identification of SCs could have been done after 6 days which was the threshold in our study.

We considered that these CD117 positive cells are in fact SCs. Some recent articles suggested that stem cells from the muscle stem cell niche lack a immunopositivity for CD117, these positive CD117 cells being considered as telocytes. This hypothesis was confirmed, according to the authors, by the fact that these cells have telopodes and an oval body shape [14]. The location of the telocytes, according to various authors was in the endomysium and perymysium, and their prolongations were in contact with
blood vessels and nerve endings [15]. Their morphology bears a striking resemblance with the cells considered by us to be SCs (see e.g. Fig. 9a&b from [15]). However, the fact that the cells identified by our study are CD56 positive suggests that our initial hypothesis of their being in fact stem cells, even if some of the cells identified have structures that could be considered prolongations, and are sometimes in contact with nerve fibers of vessels.

Limits of the study. We used a low number of cases; by increasing the number of cases we could have identified SCs at larger postmortem intervals; however, as seen in our figures, even at 6 days the cellular architecture of SCs is severely damaged, suggesting a limited usefulness of harvesting muscle SCs after six days. Many authors recommend the use of PAX7 or PAX3 as markers for satellite stem cells. However, in head musculature PAX3 is negative due to a specific developmental program [16]. Moreover, PAX7 expression diminishes when SCs enter a proliferative phase [17]. For these reasons we preferred to use CD56 and c-kit as markers for muscle SCs.

In conclusion, our study suggests that SCs can be identified up to six days after the estimated moment of death in uncontrolled environments. A positive reaction for CD117/c-kit is suggestive for SCs, contrary to recent studies that considered them to be negative for this marker.

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Conflict of interest. None.

References