Regeneration Capabilities of Rat Rectus Abdominis Muscle

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Abstract

In human diseases, skeletal muscle regeneration is a powerful, naturally-occurring process of tissue reconstruction that follows upon myofiber damage secondary to injury, which does not affect the tissue circulation and scaffold. On the other hand, the ablated tissue, in traumatology and free muscle grafting, is commonly seen to be replaced by scars. The final outcome is poor even after in situ myoblast seeding of the harvested muscle when larger amount of muscle tissue is grafted. Attempts of full-thickness auto-grafting of the rat rectus abdominis also result in a scar. In a rat model of full-thickness 24-mm-long ablation of the medial third of rectus abdominis the harvested muscle was auto-grafted in situ. We compared regenerative myogenesis in 2- 4- and 8-mm-wide lesions. We show that all the patches were well integrated with the abdominal wall and that neovascularization from the peritoneum was usually present. In the case of the 8 x 24-mm the healing process ended in scar, since the full cell population of the auto-graft (satellite cells included) initially underwent ischemic necrosis. On the other hand, in the 2- and 4 x 24 mm lesion the healing process is almost completely due to myofiber regeneration. A full-thickness RA crush injury, which extends 24 x 8 mm, that was, the maximal size of the harvested muscle in the ablation experiments, also shown full regeneration of the injured myofibers. All together, our observations strongly witness that the satellite cells of the abdominal wall muscles behave like those of any other rat muscle. When occurring, muscle regeneration seems to be the result of one wave of migration of angioblasts and then satellite cell–derived myoblasts from the muscle tissue that surrounds the graft. The spontaneous regenerative process seems to be limited to about 2 mm of damaged tissue. These volume-related constrains clearly contribute to the high rate of success in mice experiments and to the frustrating negative results of myogenic stem cell seeding into acellular patches in larger animals.

Key words: muscle, scaffold, stem cell, abdomen, satellite cell, muscular acellular matrix.

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Introduction

Beside voluntary movements, skeletal muscles maintain structural contours of the body. Muscle trauma, dystrophy and atrophy are common pathological processes that result in muscle deficiencies. Full losses of anatomically defined muscles are encountered in pathological states, such as congenital abdominal wall defects (gastroschisis and omphalocele). Attempts to functionally correct them have been employed with limited success [1, 11]. In reconstructive plastic surgery, a particular case of muscle loss is consequent to pedicle or micro-vascular muscle flaps. The need to reduce the donor site morbidity prompted us to study a rat model, which reproduces the clinical conditions occurring in humans after transplantation of rectus abdominis (RA) muscle. Our long-term aim is to tissue engineer a three-dimensional vascularized skeletal muscle using isolated myoblasts seeded into acellular matrices grafted in the site of the abdominal wall defect. Skeletal muscle regeneration is a powerful process of tissue reconstruction when myofibers die secondary to genetic or myotoxic injury. Examples of successful muscle regeneration are not only injuries by snake venom or marcaine injection into normal or ischemic muscles, but also free auto graft or even injection of minced tissue in small leg muscles of rodents [3, 4, 12, 14, 15, 19, 28]. This study presents our results in the case of full thickness ablation of the

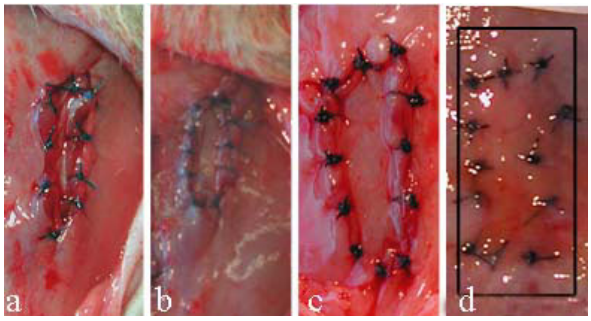

Figure 1. Surgical procedures for reconstruction of abdominal wall continuity after full-thickness ablation of the central third of the rectus abdominis. Wall reconstruction, as shown immediately after surgery of 8 x24 mm ablation and grafting (A). B and C show the case of 4- and 2- x 24 mm grafts, respectively. D, external aspect of the 8 x24 mm graft three weeks after surgery. The black frame includes the abdominal wall portion harvested to perform morphological analyses. Stitches well define the graft/muscle frame border.

RA muscle in the rat. To study its regeneration capabilities we have performed different size of full thickness defects of RA muscle, starting from a 2 x 24 mm injury. Our purpose were to better understand why it is so difficult to replicate in the RA muscle model the results of muscle regeneration in small muscles of lower leg in rats. In particular we would like to know if such a poor

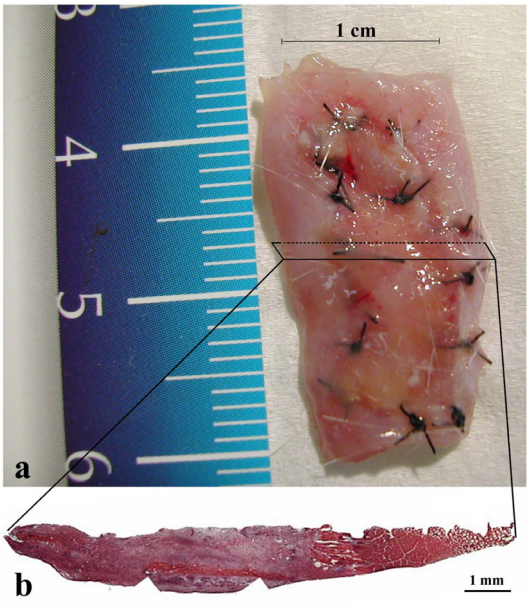

Figure 2. Reconstruction of abdominal wall continuity after full-thickness ablation of the central third of the rectus abdominis. A, Macroscopic aspect of the external surface of the harvested muscle tissue specimen, Scale bar, 1 cm. B, Muscle cross-section obtained at the middle of the muscle specimen (black plane). H-E stain. Scale bar, 1 mm.

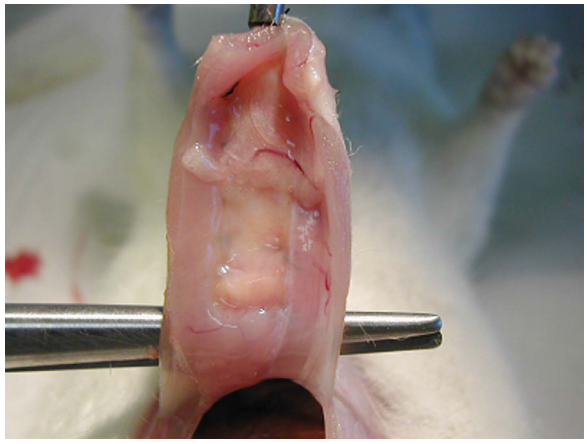

Figure 3. Peritoneal surface of the implant 3 weeks after surgery. The implant is well integrated to the abdominal wall and shows neovascularization from the peritoneal vessels.

outcome is related to a lower potential of the RA satellite cells to be activated and/or sustained in their proliferation.

Materials and Methods

Animals and surgical procedures

Male Wistar rats, weighing 250-350 g, were used. The animals were anesthetized using an intraperitoneal cocktail (ketamine hydrochloride 40 µg plus xylazine 20 µg / 100 mg of body weight). The abdominal area was shaven and aseptically prepared using povidone-iodine (Betadine) and 70% alcohol solution. The abdominal muscles were exposed through a 3-cm abdominal incision. Sterile templates measuring 24 mm long and either 2-, 4-, and 8-mm wide were used to mark and resect areas to produce full thickness defect of RA muscle. The rectangular area (2-, 4- and 8 x 24 mm) of harvested RA muscle was immediately grafted in the same place using 4.0 nylon. Figure 1, A displays the aspect of the abdominal wall immediately after grafting. The skin incision was closed with 4.0 nylon. Muscle regeneration was also induced by RA crushing, using a needle-holder to pinch a rectangular area (24 x 8 mm) of RA.

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All surgical procedures were performed in an identical fashion by a single surgeon. Three weeks after surgery, abdominal wall was exposed in anaesthetized animals. Fig 1 D shows the 8 x 24 graft. The black frame includes the abdominal wall, which is harvested to perform morphological analyses (see below). Stitches well define the graft/muscle rim border. Explanted tissue is quenched in liquid nitrogen and stored at –80° until use.

Morphologic Analyses

As shown in Figure 2, the frozen abdominal wall explant is cross-sectioned at the middle of the specimen.

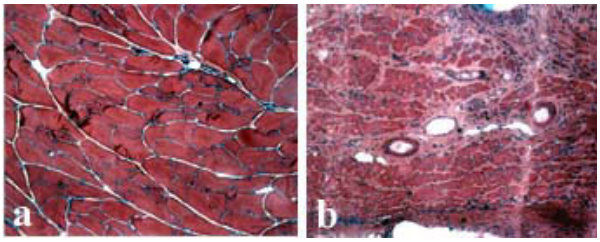

Figure 4. Myofiber regeneration after 2- and 4 x 24 mm RA grafting. H-E stain. Scale bar, 100 µm. A, 2 x 24 mm graft three weeks after surgery. The midle portion of the graft is fully paved by regenerated myofibers. B, 4 x 24 mm graft. The medial cross section of the graft is paved by regenerated myofibers, but in the middle some scar tissue is present.

Serial sections of frozen specimens are stained with haematoxylin-eosin or anti-MHCemb (Novo Castra, New Castle upon Tyne, U.K.), as reported in Rossini et al 2002 [23].

Immunohistochemistry. Cryo-sections are labelled for the presence of MHC-emb (from Novocastra, NCL-MHCd diluted 1:20) for 1 hour at room temperature. The slides are then washed twice with TBS (5 min each) and incubated with FITC-conjugated anti-mouse Ig (from Sigma, F-2266 diluted 1:200) for 1h at room temperature. The slides are washed twice with TBS (5 min

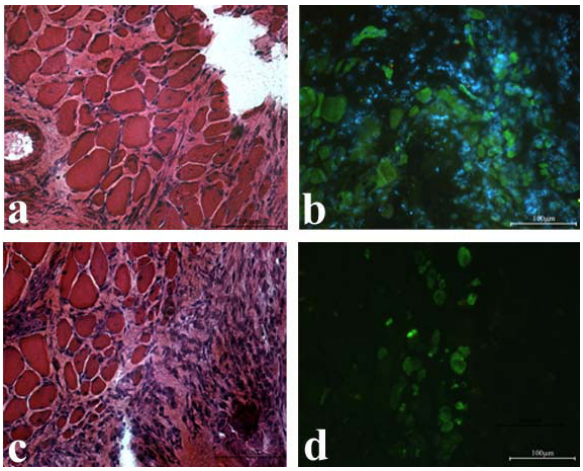

Figure 5. Myofiber regeneration after 8 x 24 mm RA grafting. Scale bar, 100 µm. A and C, H-E stain. B and D, immunostained with antibody to MHCemb. Myofiber regeneration occurred only at the borders of the graft (A and B). White arrows point to centrally nucleated large myofibers (A), among which small fibers are immunostained with antibodies to embryonic myosin (green cells in panel B) The central portion of the graft (4-6 mm) presents fibrous connective tissue and heavy infiltration of mononucleated cells among which myofibers are rare (Fig. 5, C). Only scanty cells appear positive after staining with MHCemb staining (Fig. 5, D).

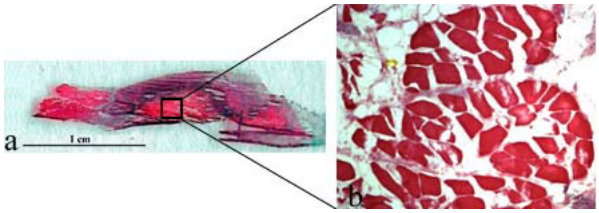

Figure 6. Ischemic myofiber necrosis of the 8 x 24 mm graft. H-E stain. A scale bar 1 cm and B scale bar 100µm One week after surgery the 8 x 24 mm grafted muscle tissue is necrotic as all muscular and non muscular nuclei underwent lysis. The graft is only partially infiltrated by inflammatory

min each) and nuclei counter-stained by Hoechst 33258. Negative controls are by omitting the primary antibody. Images are acquired by a Zeiss fluorescence microscope connected to a Leica DC 300F camera at low magnitude under the same conditions that are used to acquire a reference ruler.

Results

The external surface of the graft was always free from scar adhesion to the sub-dermis plane (Fig. 1,D); all grafts are well integrated to the abdominal wall and sustain the weight of the abdominal organs. Herniations were never seen. Neovascularization from peritoneum was always present (Fig. 3).

Figure 4 shows that at light microscopy, the core of the 2 x 24 mm graft is completely paved by regenerated myofibers (Figure 4, A). The 4 x 24 mm autograft is also almost completely paved by regenerated myofibers. The central one-two millimeters of the cross section only shown fibrous connective tissue (Figure 4, B).

On the other hand, Figure 5, show that in the case of 8 x 24 mm graft muscle regeneration occurred only at the borders of the graft (A and B). The same poor extent of muscle regeneration occurred at the cranial and caudal border of the graft.

The central portion of the graft (4-6 mm) presents fibrous connective tissue and heavy infiltration of mononucleated cells among which myofibers are rare (Fig. 5, C). Only scanty small cells appear positive after staining

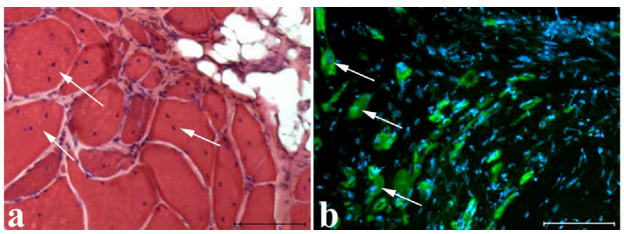

Figure 7. Myofiber regeneration after 8 x 24 mm RA crushing. Scale bar, 100 µm. A, H-E stain; B, immunostained with antibody to MHCemb. White arrows in panel A point to centrally nucleated myofibers. Many centrally-nucleated myofibers are positive to MHC-emb antibodies in panel B.

with MHCemb staining (Fig. 5, D).

Therefore, three weeks after surgery the 8 x 24 mm graft core was interested by a productive inflammatory process, which would be end in fibrous scar. Scarring is the consequence of the early ischemic necrosis of the graft. Figure 6 shows that one week after surgery the 8 x 24 mm grafted muscle tissue was necrotic. Indeed, all muscular and non muscular nuclei underwent lysis in the graft core. The graft is only partially infiltrated by inflammatory cells (Fig 6).

That the lack of muscle regeneration in the core of 8 x 24 mm autograft is not a peculiar feature of the RA myofiber population, is also confirmed by the results shown in Figure 7. A successful regenerative process follows a full-thickness RA crush injury, which extends 8 x 24 mm, that is, the size of the harvested muscle in the autograft experiments. White arrows point to the numerous centrally nucleated myofibers, which pave the crushed muscle three weeks after injury (Fig. 7, A). Accordingly, many large myofibers still behave positive to anti MHCemb monoclonals (Fig. 7, B).

Discussion

Muscle atrophy, dystrophy and trauma are the main pathological processes causing muscle deficiencies. Muscle loss is also encountered in pathological states, for instance, congenital abdominal wall defects (gastroschisis and omphalocele). A particular case of muscle loss is related to pedicled or microvascular muscle flaps in reconstructive plastic surgery. Attempts to functionally correct the donor site morbidity have been employed with limited success [9, 18].

Skeletal muscle regeneration is a powerful process of tissue reconstruction when myofibers die secondary to injuries, like exercise-induced muscle damage in normal and dystrophic muscle, partial ischemia or myotoxic agents, that spare scaffold and capillary network of muscle tissue. In the case of smallest muscles of rodent legs, functional muscle regeneration also occurs after full free grafting or even muscle mincing [3, 4, 7, 14, 15, 19, 23, 28, 29,]. On the other hand, it is a common observation in traumatology that the lost muscle is finally substituted by scar. After pyogenic myositis or trauma, muscle repair and regeneration are mutually exclusive processes [11, 20]. Even if myogenic stem cells are delivered after debridment of necrotic tissue or implantation of acellular scaffolds in the site of a harvested muscle, the final outcome is poor [16, 18, 24, 25, 27, 30].

As to basic surgical needs, the present rat model is satisfactory in reconstruction of the abdominal wall after a full-thickness ablation of RA central third. The grafts have the mechanical properties needed to support the abdominal organs. Indeed, the integrated grafts never herniated. Taking advantage of these properties, we are using a step-by-step approach to identify in this particular case the factors, which limit muscle regeneration in the graft core, in particular during the critical early phases, which would allow death or survival of autologous myogenic cells.

In the case of a large (8 x 24 mm) full-thickness RA autograft, the only regenerated myofibers observed three weeks after surgery are confined to the surrounding muscle rim to which grafts are sutured. Scar in the core of the graft is the result of a race between fibroplasias and myogenesis in which the blood vessels and the fibroblasts win, and the myogenic cells lose. The processes involved in skeletal muscle regeneration have been widely documented and are essentially similar regardless of the type of injury

In our model the harvesting of the RA is obtained by severing muscle fibers, so that the muscle damage extends to the muscle rim and the regenerative healing process is confined to a few millimetres of surviving muscle stamp at cranial and caudal border. Since the grafted tissue fully undergoes ischemic necrosis, satellite cells included (Fig. 7), the scanty muscle regeneration of the graft core is the product of lateral migration of myoblasts, which are the product of satellite cell activation and proliferation in the surrounding muscle tissue (obliquus abdominis in the case of 8 x 24 mm lesion, which in adult rat is larger than RA).

Plausibly, the satellite cells present in the graft are too poorly bathed in nutrients to survive the graft revascularization phase. Therefore, the graft undergoes repair contrary to the case of “whole” muscle graft, where a small (< 6 grams) essentially intact muscle belly is transplanted, virtually all muscle fibers regenerate completely [13, 17, 22]. Into the graft core, the few myogenic events are only identified by applying a monoclonal antibody to embryonic myosin, a well-characterized molecular marker of early myogenesis in development and regeneration [2, 4, 5, 15].

These results are not the consequence of the absence or of a peculiar inability of RA satellite cells to proliferate and differentiate [8]. Regeneration of muscles surrounding the graft is strong evidence that the satellite cells of abdominal wall muscles behave as those of any other rat muscle. Around all the grafts, i.e. at the level of the stitches, there is a 2 mm border of regenerated large myofibers with central nuclei. This seems the maximal extent of tissue that the surrounding satellite cells are able to colonize if a neovascularized layer of tissue is available. Indeed, 2- and 4-mm wide lesions heal with almost full regeneration of lost myofibers. Furthermore, three weeks after a crush lesion of the rectus abdominis, which extended to an area of 8 x 24 mm, the regenerated tissue is recognized at light microscopy by the presence of centrally nucleated large myofibers. Among them, antibodies to embryonic myosin label many early regenerated myofibers. These events are in response to physical damage and post-traumatic incomplete ischemia, which induce myofiber death/regeneration in the muscle tissue. This is the final evidence that RA satellite cells are able to proliferate and differentiated. If a muscle scaffold and a capillary

network are in part preserved at a distance of one-two millimeters, RA satellite cells are fully capable of regenerate damaged myofibers, as in any other muscle.

On the other hand, in the case of 8 x 24 mm full thickness ablation of the central third of the rat rectus abdominis, the prolonged ischemia does not allow survival of the satellite cells present in an auto-graft. Conceivably, early after the abdominal wall reconstruction myoblasts undergo ischemic apoptosis and/or necrosis, as we clearly shown here.

These RA ablation experiments, strongly suggests that coordinate time course of revascularization events and myoblast proliferation is mandatory to allow myoblast emigration into an acellular graft or a necrotic tissue. This brings to mind the protocols used by cardiac surgeons, which indeed call for skeletal muscle myoblasts to be seeded around the necrotic core of an infarcted myocardium [6, 14, 21]. The volume-related constrains we have enlightened, clearly contribute to the high rate of success in mice experiments and to the frustrating negative results of myogenic stem cell seeding into acellular matrixes in larger animals [24, 26].

The experiments here reported are preliminary of a long-term project to develop artificial myogenic scaffolds. We need to optimize procedures to increase survival of injected myogenic cells in vivo, in particular when reconstruction of clinically significant amounts of muscle are needed, a case in which revascularization and reinnervation are essential processes. Of course, the single-step approach would possibly not be successful, if major muscle defects ought to be treated. We are confident that paying attention to "guided healing" approaches, in a foreseeable future the long-lasting dream of plastic surgeons will be a clinical option.

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