FADDdel-GFP Modified Mouse Insulinoma Cells Counteract the Cytotoxicity of Reactive T Cells

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IDDM results from pancreatic beta cell destruction by islet-reactive T cells, a process that involves beta cell apoptosis. FasL-Fas pathway plays a major role in pancreatic beta cell death. Fas-associated death domain protein (FADD), the component of the tumor necrosis factor receptor type 1 (TNF-R1) and Fas signaling complexes, is involved in TNF-R1- and Fas-induced apoptosis. Inhibiting the function of FADD will lead to blocking downstream apoptosis signal, which protects pancreatic beta cells from destruction by FasL-Fas pathway. In this study we constructed eukaryotic expressing vector of fusional protein FADDdel-GFP named pFADDdel-GFP. After pFADDdel-GFP was transfected into NIT, the expression of FADDdel-GFP in NIT was detected by fluorescence microscopy and the resistance of NIT transfected with pFADDdel-GFP to cytotoxicity mediated by special T cells was detected by FACS and MTT. The results showed that NIT modified by pFADDdel-GFP obviously resisted cytotoxicity mediated by special T cells. Therefore, it may be useful in the prevention or treatment of IDDM by intervening FasL-Fas pathway. *Cellular & Molecular Immunology*. **2004; 1(5):383-386.**

Introduction

In general, type I insulin-dependent diabetes mellitus (IDDM) is considered as the selective destruction of insulin-producing cells in the pancreas, or insulitis, it is an organ-specific autoimmune disorders resulting from a complex, T cell-dependent process directed against pancreatic β cells (1, 2). A variety of immune effector cells, including CD4^{+} T cells, CD8^{+} T cells, and macrophages, as well as cytokines and other inflammatory products of these cells, have been implicated as mediators of pancreatic islet-cell destruction in type I diabetes. Pancreatic islet infiltration by macrophages and T-cells (insulitis) is a well-recognized antecedent of cell destruction in auto-immune diabetes. IDDM results from beta cell destruction by islet-reactive T cells, a process that involves beta cell

apoptosis. Therefore, preventing islet β cells cytotoxicity mediated by autoreactive T cell may have an important role in the therapy and cure of type I diabetes. However, the mechanisms by which cells die remain undefined. Recently, attention has been directed to the interaction of FasL (CD95L) on T-cells with a Fas (CD95) receptor as a possible mechanism for cell killing in type I diabetes (3, 4). Interventions that block the Fas-FasL pathway may be useful in the prevention or treatment of type I diabetes (5, 6).

Most patients receive subcutaneous insulin injections to reduce their blood glucose levels. However, strict glucose control by multiple insulin injections is associated with an increasing risk of hypoglycemia and weight gain, while a less strict glucose control is insufficient to prevent chronic complications such as nephropathy, neuropathy and retinopathy (7). One promising avenue for the treatment of insulin-dependent diabetes is the transplantation of pancreatic islets into affected individuals. Unfortunately, immune-mediated rejection of foreign islet tissue remains a major obstacle to the success of this approach (8). The FasL-Fas pathway is important for the induction of apoptosis during rejection (9-13). FADD (Fas associated protein with death domain) is a key adapter protein in the apoptosis signal transduction pathway by death receptors such as Fas, TNFR1, DR4, et al. (14-16). If the transduction

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of downstream apoptosis signal of FADD is blocked, cell damage will be decreased, thus allograft survival will be prolonged. In this study, we modified NIT with pFADDdel-GFP and detected its resistance to cytotoxicity mediated by specific T cells. This study aims to protect modified NIT from cytotoxicity mediated by specific T cells. It is helpful for further study on the pathogenesis of IDDM and the measure of prevention and cure.

Materials and Methods

Construction and identification of pFADDdel-GFP

The plasmid pEFFLAGFADDdelN79 was presented by Australian WEH1 laboratory, which contained the gene fragment of FADDdel. The target gene FADDdel was amplified by PCR using upstream primer 5'-GGA CTG ACA GAA GCT TAT CTT TCG AAA CCA CCA TG-3' and downstream primer 5'-GAA AGT CGA CTT GGA CGC TTC GGA GGT AGA T-3' at 30 cycles of $94^{o}C$ 1 min, $56^{o}C$ 1 min, $72^{o}C$ 1 min. The PCR product digested with Hind III and Sal I was inserted into vector pEGFP-N1 containing the gene of GFP. The recombined plasmid named pFADDdel-GFP was confirmed by digestion with Hind III and Not I.

Transfection, screening and detection of FADDdel-GFP in NIT

NIT (mouse insulinoma cells) were presented by Australian WEH1 laboratory. NIT were passaged on 24-well tissue culture plates full of Dulbecco's Modified Eagale's Medium (DMEM, Sigma) supplemented with 10% fetal calf serum (Sigma). Plasmids of pFADDdel-GFP were transfected into NIT by Lipofectamine™2000 (Invitrogen). G418 (600 µg/ml, Sigma) was added into the media after transfected NIT had been cultured for 72 hours at 37°C 5% CO_2. The transient and stable expression of pFADDdel-GFP in NIT was detected by fluorescence microscopy. NIT transfected with pFADDdel-GFP were named as NITf-g. NIT transfected with pEGFP-N1 were named as NITg.

Generation of effector cells

Balb/c mouse was immunized two times with NIT (5×10^6) by abdominal cavity injection. Spleen cells from immunized mouse were stimulated with 10% NIT that treated with mitomycin C (30 µg/ml, Alexis biochemicals). They were cultured in 10% FCS RPMI 1640. Then IL-2 (interleukin 2, 50 U/ml) and Con A (concanavalin A) (5 µg/ml) were added into the media in the next day. After 6 days, cells were collected and washed 3 times, which were used as effector cells.

Fas expression induced

Cytokine-treated NIT, NITg, NITf-g expressing Fas were used as target cells. Cells were cultured for 72 h in 10% FCS DMEM containing mIFN-γ (1000 U/ml, Peprotech EC LTD), mIL-1β (50 U/ml, Peprotech EC LTD) or both (1,000 U/ml and 50 U/ml, respectively).

Flow cytometric analysis

After washed twice with RPMI 1640, effector cells and
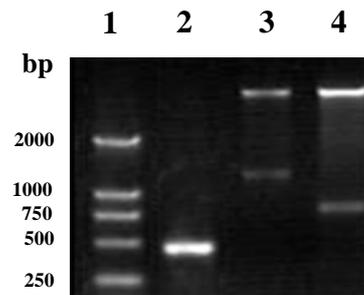
Figure 1. Identification of the recombinant pFADDdel-GFP. Lane 1, DNA marker; lane 2, PCR products of FADDdel; lane 3, pFADDdel-GFP digested with Hind III and Not I (The small fragment is 1235 bp); lane 4, pEGFP-N1 digested with Hind III and Not I (the small fragment is 763 bp). It was proved that pFADDdel-GFP contained FADDdel which is 472 bp. The recombinant pFADDdel-GFP was successfully constructed.

target cells were incubated in complete media at the (E: T) ratio of 10:1 for 4 h at 37°C 5% CO_2. Then the cultured cells were stained with PI (5 µg/ml) for 30 min at 4°C. After washed with PBS, the cells were analyzed by a flow cytometer.

MTT assays

After the cytokine treated target cells were incubated at 1×10^4 per well in 96-well culture plate for 24 h, the effector cells were added to each well in triplicate at the (E:T) ratio of 10:1 for 4 h. At the mean time, two control groups were established: target cells control and effector cells control. Cytotoxicity was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylerazolium bromide (MTT) (Gibco). The cytotoxicity was calculated as follows: Cytotoxicity (%) = (1- experimental groups'OD - effector cells control's OD)/ target cells control's OD × 100%.

Statistical analysis

Statistical analysis was performed with t test and values of $p < 0.05$ were considered significant.

Results

Construction of pFADDdel-GFP

The recombinant pFADDdel-GFP and pEGFP-N1 were digested with Hind III/Not I respectively. As shown in Figure 1, it proved that the plasmid pFADDdel-GFP was successfully constructed.

Expression of FADDdel-GFP in NIT

NITf-g and NIT without transfection were detected by fluorescence microscopy. Figure 2A showed that there was green fluorescence in cytoplasm of NITf-g. Figure 2B showed there was no green fluorescence in cytoplasm of NIT without transfection. It proved that FADDdel-GFP were successfully expressed in NITf-g.

NITf-g resistant to cytotoxicity of specific T effector cells

Specific T effector cells were obtained by immunized mice *in vivo* and mixing culture of lymphocyte and NIT *in vitro*.
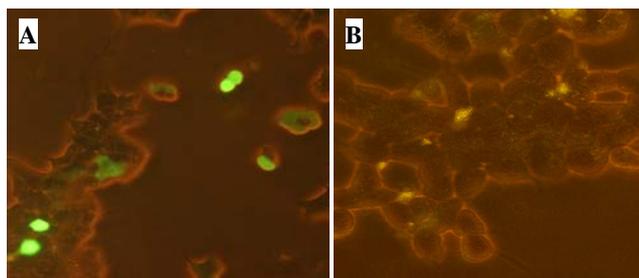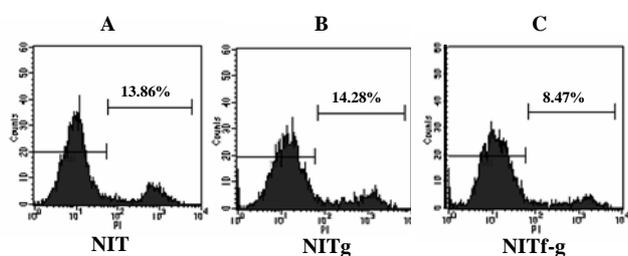
Figure 2. Expression of FADDdel-GFP in NIT. There were green fluorescence in cytoplasm of NIT transfected with pFADDdel-GFP (Figure 2A) and there were no green fluorescence in NIT without transfection (Figure 2B). It was proved that FADDdel-GFP was successfully expressed in NIT transfected with pFADDdel-GFP.

Figure 4. Different cytotoxicity mediated by specific T cells was detected with MTT. The cytotoxicity of NITf-g group is weaker than the other two groups. The cytotoxicity between NIT and NITf-g was of significant difference ($p < 0.05$) and so was it between NITg and NITf-g ($p < 0.05$), but there were no significant differences in the cytotoxicity between NIT and NITg ($p > 0.05$). It was proved that NITf-g could resist cytotoxicity mediated by specific T cells.
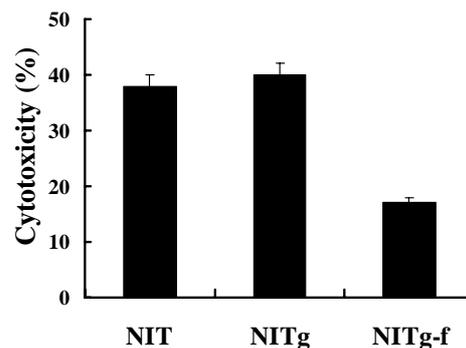
Figure 3 showed the specific T-mediated cytotoxicity detected by FACS. Figure 3A showed the percentage of lysed target cells in NIT was 13.86%. Figure 3B showed the percentage of lysed target cells in NITg was 14.28%. Figure 3C showed the percentage of lysed target cells in NITf-g was 8.47%. The results showed the percentage of lysed target cells in NITf-g was less than that in NIT and NITg. There were no significant differences in the percentage of lysed target cells between NIT and NITg. Figure 4 showed the specific T-mediated cytotoxicity detected by MTT. The results indicated the cytotoxicity of NITf-g group was the weakest among the 3 groups. Cytotoxicity assays revealed that transfectants NITf-g could resist cyotoxicity mediated by specific T effector cells. NIT or NITf-g as target cells showed different susceptibility to T cell-mediated cytotoxicity ($p < 0.05$), and so was it between NITg and NITf-g, but there were no significant differences in the cytotoxicity between NIT and NITg ($p > 0.05$).

Discussion

IDDM is thought to be an autoimmune insulitis caused by massive cytotoxic T cell infiltration. Evidence suggests that pancreatic β-cell destruction is caused by autoreactive

Figure 3. Susceptibilities of target cells to T cell-mediated cytotoxicity were detected by FACS. Target cells and T cells were co-cultured for 4 h, then stained with PI and analyzed by flow cytometry to determine cytotoxicity. The numbers above the bars indicated the percentage of lysed cells. The percentage of lysed target cells in NITf-g (C) was less than that in NIT (A) and NITg (B).

T-cells (17-19). How to protect pancreatic β-cell including tansplanted pancreatic β-cell from destructing is a deserving problem. In this study, we modified NIT with FADDdel-GFP and suppressed intracellular apoptosis signal transduction through Fas/FasL, which resist specific T mediated cytolytic effect.

Fas expression on β-cells plays an important role in the development of spontaneous autoimmune diabetes. Fas expression was detected selectively on diabetic pancreatic β-cells but not on β-cells in normal human pancreas. Also, apoptosis was detected in the Fas$^+$ β-cells located close to the FasL$^+$ T-cells infiltrating the islets (20). Induction of Fas receptor expression on β-cells by cytokines may be the mechanism that permits β-cell destruction by Fas ligand-expressing cells in the islets (21). FADD is best known as an intracellular factor that, upon ligand binding, is recruited to the cytoplasmic domain of Fas, a member of the TNF receptor (TNFR) superfamily (22). Fas and FADD associated through a conserved motif known as the death domain (DD), which is found in both the Fas cytoplasmic tail and the FADD protein (21). FADD can recruit the initiator caspase-8 to form the death-inducing signaling complex (DISC) through a second element known as the death effector domain (DED), presented in FADD and in the prodomain of the caspase (23). Therefore, the formation of DISC is important in conduction of intracellular apoptosis signal. If DED of FADD is deleted, the formation of DISC will be interfered and the downstream apoptosis signal will be suppressed.

NOD mice spontaneously develop IDDM which resembles human IDDM (24, 25). An insulinoma cell line, NIT, having the nonobese diabetic (NOD) genotype, can transfer of culture *in vitro* and secrete insulin. So we took NIT as the cell model of IDDM study. It was proved that GFP report gene did not influence the function of cells. FADDdel and GFP were fused, which made it easier to detect the expression of FADDdel. And the function of FADDdel was not influenced. Because the amino acid sequence and stereo structure of DD in FADDdel-GFP had

no change, FADDdel-GFP in NIT could competitively bind to DD of Fas with normal FADD in cytoplasm, which competitively suppressed the function of normal FADD. So T cell mediated apoptosis was effectively inhibited. In our study, we constructed successfully eukaryotic expressing vector of fusional protein FADDdel-GFP and transfected FADDdel-GFP into NIT. The ability of resistance of NIT transfected with FADDdel-GFP to T cell mediated apoptosis was reinforced and the lysed cells decreased obviously. This study will help to explain why physiologic mechanisms of immune protection become subverted to produce IDDM, and hopefully lead to better ways to treat IDDM.

We also observed that NIT transfected with FADDdel-GFP could not block T mediated cytotoxicity completely, as there existed other cellular damage mechanisms such as perforin and granzyme. Thus, suppressing Fas/FasL method cannot completely prevent the development of apoptosis. Although it was shown that NIT transfected with Wee1 gene could resist to T-mediated cytotoxicity in our study, it couldn't block cell damage absolutely (26). Damage of islet cells is due to multiple factors. Therefore, we should protect islet cells in several ways, which may be more promising way to treatment of diabetes.

Acknowledgements

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