Reconstruction of Ablated Rat Rectus Abdominis by Muscle Regeneration

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Skeletal muscle regeneration is a powerful, naturally occurring process of tissue reconstruction that follows myofiber damage secondary to myotoxic injury that does not normally affect the tissue circulation and scaffold. The ablated tissue, in traumatology and free muscle grafts, is frequently replaced by scars. The final outcome is poor even after in situ myoblast seeding of the harvested muscle. The goal of this study was to identify protocols to reconstruct muscle tissue, even in such adverse environments. The authors applied a step-by-step approach to identify factors favoring the survival of autologous satellite cells and, thus, muscle regeneration. In a rat model of full-thickness rectus abdominis muscle ablation, autologous myoblasts were isolated from the explanted rectus abdominis and seeded in a homologous acellular matrix immediately after wall reconstruction (group 1, five animals). In group 2 (five animals), the ablated rectus abdominis was autografted in situ. In a third group of five rats, Marcaine was injected into both the autograft and the surrounding abdominal wall muscle. Three weeks after surgery, serial cross-sections of the reconstructed abdominal wall were stained with hematoxylin and eosin or embryonic myosin antibody, a well-characterized molecular marker of early myogenesis in development and regeneration. Percentages of the patch area covered by regenerated myofibers were determined by morphometry.

When autologous myoblasts were seeded in a homologous acellular matrix, the only myofibers observed to regenerate were those along the border of the patch. Autografting of the middle third of the rectus abdominis muscle similarly resulted in scar formation. The few muscle cells in the graft core were scanty myoblasts that could be detected only by monoclonal embryonic myosin antibody. Although negative for myofiber regeneration, the results in both cases confirmed the mechanical patency of the patches with regard to abdominal organ support. Myofibers were successfully regenerated in the graft by injecting Marcaine into both the autograft and the surrounding muscles. Three weeks after surgery, the patch was paved with young, centrally nucleated myofibers intermixed with young myofibers and myotubes expressing embryonic myosin. The difference in percentage of patch area covered by regenerated myofibers in group 3 (Marcaine injection around the patch, 81.6 ± 3.0 percent) (mean ± SD) versus either group 1 (Myoblast-seeded acellular patch, 18.0 ± 3.0 percent) or group 2 (Autograft, 25.8 ± 7.0 percent) was statistically significant on independent *t* test analysis ($p < 0.0001$). Even an acellular matrix showed some myofiber regeneration after surrounding muscles had been injected with Marcaine. This is the first successful evidence of muscle reconstruction after full-thickness ablation of the middle third of the rectus abdominis. Muscle regeneration seems to be the result of successive waves of migration of angioblasts and then satellite cell-derived myoblasts from the muscles surrounding the patch. The results strongly suggest that vascularization of the scaffold and successive coordinate proliferation of the seeded cells are required for myoblasts to be able to migrate into the patch and differentiate up to myofiber stage. (*Plast. Reconstr. Surg.* 114: 1509, 2004.)

Skeletal muscles, besides allowing voluntary movements, maintain the structural contours of the body. Muscle trauma, dystrophy, and atrophy are common pathological processes that result in muscle deficiencies. Skeletal muscle regeneration is a powerful process of tissue reconstruction that occurs when myofibers "die," secondary to myotoxic injury, which, instead, usually spares the tissue circulation and scaffold. Examples of successful muscle regeneration may be occasioned not only by injections of snake venom or Marcaine in normal or ischemic muscles but also by free autografts and even by the injection of minced tissue in rodent leg muscles.[1–4] In traumatology and free muscle grafts, however, scars are commonly seen replacing the ablated tissue.[5]

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The complete loss of anatomically defined muscles may be determined by certain pathological states, such as congenital abdominal wall defects (gastroschisis and omphalocele). Attempts to correct them functionally have encountered limited success.[6] The final outcome is poor even after myoblast seeding in the site of the harvested muscle.[7–9]

In reconstructive plastic surgery, the harvesting of pedicle or microvascular muscle flaps poses a particular problem of muscle loss. The need to reduce donor-site morbidity prompted us to study a rat model that reproduced the clinical conditions occurring in humans after rectus abdominis muscle transplantation.

This study presents the case results of full-thickness ablation of the rectus abdominis muscle in rats. An acellular matrix seeded with autologous satellite cells immediately after wall reconstruction and even a free autograft of the middle third of the rectus abdominis muscle are shown to have resulted in scarring, whereas injections of Marcaine in both the graft and the surrounding muscles successfully drove the healing process to muscle regeneration.

The long-term aim of our project is to tissue engineer a three-dimensional vascularized skeletal muscle using isolated myoblasts seeded into acellular matrices implanted in the site of the abdominal wall defect.

Materials and Methods

Homologous Acellular Muscle Matrixes

Rectangular, 24 × 8-mm, full-thickness patches of rectus abdominis muscle were harvested from syngenic donors to prepare acellular matrixes as described by Sicard et al.[10] In brief, the patches were subjected to three cycles of freezing (immersion in liquid nitrogen) and heating (in a boiling water bath) to inactivate the cells, in particular, the myogenic cells (muscle satellite cells). The acellular matrixes were maintained in sterile condition at −80°C until surgery.

Animals and Surgical Procedures

The animal protocol used in this study was approved by the institutional animal care committee of our university. Wistar rats weighing 250 to 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 shaved and aseptically prepared using povidone-iodine (Betadine) and 70% alcohol solution. The abdominal muscles were exposed through a 3-cm abdominal incision. A sterile template measuring 24 × 8 mm was used to mark and resect an area, producing a full-thickness defect of the middle third of the rectus abdominis muscle (Fig. 1, *above*).

The animals ($n = 15$) were divided in three groups. Group 1 ($n = 5$) had the abdominal muscle defect repaired with acellular matrix seeded with autologous myoblasts. In the site of the rectus abdominis ablation, a 24 × 8-mm acellular patch was sutured and injected with satellite cells isolated from the rectus abdominis as described by Cantini et al.[11] The final pellet, containing 1,000,000 cells, was resuspended with 600 μl of phosphate-buffered saline, and the cell suspension was inoculated into the acellular matrix with a 25-gauge needle. In group 2 ($n = 5$), the autograft group, the rectangular area (24 × 8 mm) of rectus abdominis muscle was harvested and immediately grafted in situ using 4.0 nylon. Group 3 ($n = 5$) underwent the injection of Marcaine 0.5% into the patch and surrounding abdominal wall muscles. At the time of surgery, the autografts were injected with 0.3 ml of Marcaine 0.5%. The surrounding muscles were also infiltrated with four 0.3-ml Marcaine 0.5% injections. In all three groups, the skin incision was closed with 4-0 nylon, and the surgical procedures were performed by the same surgeon.

Analyses of Muscle Biopsy

Three weeks after surgery, the abdominal wall of the anesthetized animals was exposed and a rectangular area of approximately 28 × 12 mm was removed such that some surrounding muscle tissue was included in the muscle specimen. The harvested tissue was soaked in liquid nitrogen and stored at −80°C until ready for use. The reconstructed abdominal walls were cross-sectioned at the middle of the specimen, and serial sections were stained with hematoxylin and eosin or anti-MHCemb (Novo Castra, Newcastle upon Tyne, United Kingdom), as reported by Rossini et al.[12] Percentage of patch cross-sectional area covered by regenerated myofibers was determined by morphometry.

Statistical Analysis

The percentage area of regeneration was analyzed for statistical significance, using the in-

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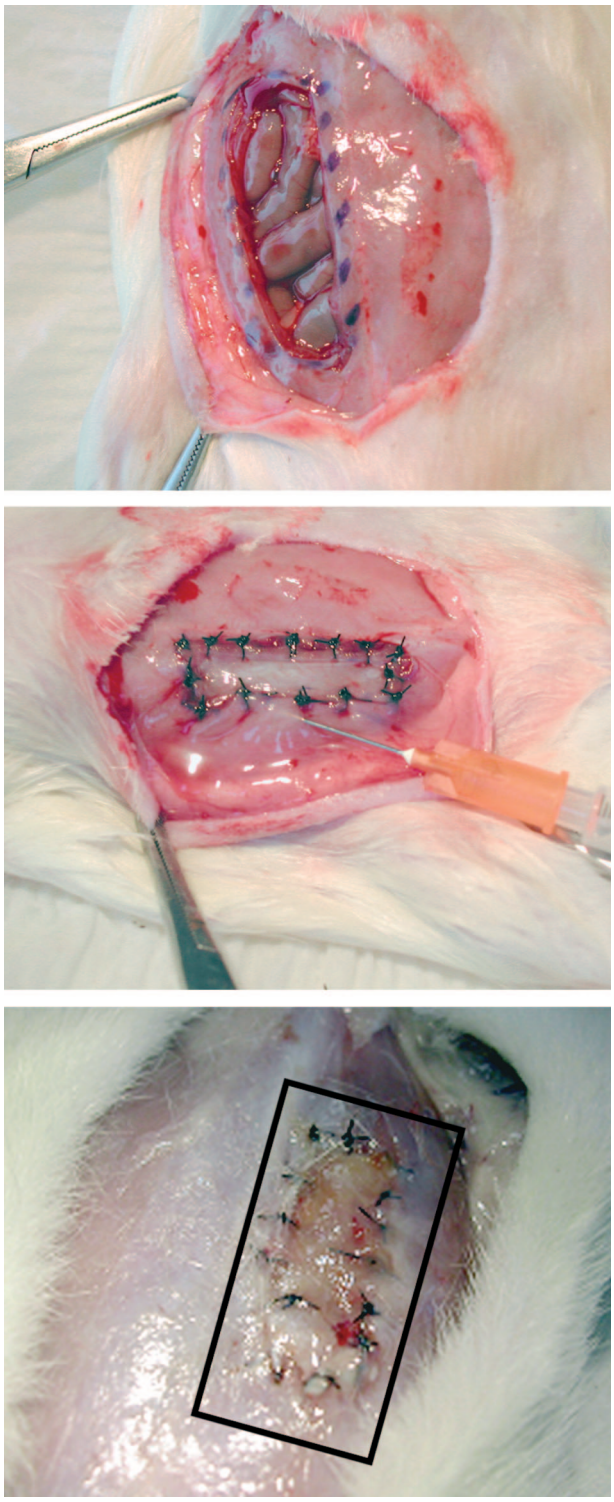

FIG. 1. Surgical procedures for the reconstruction of abdominal wall continuity after full-thickness ablation of the middle third of the rectus abdominis muscle of a rat. (*Above*) An ablation template lies on the middle third of the rectus abdominis (measuring 8 × 24 mm). (*Center*) Wall reconstruction, as shown immediately after surgery. (*Below*) External aspect of the implant 3 weeks after surgery. Stitches well define the borders between the patch and the muscles of the abdominal wall.

dependent *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the macroscopic aspect of a patch 3 weeks after surgical reconstruction of the abdominal wall of the rat. Stitches well define the borders between the patch and the abdominal wall muscles. All three types of patches were well integrated with the abdominal wall and neovascularization from the peritoneum was usually present. The external surface of the patches was always free from scar adhesion to the subdermal plane. No herniation was observed, and it was concluded that all three types of patches were able to sustain the abdominal organs.

In Figure 2, a homologous acellular matrix that had been injected with autologous satellite cells is shown, under light microscopy, to present loose connective tissue and heavy infiltration of mononuclear cells. Myofibers are absent (Fig. 2, *above, left*). Only scanty small cells appear positive after staining with a monoclonal embryonic myosin antibody (myoblasts, the green cells in Fig. 2, *above, right*). Figure 2, *below*, demonstrates how the lack of muscle regeneration is not related to the nature of the scaffold seeded with myogenic cells. The core of an rectus abdominis autograft is substituted by reparatory connective tissue infiltrated by mononucleated cells (Fig. 2, *below, left*), among which rare myogenic cells are present (anti-MHCemb–positive cells in Fig. 2, *below, right*). On the other hand, a 1-mm rim of regenerated myofibers was always present around all the patches, i.e., at the level of the stitches. Among them, many young regenerated myofibers are labeled by embryonic myosin antibodies (results not shown).

Figure 3 shows the successful regeneration of an autografted rectus abdominis when Marcaine 0.5% was injected into both the implant and the surrounding abdominal wall muscles at the time of surgery. The patch may be identified under low magnification by its reduced thickness in comparison with the rim of surrounding abdominal muscles (Fig. 3, *left*). Under higher magnification (Fig. 3, *center*), it appears paved with polygonal myofibers, some of which are centrally nucleated. In Figure 3, *right*, anti-MHCemb immunofluorescence confirms that the regenerative process is ongoing in the patch. The cross-section area of the Mar-

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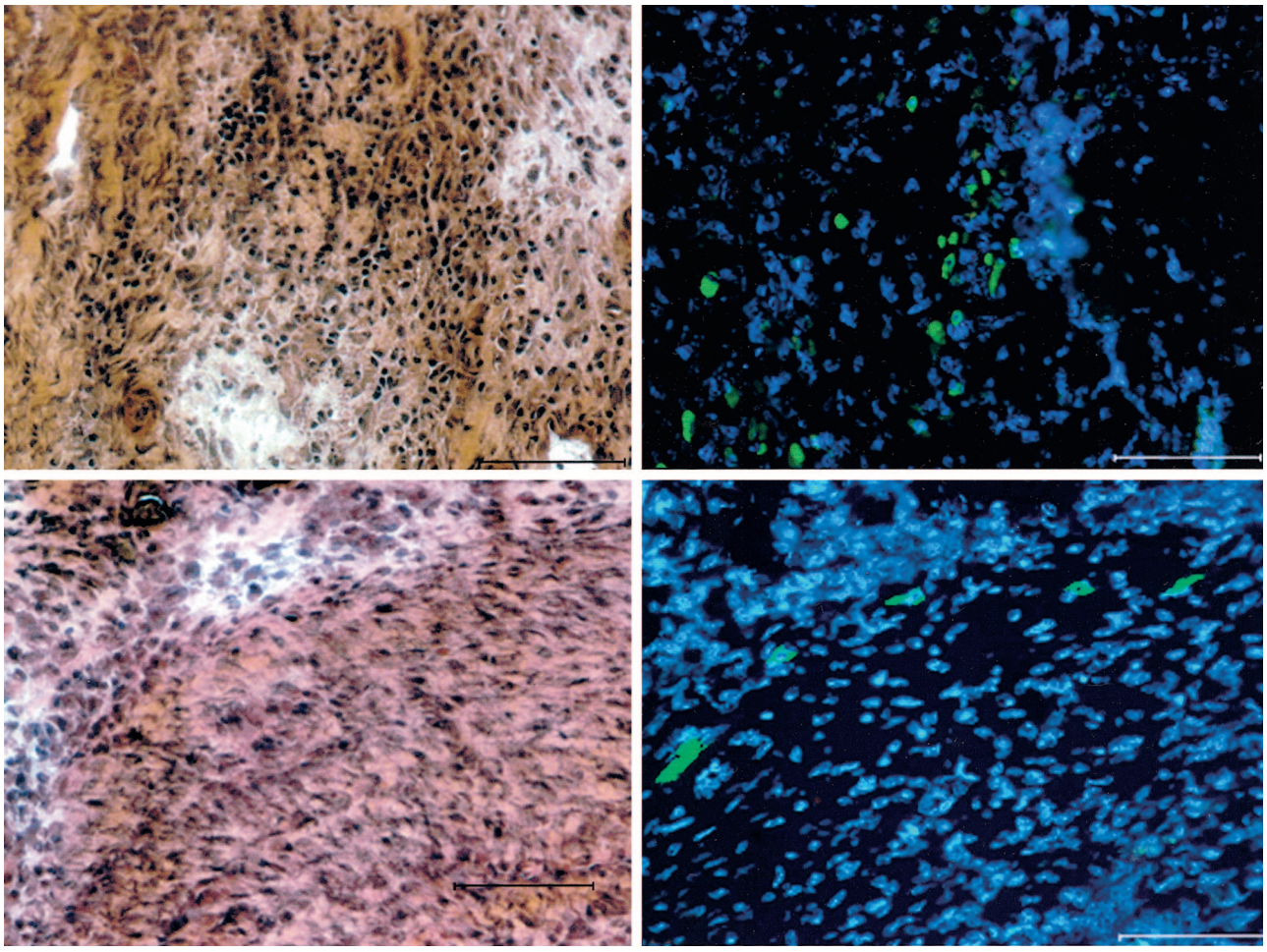

FIG. 2. Reconstruction of rectus abdominis defect with either homologous acellular matrix seeded with autologous myoblasts or rectus abdominis autograft. (*Left, above and below*) Hematoxylin and eosin stain; (*right, above and below*) immunostaining with MHCemb antibody. (*Above*) Acellular matrix seeded with autologous myoblasts. (*Below*) Rectus abdominis autograft. Scale bar, 100 μm. Implants are substituted by connective tissue heavily infiltrated by mononuclear cells (*left*), among which myoblasts or myotubes are rare (*green cells, right*).

caine-treated specimens was almost entirely covered with young myofibers (Fig. 4).

The difference in percentage of patch area covered by regenerated myofibers between group 3 (Marcaine injection around the patch, 81.6 ± 3.0 percent, mean ± SD) versus either group 1 (Myoblast-seeded acellular patch, 18.0 ± 3.0 percent) or group 2 (Autograft, 25.8 ± 7.0 percent) was statistically significant upon independent *t* test analysis ($p < 0.0001$).

Figure 5, *above*, shows that Marcaine infiltration of both an acellular matrix and the surrounding abdominal wall muscles was able to induce proliferation of myoblasts, which then migrated into the patch. Figure 5, *below*, confirms the presence of small myofibers, which tested positive on anti-MHCemb staining.

DISCUSSION

Skeletal muscle regeneration is a powerful process of tissue reconstruction occurring after myofiber death secondary to injuries that spare the scaffold and capillary network of muscle tissue, like exercise-induced muscle damage in normal and dystrophic muscle, or myotoxic agents. In the case of the smallest leg muscles in rodents, functional muscle regeneration also occurs after full free autografting or even muscle mincing.[1–5,9,13–16]

In traumatology, lost muscle is commonly observed to be eventually replaced by scar tissue.[6,17] Muscle loss is also encountered in pathological states, such as congenital abdominal wall defects (gastroschisis and omphalocele). A particular case of muscle loss is related to pedicled or microvascular muscle flaps in

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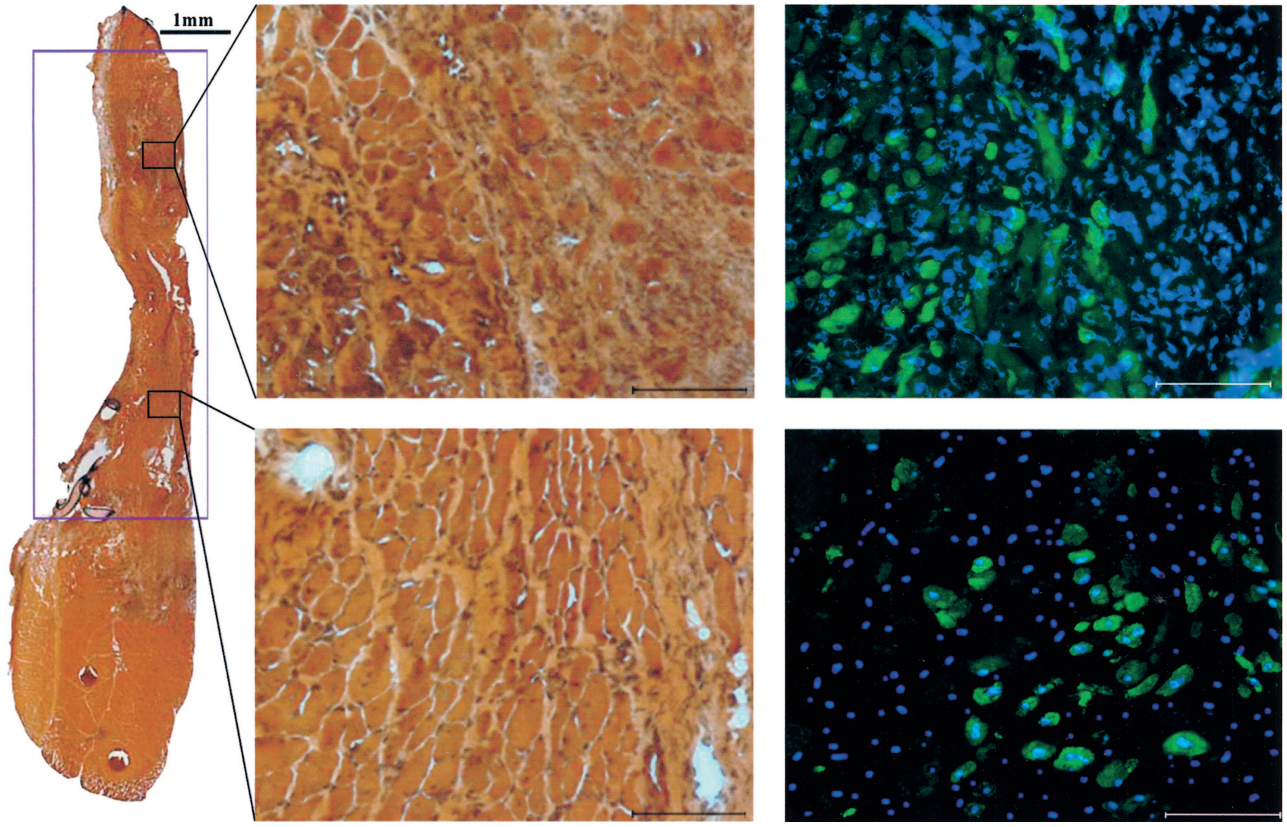

FIG. 3. Marcaine-induced myofiber regeneration in rectus abdominis autografting. (*Left and center*) Hematoxylin and eosin stain; (*right*) immunostaining with MHCemb antibody. (*Left*) Scale bar, 1 mm. (*Center and right*) Scale bar, 100 μm. (*Left*) Low magnification view of the cryosection. (*Center*) High-magnification views of the implant near the muscle rim. (*Right*) Autograft core. The graft is paved with polygonal myofibers, some of which are centrally nucleated. Embryonic myosin antibodies reveal that 3 weeks after surgery the regenerative process is still active in the patch (*green cells*).

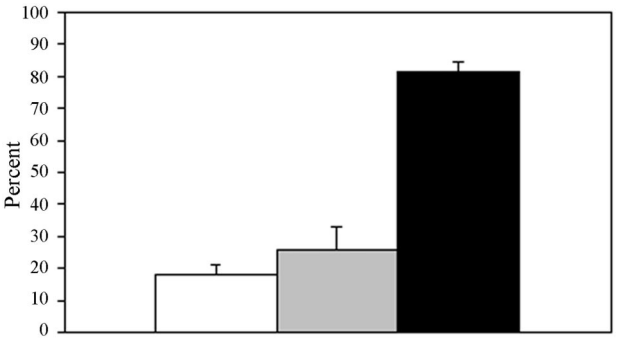

FIG. 4. Effect of Marcaine injection on muscle regeneration after full-thickness ablation and reconstruction of the middle third of the rectus abdominis. Percentage of patch cross-sectional area covered by regenerated myofibers. Group 1 (myoblast-seeded acellular patch, ▨), 18.0 ± 3.0 percent (mean ± SD); group 2 (autograft, ▨), 25.8 ± 7.0 percent; group 3 (Marcaine injection around the patch, ■), 81.6 ± 3.0 percent. Group 1 versus group 2, $p = 0.05$. Group 3 versus either group 1 or 2, $p < 0.0001$.

reconstructive plastic surgery. Attempts to correct donor-site morbidity functionally have been made with limited success.[18] Clinically nondegradable materials such as Teflon or Marlex are used, despite the concomitant foreign-body reaction, with the risk of implant rejection. Prosthetic materials present well-known complications such as infection and the need for multiple surgeries. In these settings, homologous biomaterial should reduce complications and shorten recovery times.[19] Because of the very high metabolic demand of muscle tissue and its dependence on motoneuron activity, there is general agreement that it cannot be successfully free grafted and that free muscle grafting, in humans and other large mammals, is not a clinically reliable method. Considerable further research will be required to advance beyond the presently successful microsurgical approaches. As to basic surgical needs, the present rat model is satisfactory in abdominal wall reconstruction after full-thickness ablation of the rectus abdominis central third. The cellular or acellular rectus abdominis implants have the mechanical properties required to support the abdominal organs. Indeed, the integrated patches never herniated.

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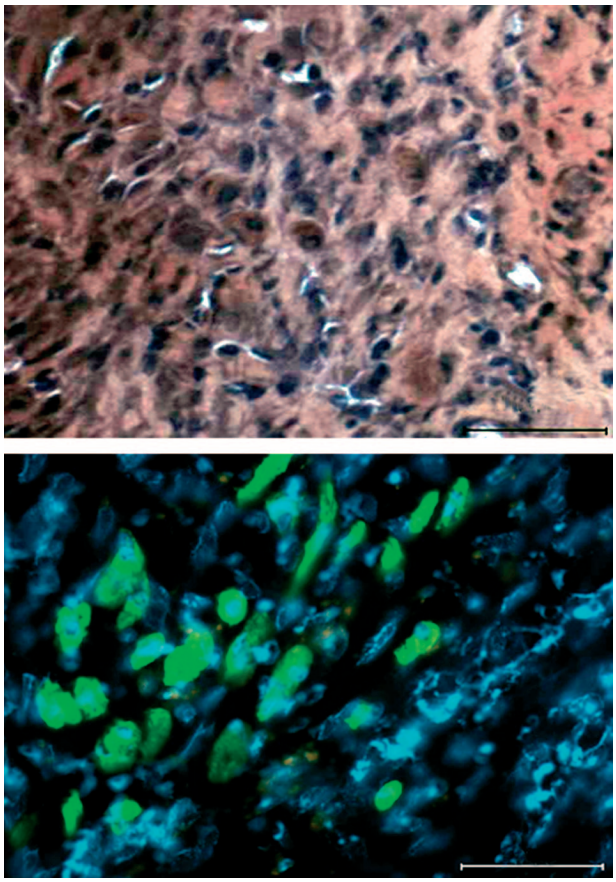

FIG. 5. Effect of Marcaine injection on reconstruction of rectus abdominis defect with homologous acellular matrix. (*Above*) Hematoxylin and eosin stain; (*below*) immunostaining with MHCemb antibody. Scale bar, 100 μm. Implants are substituted by vascularized connective tissue and regenerated myofibers (*green cells, below*).

Taking advantage of these properties, a step-by-step approach was used in the study to identify the factors that may favor muscle regeneration in the patch, particularly during the critical early phases, determining the death or survival of the autologous myogenic cells. In the present series of experiments, besides the case of the autologous myoblasts seeded immediately into the homologous acellular rectus abdominis matrix and even that of rectus abdominis autografting, the only myofibers observed to have regenerated 3 weeks after surgery were confined to the surrounding muscle rim, onto which the patches were sutured. Our results are in agreement with other reports in the literature showing that when myogenic cells are delivered after implantation of acellular scaffolds in the site of a harvested muscle, the final outcome is poor.[7,8,10,20–23] Scarring is the result of a race between fibroplasias and myogenesis, in which the blood vessels and the fibroblasts win and the myogenic cells lose.

In instances of full-thickness ablation of the central third of the rat rectus abdominis, seeded autologous myoblasts or even satellite cells present in an autograft seem unable to survive. Conceivably, soon after abdominal wall reconstruction, the myoblasts undergo ischemic apoptosis and/or necrosis. The few myogenic events can only be detected by applying an embryonic myosin monoclonal antibody, a well-characterized molecular marker of early myogenesis in development and regeneration.[4,5]

This first series of negative results is related to the initial planning of the experiment. Assuming that the outcomes of free muscle autografting of the small leg muscles could be replicated with the abdominal wall,[1–5] a one-step intervention aimed at minimizing surgical requirements was designed. These results are not the consequence of a lack in the rectus abdominis of satellite cells or of a peculiar inability of theirs to proliferate and differentiate.[23] The muscle regeneration around the patch bears strong witness that the satellite cells of the abdominal wall muscles behave like those of any other rat muscle, responding to physical damage and posttraumatic transient ischemia, which induce myofiber death and regeneration in the muscle tissue surrounding the implants.

The injection of Marcaine into both the muscle autograft and the surrounding muscle rim, however, promotes proliferation, differentiation, and migration of the myoblasts into the implant beginning 2 to 3 days after surgery.[2–5] Thus, 3 weeks after surgery, the patch could be seen to have filled with young, centrally nucleated myofibers intermixed with myotubes still expressing embryonic myosin. Even an acellular homologous matrix showed a higher rate of myofiber regeneration when the surrounding muscles were injected with Marcaine. This result 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.[24–26]

This is the first evidence of a scaffold being colonized by myofibers after abdominal wall repair following full-thickness ablation injury by implantation of an acellular matrix, whether such is the outcome of preimplantation treatments or of prolonged ischemia of the autograft. These results strongly suggest that the implementation of myoblast emigration into the implants

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requires coordinate time frames for revascularization and myoblast proliferation.

The experiments reported here are the preliminary results of a long-term project involving the development of artificial myogenic scaffolds. Procedures will have to be optimized to increase the survival of injected myogenic cells in vivo, particularly when reconstruction of clinically significant amounts of muscle is required, making revascularization and reinnervation essential processes. The single-step approach might logically not be successful where major muscle defects need to be treated. We are confident that if this "guided healing" approach can be fine-tuned, the reconstruction of ablated muscle—that long-standing dream of plastic surgeons—will be a clinical option in a not-too-distant future.

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