Creating Immune-Privileged Sites within Skeletal Muscle: Apoptosis of FasL-Transfected Myoblasts Prevents the Formation of FasL-Expressing Muscle Fibers In Vivo

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Abstract

It has been suggested that over-expression of FasL could provide immune-privileged sites where transfected genes or/and grafted cells could exert their therapeutic action without inducing an immune response. Given the good transfectability of skeletal muscle, such tissue has been considered as a prime candidate for FasL engineerization. However, attempts to create FasL-expressing myoblasts have so far generated conflicting results. In this paper we set out to further investigate such approach, both *in vitro* and *in vivo*. We have designed a chimeric expression plasmid in which the addition of a GFP moiety to the C-terminal of FasL coding sequence rendered the final protein incapable of inducing apoptosis. Such construct has then been used for *in vitro* and *in vivo* transfection of rat muscle cells and its effects were compared with those obtained with a plasmid expressing normal FasL. The vast majority of primary myoblasts transfected *in vitro* with the FasL-expressing plasmid underwent apoptosis within the first 48 hours. Conversely, transfections with the FasL-GFP plasmid failed to induce cell death and allowed the formation of FasL-GFP positive myotubes, thereby demonstrating the inability of the chimeric product to activate the FasL-Fas pathway. *In vivo*, muscles co-injected with LacZ- and FasL-encoding plasmids developed a leukocytes infiltrate and transfected fibers were rapidly cleared out. When muscles were injected with a mixture of FasL-GFP and LacZ plasmids, transfected fibers persisted for at least two weeks. The results presented here demonstrate that Fas-FasL interaction is responsible for both the apoptosis of FasL transfected myoblasts *in vitro* and disappearance of FasL-engineered muscle fibers *in vivo* when transfections are carried out in regenerating muscles. However, our findings also suggest that it might be possible to obtain FasL-engineered mature muscle fibers by appropriately regulating the expression of the exogenous DNA during muscle differentiation.

Key words: FasL, immune-privileged site, gene therapy, muscle.

Basic Appl Myol 12 (3): 101-108, 2002

The ability to express an exogenous gene efficiently and steadily in muscle tissue would allow developing gene therapy protocols for the treatment of many diseases. These include not only muscular dystrophies but also the many conditions in which muscle fibers could be used for the synthesis and secretion of a therapeutic protein.

Several approaches are available to efficiently introduce foreign genes into muscle, e.g., myoblast transplantation [4, 9, 10, 20, 21] and direct injection of naked DNA in the presence of muscle regeneration or electroporation [32, 33]. Viral vectors [1, 8, 23] and, to a lesser extent, lipid-based vectors [24] have also been successfully used. All these approaches, however, have to deal with the problems arising from the local inflammatory and/or immune response elicited against the foreign DNA product, the viral proteins or the transplanted cells. A way of controlling these phenomena would therefore be highly beneficial for muscle gene therapy.

It is well known that the Fas-FasL interaction is involved in the cell death signal (apoptosis) in several cell populations, such as infected cells, tumoral cells and grafted tissue. The system also controls the T-cell expansions during immune responses, like the production of pluri-activated CTL, and plays a central role in B cell regulation [15, 17, 18, 27]. Loss-of-function mutations in

the Fas-FasL system result in lymph-proliferation and auto-immunity [5, 12]. Fas receptor (Fas, APO-I, CD95) has been found in a variety of cell types such as hepatocytes, B and T lymphocytes and, more recently, in myoblasts [29]. The transmembrane type II Fas ligand protein (FasL, CD95L) is constitutively expressed in Sertoli cells and in the epithelial cells of the anterior chamber of the eye, where it contributes to maintain an immune-privileged environment by destroying the inflammatory cells that infiltrate these tissue [7, 19]. These observations led to the hypothesis that FasL over-expression in a selected tissue could provide an immuno-privileged site, which, in turn, could protect allogeneic cell grafts and/or allow for the safe expression of a foreign gene. In this regard, in a mouse model it has been reported that testicular tissue transplanted under the kidney capsule in allogeneic animals can survive indefinitely, whereas testis grafts from a FasL deficient strain (*gld*) are rejected [2, 14, 30]. Also, Lau and colleagues reported that a mixture of FasL over-expressing murine myoblasts and pancreatic islets could be successfully grafted into allogeneic animals [16]. On the other hand, these findings could not be confirmed by Kang and colleagues who, in similar experiments, reported rapid death of FasL-engineered myoblasts *in vitro* and rapid rejection of islets grafted *in vivo* together with transfected myoblasts [13]. In order to elucidate the role of Fas-FasL interaction in the survival of transfected muscle cells, we have designed a plasmid encoding for a chimeric FasL-GFP protein in which the interaction of FasL with its receptor is prevented by the GFP moiety added at the 3' end. Here we report the results obtained with two experimental models, cultured primary rat myoblasts and regenerating rat muscle, which have been transfected with different combinations of FasL, FasL-GFP and LacZ -expressing plasmids.

Materials and Methods

Plasmid construction

pCMVLacZ was from Clonetech; pCIneo was from Promega. A Bluescript II SK(+) plasmid containing the murine FasL cDNA was a generous gift of Prof. Nagata (Osaka, Japan). pCIneoFasL, in which the 940 base pair FasL cDNA is under the control of a the CMV early promoter/enhancer, was previously designed in our laboratory [6].

The pCIneoFasL-GFP plasmid was prepared using a HA1-hEGFP cDNA (S65T mutant, kindly provided by Dr. Pozzan, 25], that was excised from the parental plasmid with Hind III and Not I. The 780 bp HA1-hEGFP fragment was then ligated to the FasL cDNA fragment obtained by digesting the original FasL plasmid with Hind III ad Xba I. The Hind III digestion removed the stop codon of the FasL cDNA, thereby allowing to link the GFP coding sequence at its C-terminal end. The chimeric cDNA was finally inserted in the pCIneo vector between the XbaI and Not I sites.

Primary muscle cell cultures and transfections

Rat primary myoblasts were obtained from newborn animals by trypsin digestion and maintained in DMEM supplemented with 10% FCS as previously described [4]. Cells were seeded in 24 well plates at a density of $2x10^5$ cells/well and fusion was obtained by replacing the FBS with 2% Horse Serum (HS).

Immunocytochemistry analyses were performed on cells cultured on glass coverslips coated with gelatine. All culture media were supplemented with 100 U/ml penicillin and 100µg/ml streptomycin. Primary myoblast cultures were transfected with the indicated plasmids by standard calcium phosphate procedure as described in [3, 6]. Cells were left in contact with the DNA/$Ca_3(PO_4)_2$ particles for 10 hours, after which they were rinsed three times with PBS and re-fed with fusion medium. Except where otherwise indicated, 4 µg DNA per well was used for the experiments.

In vitro cell viability and apoptosis (TUNEL)

Cell cultures were stained with both Hoechst 33258 (1.5 µg/ml) and Propidium Iodide (PI, at 4 g/ml) for 10 min. After two washes in PBS, cultures were observed by fluorescence microscopy. All nuclei stained blue (Hoechst), but only cells with damaged plasma membrane had internalized the PI and therefore showed red fluorescence. The overlap of the two fluorochromes in the nuclei of distressed or dead cells led to the emission of a bright white signal. For detection of apoptosis, cultures were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. DNA fragmentation was revealed by in situ nick/end labeling TUNEL (In Situ Cell Death detection Kit; Boehringer Mannheim), performed according to [25]. For quantitative analyses, 6 different fields were photographed in each well and PI and TUNEL-positive cells were counted. Each condition was evaluated in three independent experiments. In each experiment, an equal number of transfected wells were used for PI/Hoechst staining (cell viability) and for TUNEL staining.

In vivo muscle transfection

Tibialis Anterior muscle of adult Wistar male rats (250 gr) was used for *in vivo* transfection. Briefly, surgically exposed muscles were injected with 200 µl of a 0.5% marcaine aqueous solution. After 4 days, regenerating muscles were injected with 50 µg DNA of the indicated plasmids in a total volume of 100 µl. Right TA muscles were injected with pCMVLacZ plus pCIneoFasL or pCMVLacZ plus pCIneoFasL-GFP plasmids, whereas left TAs were injected with pCMVLacZ alone. After marcaine treatment, skeletal muscle regenerates *via* activation of quiescent satellite cells that originate a great number of proliferating myoblasts. During this process, the uptake of injected foreign DNA (plasmid DNA) in the fibers is greatly enhanced [9]. Animals were sacrificed at 7 and 14 days after DNA injection. Harvested muscles were cut at the middle belly in 10 µm thick slices and

serial sections were H&E stained for morphology or processed for immunohistochemistry and ß-gal activity (see below). All surgical procedures were carried out in compliance with the local regulations for handling laboratory animals, using Ketamine and Xilazine for anesthesia and ossitetracycline as a post-surgery antibiotic.

ß-gal staining and immunohistochemistry

For ß-gal staining, muscle sections were fixed for 10 minutes in 2% paraformaldehyde. Samples were rinsed in PBS and incubated overnight with a solution containing 1mg/ml X-gal in DMSO, 5 mM potassium ferrycianide, 5 mM potassium ferrocianide, 2mM MgCl2, 0.02% NP-40 and 0.01% sodium deoxycolate at 37°C.

For FasL staining, samples were labelled with an anti-FasL rabbit polyclonal Ab that recognizes the C-terminal portion of the protein (Santa Cruz, USA). An anti-rabbit IgG rhodamine-conjugated secondary antibody was used to reveal FasL-positive cells. Nuclei were counterstained with Hoechst 33258. FasL-GFP fusion-protein was also revealed by green auto-fluorescence of GFP portion.

Results

In vitro experiments

Primary myoblasts were transfected in parallel with pCIneoFasL or pCIneoFasL-GFP and then analyzed at different time points. Twenty-four hours after the end of transfection (i.e., after removing the transfection mixture), both experiments showed expression of FasL protein, as visualized by an anti-FasL antibody (Figure 1a-c). Cultures transfected with the pCIneoFasL vector, however, showed extensive cell damage, characterized by rounded shape and detachment from the bottom of the dish. Increased cell death was demonstrated by the PI-Hoechst survival test, in which a lower number of PI-positive cells were found in mock and FasL-GFP transfected cultures (Fig. 1 d-f). This finding was confirmed by TUNEL analyses, that showed how apoptotic nuclei were much more numerous in FasL-transfected cultures as compared to control and FasL-GFP transfected cultures (Fig. 1 g-i).

The results of quantitative analysis of cell viability and apoptosis are summarized in Table 1. Cell counts showed that 23% of cells of FasL transfected cultures presented cell damage (PI-positive), almost twice as much of what

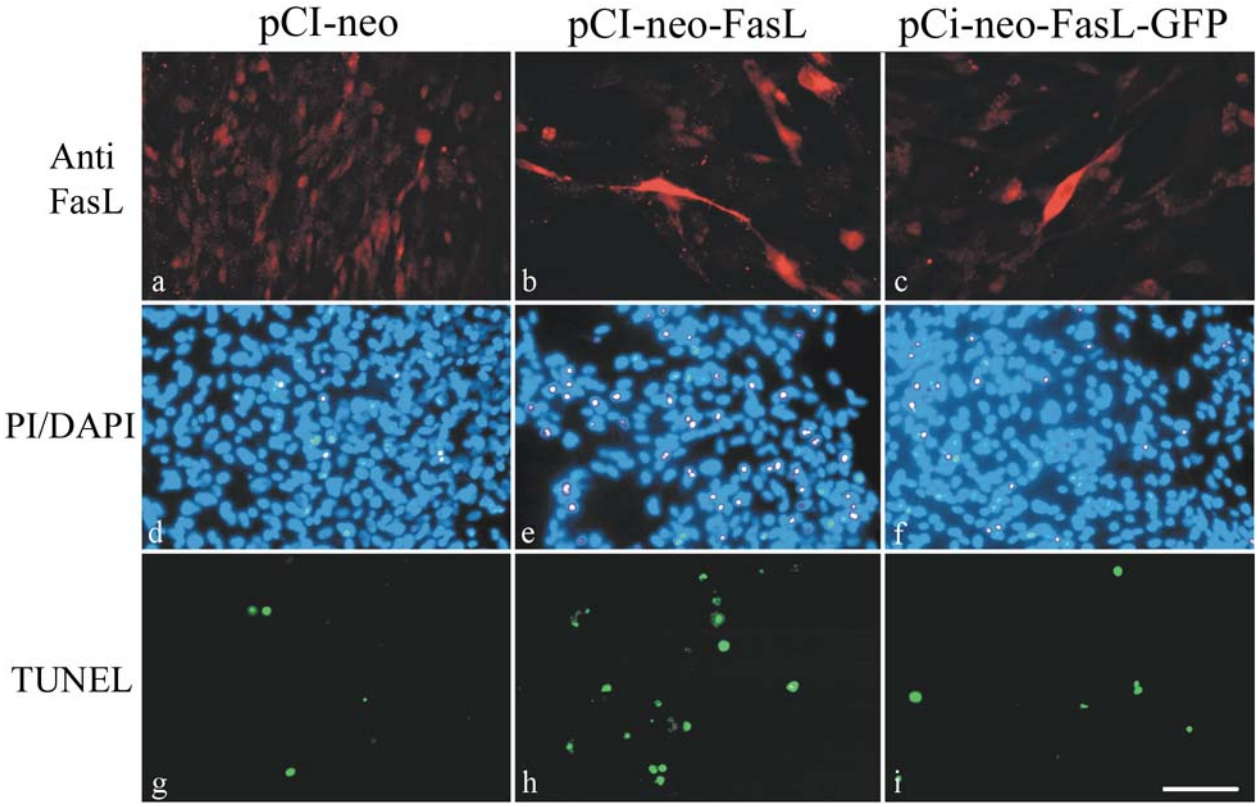

Figure 1. Primary rat muscle cells cultured for 18 hrs after transfection. Transfections were carried out with pCIneo plasmid (panels a, d, g), pCIneoFasL plasmid (panels b, e, h) and with pCIneoFasL-GFP plasmid (panels c, f, i). An anti-FasL antibody was used to reveal FasL protein (a, b, c). Cell viability is shown in panels d, e and f; PI positive signal indicates cell death. Apoptosis is shown in panels g, h and i: green fluorescence (TUNEL) indicates DNA fragmentation. Bar 58 µm.

Table 1

	PI/Hoechst +ve cells	TUNEL +ve cells
Control	12 ± 0.9	2.5 ± 0.2
FasL-GFP	13 ± 1	3 ± 0.2
FasL (4 µg/well)	23 ± 1.2	14 ± 1.2
FasL (2 µg/well)	22 ± 1.3	14 ± 1.4
FasL (1 µg/well)	23 ± 1.2	15 ± 1.2

found in FasL-GFP and sham-transfected cells. The cell damage found in controls was most likely due to the intrinsic toxicity of calcium-phosphate transfection.

The difference between FasL, FasL-GFP or sham-transfected cells was even more striking when we considered the TUNEL results. In fact, 14% of nuclei were apoptotic in cultures transfected with pCIneoFasL, compared to just 2.5-3% found in cells transfected with pCIneoFasL-GFP or pCIneo. In order to verify if lower transfection efficiency could improve the survival rate amongst FasL expressing cells, experiments were carried out using decreasing amount of pCIneoFasL. As shown in Table 1, lowering the amount of pCIneoFasL plasmid DNA from 4 to 1 µg per well did not yield any appreciable change in the level of apoptosis.

It is important to note that in primary cultures transfected with pCIneoFasL we observed the presence of what appeared to be FasL-resistant myoblasts. Specifically, after the massive cell death occurring within the first 2-3 days after transfection, the cultures eventually recovered and showed the formation of some myotubes. Most of them were actually FasL-negative, thereby suggesting that they originated from myoblasts that had not been transfected at all. However, some myotubes were instead FasL-positive and hence had to derive from transfected myoblasts that had escaped apoptosis (Figure 2). As expected, FasL-GFP transfected cultures yielded a number of myotubes comparable to that obtained in mock-transfected controls and some of them showed positive stain for both FasL and GFP (Figure 2).

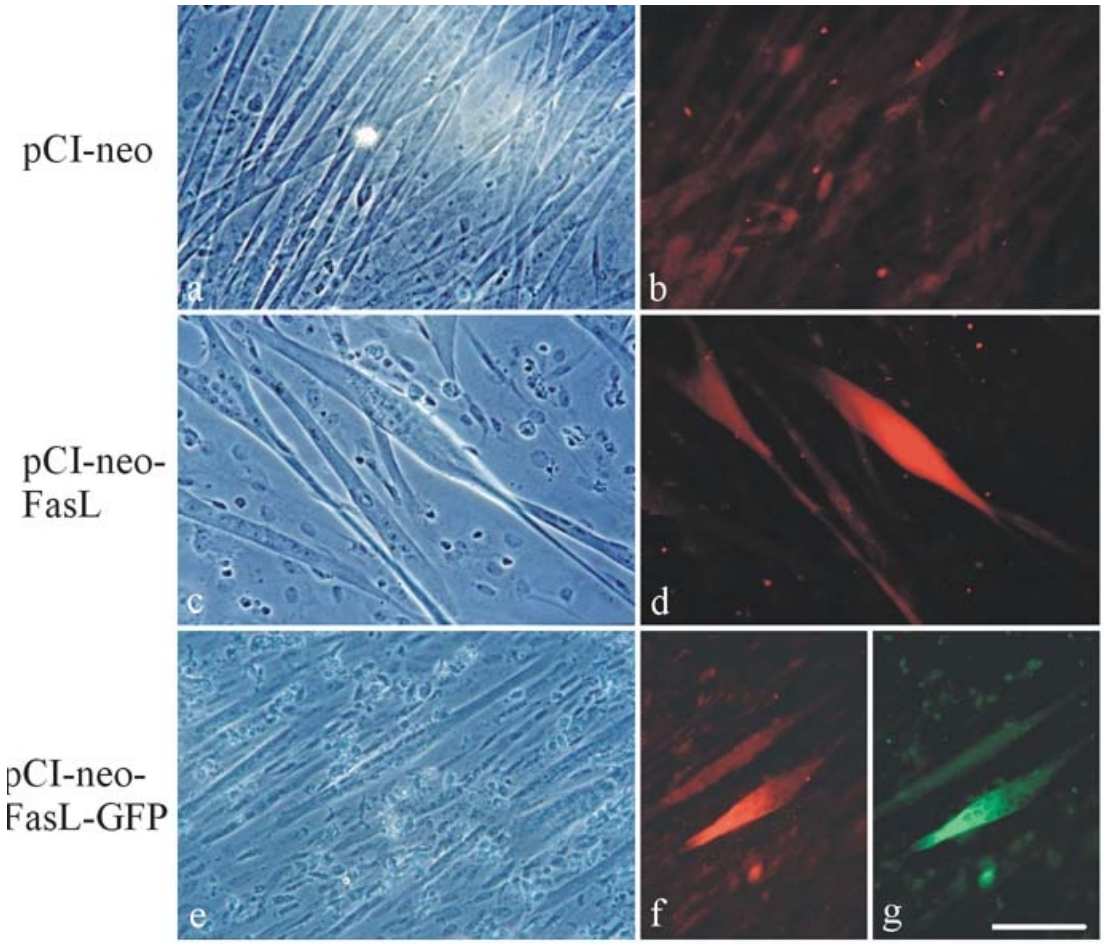

Figure 2. Primary rat muscle cells transfected and then cultured for 5 days. Transfections were carried out with pCIneo plasmid (panels a, b), pCIneoFasL plasmid (panels c, d) and pCIneoFasL-GFP plasmid (panels e, f, g). Only few myotubes, derived from fusion of transfected myoblasts escaped from FasL-induced death, show FasL expression (c, d) as compared to control (a, b). The FasL-GFP chimera is shown in panels f, g. Panels a, c, e: phase contrast; panels b, d, f: anti-FasL antibody; panel g: GFP green fluorescence. Bar 29 µm.

In vivo experiments

The aim of our *in vivo* transfections was to determine if it was possible to obtain skeletal muscle fibers engineered to express the FasL protein by means of the "naked DNA" injection technique. To this aim, we injected marcaine pre-treated TA muscles of adult rats with either a mixture of pCIneoFasL and pCMVLacZ plasmids, pCMVLacZ alone or with a mixture of pCMVLacZ and pCIneoFasL-GFP. Transfected muscles were then analyzed at different times for the expression of ß-galactosidase, FasL and GFP. As expected, one week after transfection with Lac Z alone, large blue fibers were seen throughout the regenerating muscle (Figure 3, panel a). The counter-lateral TA muscles, transfected with a mixture of LacZ and FasL plasmids, also showed positive fibers (Figure 3, panel c), but these were surrounded by an obvious cell infiltrate, typical of the inflammatory response. Two weeks later, muscles injected with LacZ alone still showed positive fibers (Figure 3, panel b), whereas in the co-transfected LacZ/FasL muscles the regenerating areas were completely infiltrated by mononucleated cells and positive ß-gal fibers were nearly absent (Figure 3, panel d and inset). The results obtained when using a mixture of LacZ and FasL-GFP plasmids confirmed the findings of the *in vitro* experiments, in that both at one and three weeks after transfection the amount of blue fibers was comparable to that obtained with the LacZ alone (Figure 3, panels e and f).

The actual expression of FasL and FasL-GFP was

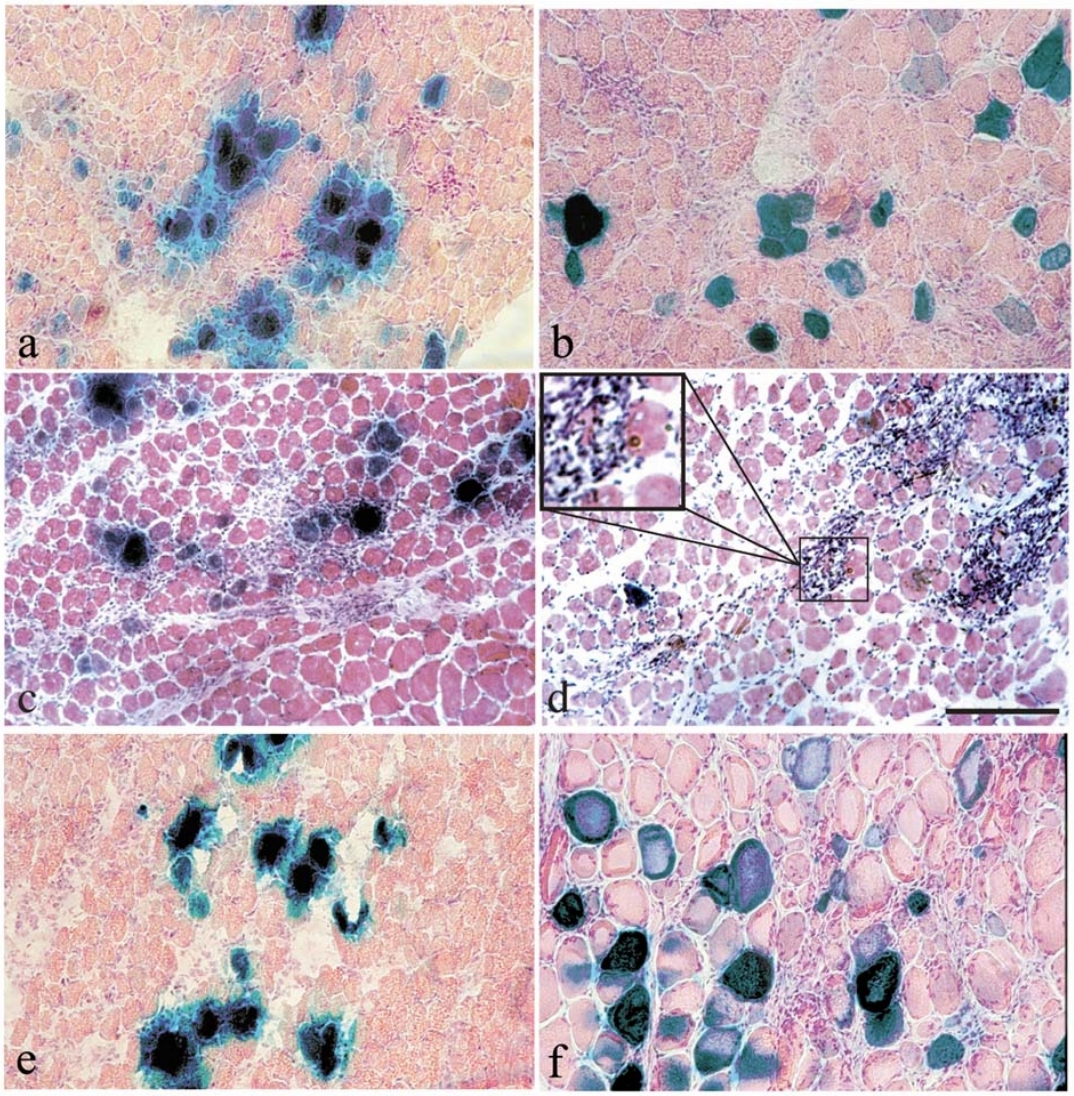

Figure 3. TA muscles transfected with: pCMVLacZ plasmid alone (panels a, b), or with a mixture of pCMVLacZ and pCIneoFasL (panels c, d). After 7 (a, c) and 15 (b, d) days muscle sections were stained for (-galactosidase activity (blue staining). The presence of leukocyte infiltrate around the positive fibers is evident in FasL-LacZ co-transfected muscles (panel c, d and insert). Bar 72 µm.

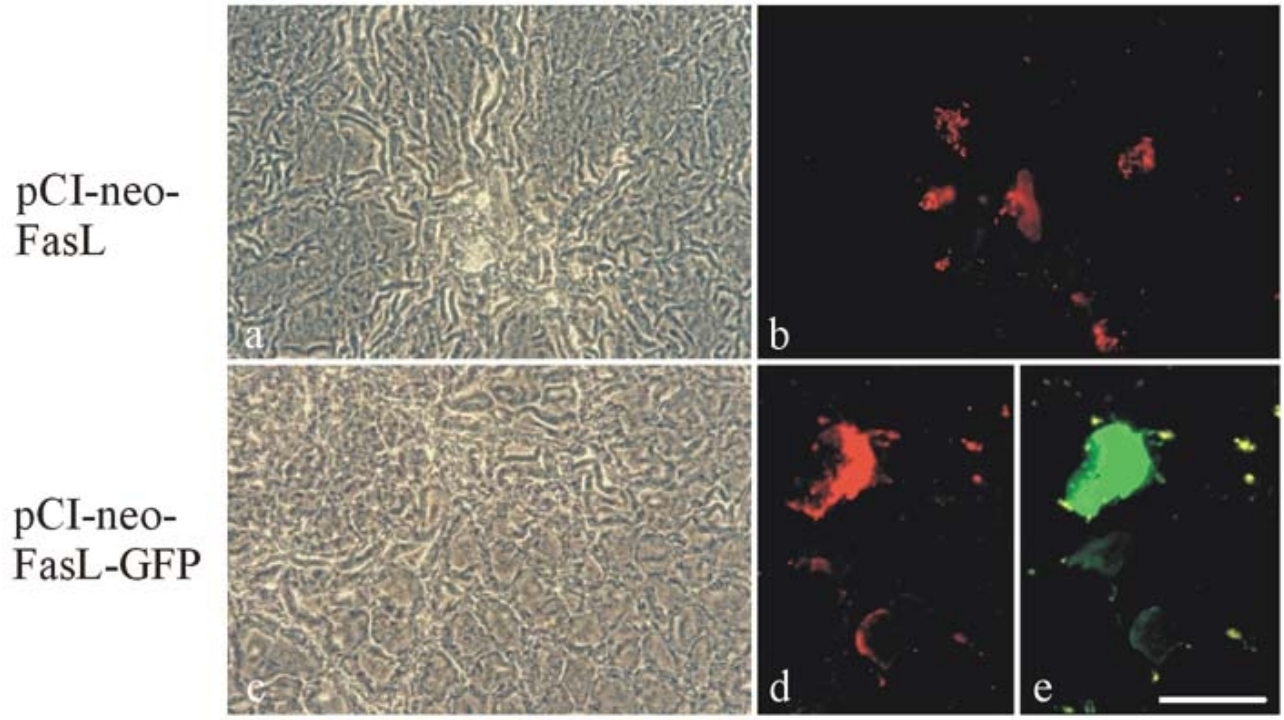

Figure 4. TA muscles transfected with: pCIneoFasL (panels a, b) and pCIneoFasL-GFP (panels c, d, e) plasmids. After 7 days muscle sections were stained with an anti-FasL antibody (panels b, d); GFP protein was revealed for the green fluorescence (panel e). Bar 29 µm.

verified by immunohistochemistry. As shown in Figure 4, seven days after transfection both FasL positive and FasL-GFP positive fibers were clearly detectable, even though FasL signal was not as strong as that of FasL-GFP. Also, the size of FasL positive fibers appeared to be smaller than those FasL-GFP positive.

Discussion

In the recent years several reports showed how Fas-FasL interaction is the key step involved in the triggering of apoptosis in several cell types, such as infected cells, tumoral cells and grafted tissue. Also, this system plays a central role in B cell regulation and in CTL-mediated cytotoxicity [5, 12, 15, 17, 18, 27]. These observations led to the hypothesis that FasL over-expression in a certain tissue could prevent the immune response against foreign proteins and/or grafted cells. Following this line of reasoning two distinct groups tried using engineered syngeneic myoblasts as a source of FasL to prevent the rejection of grafted pancreas islets. Their results were, however, quite contrasting, in that one study reported the successful production of FasL-expressing myoblasts [16] whereas the other excluded such possibility [13]. Also, we have previously shown that in established mouse myoblasts expression of FasL lead to massive cell death. In the present study we set out to define the suitability of skeletal muscle as a source of FasL, both after *in vitro* transfection of primary myoblasts and after *in vivo* direct plasmid DNA transfer in regenerating muscle.

In our hands, primary myoblasts cultures transfected with FasL-expressing plasmid displayed clear signs of sufferance. The percentage of cell death following transfection was almost twice that found in sham-transfected cells and TUNEL analysis demonstrated that the number of apoptotic cells increased by 5 fold compared to controls.

In order to better understand the role of Fas-FasL interaction in an *in vivo* model (see below), we designed a novel chimeric plasmid in which the interaction of FasL with its receptor was prevented by the presence of a GFP moiety at the C-terminal. The rationale for this approach was that published data had shown that it is FasL C-terminal region that interacts with Fas receptor [28]. The levels of cell death and apoptosis obtained in transfections carried out with the chimeric construct were comparable to those obtained in sham-transfected cells, thereby confirming that the chimeric product was not able to trigger the apoptotic cascade.

It was interesting to note that using a lower amount of pCIneoFasL plasmid for transfections, thereby decreasing the number of transfected cells, did not diminish the amount of apoptotic cells. This finding suggested that apoptosis was not directly related to the expression of FasL within each cell but, rather, that FasL secreted from the transfected cells was sufficient to also kill “bystanders” myoblasts. At the same time,

though, we observed that despite the massive cell death it was still possible to obtain FasL-positive myotubes, thereby providing a possible explanation for some of the successful experiments reported by Lau [16]. Specifically, our findings suggested that in a primary myoblast population there are some myogenic cells that, possibly due to low Fas expression, are resistant to FasL-induced apoptosis. Lau and colleagues, who cultivated their cell cultures in the presence of soluble FasL, probably selected this sub-population and therefore were able to obtain stable FasL-expressing myoblasts.

Our in vivo experiments also indicated that FasL expression was not compatible with myoblast survival. Indeed, in our hands regenerating muscles injected with a mixture of FasL and LacZ-expressing plasmids were soon invaded by an infiltrate of mononuclear cells and ß-gal positive fibers were quickly cleared out. These results are in disagreement with the hypothesis of a FasL-mediated immune protection put forward by Lau and colleagues [16] and confirm the data of Kang [13] and Turvey [31]. On the other hand, it is important to notice that in our experiments we did not encounter the acute rejection, with the characteristic massive PMN leukocyte infiltrate, described in Kang's paper. This finding is likely due to the fact that for our transfections we used naked DNA plasmid instead of a viral vector.

We therefore hypothesize that during the muscle regeneration FasL protein is responsible for the apoptosis of myoblasts (via Fas-FasL interaction) and that the consequent massive necrosis has a chemotactic effect on macrophages and lymphocytes. In this regard, it is important to notice that FasL is a secretable protein and therefore can act also on myoblasts that have not actually been transfected at all or, possibly, have been transfected only by the pCMVLacZ plasmid (see below). In is important to note that in all the experiments of co-transfections with a pCMVLacZ coding plasmid there was a noticeable difference between the number of ßgal positive fibers positive for LacZ-of and those positive for FasL-GFP, the former being by far more abundant. The reason for such apparent inconsistency likely lays in the fact that FasL-GFP protein is continuously secreted whereas the ß-galactosidase is accumulated into the cells and therefore can be more easily detected.

In summary, the early death of myoblasts caused by FasL expression in regenerating muscle prevents the formation of FasL-positive fibers. The consequent necrosis attracts macrophages and lymphocytes in the area and this, in turn, favours a more rapid disappearance of the other exogenous gene product (i.e., the ß-galactosidase produced by the pCMVLacZ plasmid). The fact that rare small FasL-positive fibers could be found in the early phases of the experiments was likely due to the presence of a small sub-population of myoblasts that, as observed in vitro, were resistant to FasL-induced apoptosis. At later stages, the "dilution" of these myoblasts into the newly formed fibers led to the disappearance of the signal with anti-FasL antibodies. Recent data, on the other hand, showed that mature myofibers are particularly resistant to FasL-mediated apoptosis thanks to high expression of caspase 8 inhibitors [11]. This considered, our results leave open the possibility that regulated FasL expression (i.e., under control of a differentiated muscle promoter) might indeed be used to create immune privileged areas in skeletal muscle. Also, bypassing the regenerating phase by introducing the DNA in adult fibers by electroporation could also lead to persistent FasL expression, provided that the procedure is be optimized to prevent muscle damage that would also lead to satellite cell-mediated regeneration.

Acknowledgments

We thank Prof Nagata (Osaka, Japan) for the FasL cDNA. This work was supported by Fondazione Telethon of Italy (grants A095 to MC and A142 to LV), and Italian MURST (grant 60% to MC). We thank Associazione Industriali Vicenza for EG fellowship.

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