Proliferating Myoblasts Transfected with FasL cDNA Can Escape from Apoptosis

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Abstract

An important but all too often neglected aspect of successful gene therapy is the attenuation or elimination of the local inflammatory response and immune reaction against transferred DNA products and cell transplantation. Expression of the FasL protein, either constitutively or after induction in certain cells, induces apoptosis when it interacts with the Fas cell-surface receptor (APO1, CD95) and the interaction Fas-FasL on T cells is thought to play a major role in the maintenance of immunological homeostasis and peripheral tolerance. Recently attention has focused on the creation of loci of immune privilege (FasL overexpressing loci) where transfected cells can be placed. During attempts to produce myotubes expressing the apoptosis-inducing FasL protein under the control of a CMV promoter, mainly myoblasts were unable to survive the initial transfection. Investigation of the presence of Fas led us to determine that the mRNA for Fas is upregulated during differentiation program of myoblasts corresponding to the peak of cell death in transfected cells. When the cultures are prolonged, some myoblasts can survive and proliferate and are able to form some FasL positive myotubes. Our findings shed light on the conflicting results obtained by two recent studies on FasL engineered myoblasts and open new perspectives regarding the possibility to induce forced expression of FasL in muscle cells.

Key words: gene therapy, myoblasts, FasL, immuno-privilege.

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Gene transfer is the basis of therapeutic strategies in many different tissues and the muscle tissue has characteristics that allow it to be used for treatment of diseases in addition to primary myopathies. The presence in the muscle tissue of a stem cell population, which is responsible for regeneration, has allowed the grafting of genetically engineered myoblasts into muscle. In addition the postmitotic nature of muscle fibres allows stable expression of transferred genes, even if they are not integrated into chromosomal DNA [4, 22, 29, 30]. For the final success of gene therapy it is important that the local inflammatory response and immune reaction against DNA products, viral proteins, cationic-lipid vectors, transplanted cells or tissues are reduced or eliminated.

It is well known that the Fas-FasL interaction is involved in immunological tolerance. The transmembrane type II Fas ligand protein (FasL, CD95L) is normally expressed on a limited number of cells such as lymphocytes, Sertoli cells, and epithelial cells in the anterior chamber of the eye [8, 18, 27]. On the contrary the Fas receptor (Fas, APO-I, CD95) is expressed on a variety of cell types such as hepatocytes, B and T lymphocytes and myoblasts [11]. The signal for cellular death, induced by the Fas-FasL interaction plays a central role in T and B cell regulation [1, 5, 31], and in CTL-mediated cytoxicity [10]. Loss of function mutations in the Fas-FasL system result in lymphoproliferation and autoimmunity [7, 20, 23, 24]. FasL expressed on the membrane of activated leukocytes induces a death signal (programmed cell death) on several cells such as infected cells, tumoral cells, and it is responsible of the rejection of grafted tissue. Fas and FasL can also be co-expressed on cells such as pluriactivated CTL [14, 17, 18, 19]. FasL overexpression in engineered tissue has been proposed to be responsible for the immunoprotection of grafted tissues [16, 28]. It has been hypothesized that the engineered tissue which overexpresses FasL would be an immunologically privileged site which protects gene

or allogeneic cell grafts [3, 6]. The presumed immunoprivilege conferred by exogenous expression of FasL in this system appeared to be similar to the naturally occurring immune protection described in the anterior chamber of the eye, in the mouse testis and in several tumors [2, 8, 9, 21, 26].

In this regard, it has been demonstrated that the testicular tissue transplanted under the kidney capsule in allogeneic mice survives indefinitely because of high expression of FasL by testicular Sertoli cells. The testis grafts from *gld* mice which lack of FasL are rejected [2]. In addition, a mixture of testis and pancreatic islets grafted into kidney also permit the survival of islets by the FasL released by testis [13]. Renal allografts transfected to overexpress FasL prolong the survival of tissue graft [28].

It has also been reported that the graft of a mixture of myoblasts overexpressing FasL and pancreatic islets into allogeneic animal regulates the T lymphocyte mediated immunoresponse by the Fas-FasL interaction. However, different groups have found completely conflicting results. Lau [16] demonstrated prolonged islet allograft survival when myoblasts overexpress FasL. On the contrary, Kang [11] observed death of engineered FasL myoblasts *in vitro* and rapid rejection of both FasL positive myoblasts and islets when grafted into animals [12]. Thus, the role of FasL is more complex than initially realised. In light of these results, we have reinvestigated this problem utilising mouse C2C12 myoblasts.

Materials and Methods

pCIneo FasL construction

The Bluescript II SK(+) plasmid containing the murine FasL cDNA was a generous gift of Professor Nagata. An expression pCIneo vector was used to clone cDNA FasL. It contains an expression cassette with the cytomegalovirus (CMV) early promoter/enhancer (Promega). The 940 base pair FasL cDNA was isolated by restriction digestion and inserted into the polylinker region via a XbaI site.

Immunoprecipitation and Western blotting

For detection of FasL, cell lysates of transfected myoblasts were prepared in radioimmunoprecipitation buffer (RIPA) (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 12 mg/ml PMSF, 30 ml/ml aprotinin, 1 mM leupeptin) and immunoprecipitated with 2 mg of rabbit polyclonal antibody anti-FasL (C178) (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A). After 1 h incubation at 4°C, 20 ml of protein A/G agarose conjugated (Santa Cruz Biotechnology) was added to each immunoprecipitate. After 12 h at 4°C, the samples were centrifuged and the pellets washed three times in PBS. The immunoprecipitates were resolved by SDS-PAGE, Western blotted and were examined with rat anti-mouse FasL monoclonal antibody (H11, Alexis Biochemicals, Switzerland). The second antibody was a peroxidase-labeled rabbit anti-rat antibody. The blots were then developed with BM Chemiluminescence Blotting Substrate POD (Boehringer Mannheim) and exposed to film for 5 min.

Muscle cell cultures

C2C12 myoblasts were obtained from the European Collection of Animal Cell Cultures (ECACC UK) and maintained in DMEM supplemented with 10% FCS. Fusion was obtained by substitution of FCS with HS 2%. The cell suspension was cultured in plastic 12 well plates at the density of $3 \cdot 10^5$ cells in F12 medium supplied with 10% FCS. For immunoblot analysis cultures were grown in 25 cm^2 plastic flasks. All cultures were added with 100 U/ml penicillin and 100 mg/ml streptomycin. For the immunocytochemistry, wells contained a glass coverslip coated with collagen.

Immunocytochemistry

Unfused (48 h) and differentiated fused muscle cell cultures (5 days) were labelled with an anti-FasL mAb (Santa Cruz, USA). An anti-mouse IgG rhodamine-labelled secondary antibody was used to reveal positive FasL cells.

Transfection of cells

C2C12 myoblasts were transfected by calcium phosphate procedure [4].

Cell viability

Cells were labelled both with Hoechst No 33258 and Propidium Iodide (PI); DNA fragmentation (apoptotic nuclei) were revealed by *in situ* nick/end labelling TUNEL (In Situ Cell Death Detection Kit-POD - Boehringer Mannheim) [25].

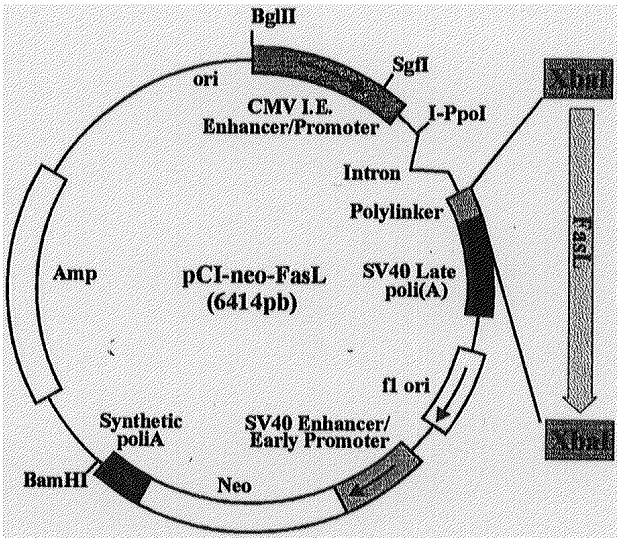

Figure 1. pCIneo FasL vector. The 940 base pair FasL cDNA was isolated by restriction digestion and inserted into the polylinker region via a XbaI site.

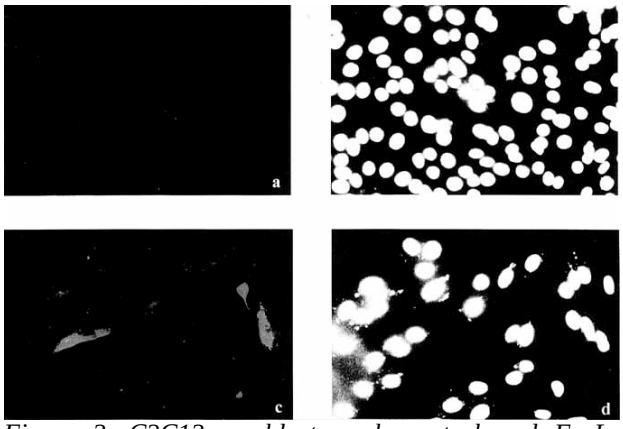

Figure 2. C2C12 myoblasts: a-b control, c-d FasL transfected cells (x400). Panel c shows some FasL + cells.

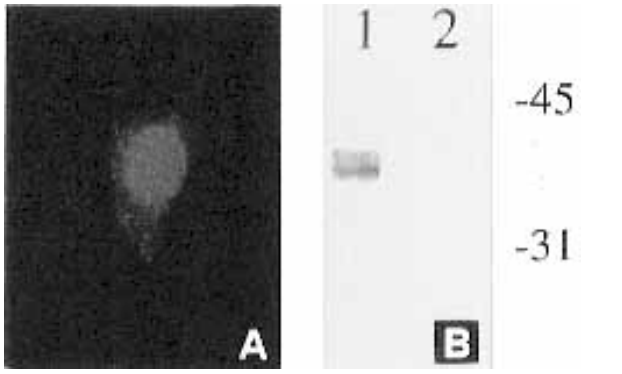

Figure 3. Immunoprecipitation and Western blotting. Panel A: FasL transfected C2C12; panel B: lane 1- transfected cells, lane 2- control cells.

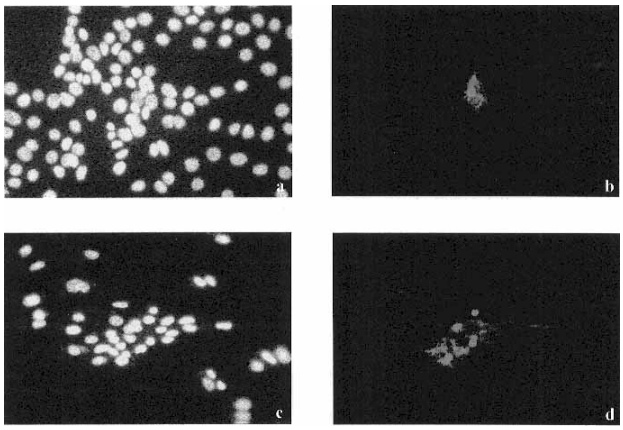

Figure 4. C2C12 myoblasts: a-b control, c-d FasL transfected cells (x400). Panels b-d show PI positive cells.

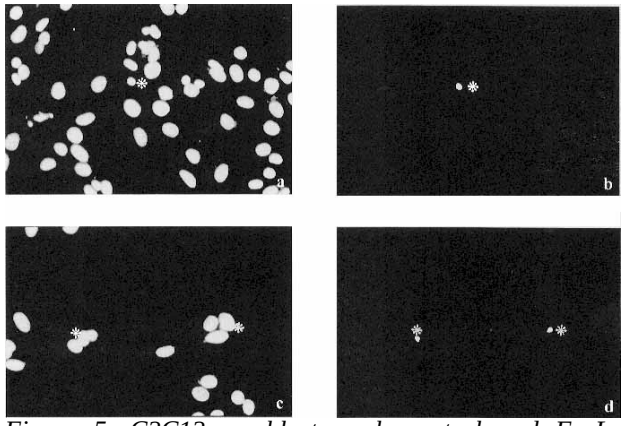

Figure 5. C2C12 myoblasts: a-b control, c-d FasL transfected cells (x400). Panels b-d show TUNEL positive cells.

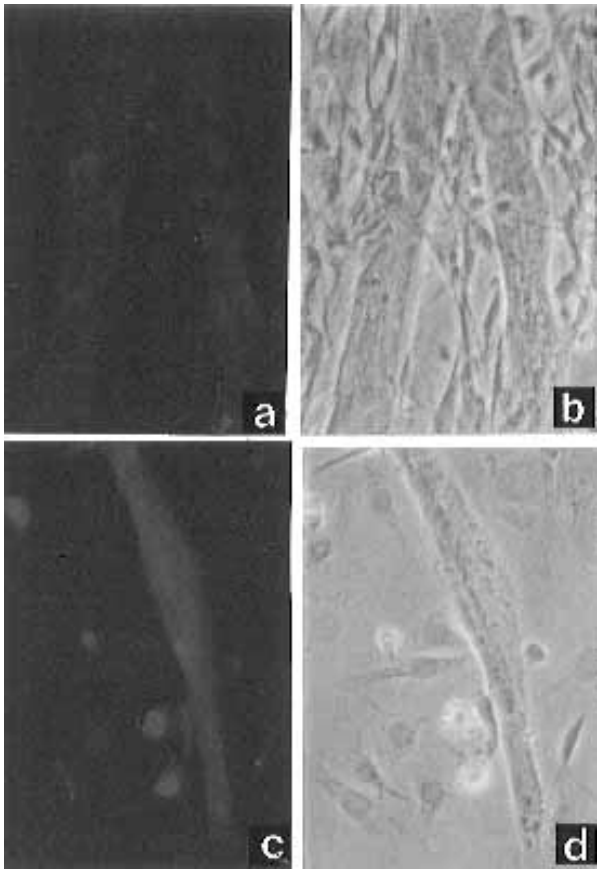

Figure 6. C2C12 myoblasts after cell fusion: a-b control, c-d transfected cells (x400). Panel c shows survived myotubes overexpressing FasL protein. Panels a and c: anti-FasL immunostaining; panels b and d: phase contrast.

Results and Discussion

C2C21 myoblasts were transfected with pCIneo FasL vector. This vector contains an expression cassette with the constitutive cytomegalovirus (CMV) early promoter/enhancer (Promega). The 940-base pair FasL cDNA was isolated by restriction digestion and inserted into the empty pCIneo did not show any death signals (fig. 5a, b). Since it is known that the death signal is related to the amount of Fas, we then monitored the expression of mRNA Fas in a control C2C12 culture. The mRNA Fas level was low during early proliferation of C2C12 myoblasts, was overexpressed just before fusion and returned to low levels in myotubes (data not shown). In

light of these results, we think that the chance of myoblast survival depends on the relative levels of expression of Fas and FasL.

To check the possibility to select Fas L resistant myoblasts, instead of adding FasL to the culture medium, we have prolonged the culture time of FasL-transfected cells and we observed in both C2C12 and primary myoblast cultures the formation of myotubes after the initial massive cell death. There is also the possibility that the survival and fusion of myoblasts to form myotubes could be related to the elimination of all FasL transfected cells during the initial phases of culturing by the induced Fas-FasL death signal. However, these surviving muscle cultures demonstrated (by staining with an anti-FasL antibody) that some myotubes express the FasL protein on the cell surface (fig. 6 c,d). This result can be compared with data obtained by Lau [16]. Since Fas and FasL can also be coexpressed in the same cell, Fas and FasL levels would play an important role for the survival of cells [24]. Indeed, as shown by Lareggina and co-workers [15], the FasL expression under the control of a regulated promoter can allow the survival of transfected FasL cells. Recently we observed that macrophages underwent apoptosis when co-cultured with proliferating myoblasts. These observations could explain the elimination of infiltrating macrophages from the site of damage by the Fas/FasL death signal by myoblasts (Sandri et al.: manuscript in preparation). Taken together the results reported here leave open the possibility that regulated FasL expression of engineered myoblasts may affect attenuation of an immune response in the context of gene therapy via myoblasts.

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