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1,25-dihydroxyvitamin D_3 protects human pancreatic islets against cytokine-induced apoptosis via down-regulation of the fas receptor

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Beta cell loss occurs at the onset of type 1 diabetes and after islet graft. It results from the dysfunction and destruction of beta cells mainly achieved by apoptosis. One of the mediators believed to be involved in beta cell apoptosis is Fas, a transmembrane cell surface receptor transducing an apoptotic death signal and contributing to the pathogenesis of several autoimmune diseases. Fas expression is particularly induced in beta cells by inflammatory cytokines secreted by islet-infiltrating mononuclear cells and makes cells susceptible to apoptosis by interaction with Fas-ligand expressing cells. We have previously demonstrated that 1,25 $(OH)_2$ D_3, the active metabolite of vitamin D, known to exhibit immunomodulatory properties and prevent the development of type 1 diabetes in NOD mice, is efficient against apoptosis induced by cytokines in human pancreatic islets in vitro. The effects were mainly mediated by the inactivation of NF-kappa-B. In this study we demonstrated that 1,25 $(OH)_2$ D_3 was also able to counteract cytokine-induced Fas expression in human islets both at the mRNA and protein levels. These results were reinforced by our microarray analysis highlighting the beneficial effects of 1,25 $(OH)_2$ D_3 on death signals induced by Fas activation. Our results provides additional evidence that 1,25 $(OH)_2$ D_3 may be an interesting tool to help prevent the onset of type 1 diabetes and improve islet graft survival.

Key words: apoptosis; Fas regulation; human pancreatic islets; Vitamin D_3.

Introduction

Although islet transplantation is becoming an acceptable alternative for the treatment of type 1 diabetes, it is currently limited by the shortage of donors and the substantial dysfunction and destruction of islets. Apoptosis is the main cause of this destruction and occurs during the first few days of transplantation.[1,2]

One of the mediators believed to be involved in beta-cell apoptosis is Fas (Apo-1, CD95, TNFRSF6), a 45 KDa transmembrane cell surface receptor and a member of the tumor necrosis factor (TNF) receptor family.[3] In the last few years, a great deal of attention has been paid to the role of Fas and its ligand Fas-L, in the regulation of immune responses.[4] The Fas/Fas-L system contributes to the pathogenesis of several organ-specific autoimmune diseases through effector cells that cause direct damage via activation of the Fas apoptotic pathway.[5]

Normal human pancreatic beta-cells do not express Fas,[6] unless they undergo cytokine treatment.[7,8] Beta cells from patients with recent onset type 1 diabetes also express Fas on their surface[9] and are thus susceptible to Fas-L-induced apoptosis mediated by tissue-infiltrating Fas-L-positive T lymphocytes[5,10] and possibly by Fas-L-expressing beta cells themselves.[11]

At a higher level of activation and fine regulation during an immune response, the activated Fas receptor can induce phenotypical and functional maturation of dendritic cells, secretion of proinflammatory cytokines such as IL-1β and TNF-α and preferential T cell polarization into a Thl phenotype.[12]

1,25$(OH)_2D_3$ is a secosteroïd hormone that activates the nuclear vitamin D_3 receptor (VDR). The immunosuppressive activities of 1,25 $(OH)_2$ D_3 have been well studied in different models of autoimmune disease and in experimental organ transplantation[13,14] which demonstrated that Fas-L gene expression was down-regulated in T lymphocytes.[15]

We have previously shown the efficiency of 1,25 $(OH)_2$ D_3 against apoptosis induced by cytokines *in vitro* in human pancreatic islets.[16] The effects were mainly mediated by the induction of the antiapoptotic A20 protein expression and the inactivation of the transcription factor NF-kappa-B. In this study, we focused on the expression of Fas as an additional target of the 1,25 $(OH)_2D_3$ anti-apoptotic effect.

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Materials and methods

Human islet processing

Human pancreases (mean age: 41 ± 3 years, $n = 1$) were harvested from adult brain-dead donors in accord with French Regulations and with the local Institutional Ethical Committee. Pancreatic islets were isolated after ductal distension of the pancreas and digestion of the tissue with liberase (Roche Molecular Biochemicals, Mannheim, Germany) as described in.[17] Islet number was determined on samples of each preparation after dithizone staining and expressed as number of equivalents to islets with a 150 μm diameter (IE). Semi-purified islets were cultured for 24 h in CMRL 1066 medium containing 0.625% human serum albumin (Gibco BRL, Life Technologies, Cergy Pontoise, France). Human beta cell processing consisted of a 48 h treatment at 37°C with cytokines: IL-lβ (50 lU/ml) + IFNy (1000 lU/ml) + TNFα (1000 lU/ml), (Valbiotech, AbCys, Paris, France) with or without 1,25-$(OH)_2D_3$ used at physiological (10^{-8} M) and pharmacological (10^{-6} M) concentrations as described in.[17] (1,25-$(OH)_2D_3$ was a generous gift from Hoffman La Roche, AG, Basel, Switzerland).

Nitrite determination

Pancreatic islets were incubated with 1,25 $(OH)_2D_3$ with or without cytokines for 48 h. Culture media were collected for nitrite determination. Nitrite assay is based on the reaction of nitrite with 2,3-diaminonaphtalene (Sigma-Aldrich chemicals) to form the fluorescent product 1-(H)-naphtotriazole as previously described.[18]

Nuclear chromatin staining

Bisbenzimide (Hoechst 33342, 10 μg/ml, Calbiochem) which enters cells with intact or damaged membranes and stains DNA was used to detect differences between normal and apoptotic nuclei in human islet cells. After stimulation with cytokines ± 1,25-$(OH)_2D_3$, human islets were distended with trypsin-EDTA as described in[16] and cytocentrifugated at 700 rpm before being stained. Cells were examined by fluorescence microscopy (excitation/emission wavelengths 355–465 nm). Apoptotic cells, identified by the presence of condensed or fragmented nuclei, were estimated by differential counting of 300–400 cells in each experimental condition.

Cell lysis and western blotting

After being stimulated, human islet cells were lysed for 30 min on ice in 50 μl of lysis buffer (20 mM tris acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM beta-glycerophosphate, 1 mM dithiothreitol, 1 mM benzamidine, and 4 μg/ml leupeptin, all reagents were from Fluka, Sigma). The detergent-insoluble material was pelleted by centrifugation at 15000 rpm for 5 min at 4°C. The supernatants containing whole cell lysate were either immediately used for Western blotting or stored at −80°C.

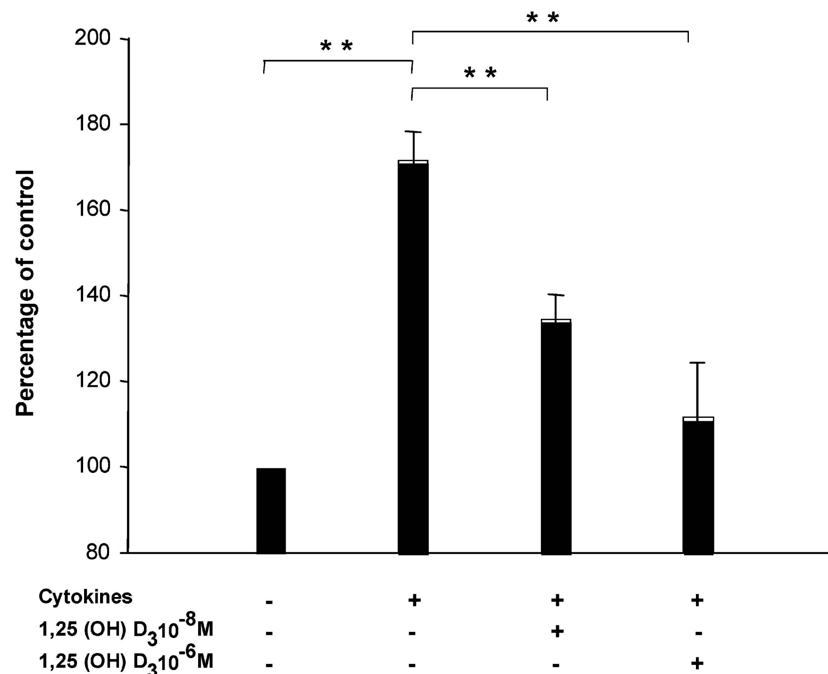
For blotting, 20 μl (50 μg) of protein were added to 20 μl SDS sample buffer (125 mM Tris-HC1, pH 6.8, 25% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue) and boiled for 5 min. SDS-PAGE (10%) was performed, and Western blotting carried out according to standard protocols.[19] Rabbit polyclonal antibody to Fas, 1:250 dilution, (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used. Western blot detection was achieved using the enhanced chemoluminescence plus reagent (ECLplus™, Amersham Pharmacia Biotech, Orsay, France).

Analysis of Fas mRNA expression by quantitative RT-PCR

Samples of 2000 to 3000 islets per islet preparation were distributed over the various conditions. After 48 h of culture, total RNA was extracted with the Nucleospin® RNA II kit (Macherey Nagel, Hoerdt, France) including DNase treatment and quantified with the RiboGreen® RNA Quantification kit (Molecular Probes). First strand cDNA was synthesised from 1 μg RNA using M-MLV (Gibco BRL) primed with random hexamers (Roche Diagnostics), in an equal volume for each islet preparation sample. The amplification was achieved with specific primers of the target sequence of Fas: sense 5-CAC AGA CCA CCT GCT TCT GA-3, antisense 5-TCC GTC GTG GAG TAA CAG TG-3 and carried out in a 25 μl volume of Ix SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) containing 300 nmol/1 of primers and 3 μl cDNA ten fold diluted after reverse transcription. After denaturation for 10 min at 95°C, solutions underwent 40 cycles of amplification in an ABI PRISM 7700 Sequence Detection System (PE-Biosystems, France). Amplification parameters included 30-s denaturation at 94°C and a 1-min annealing step at 60°C. Detection of PCR products was monitored at each cycle by measuring the increase in fluorescence caused by the binding of SYBR® Green to double strand DNA. The threshold cycle (Ct) reflects the point at which a sufficient number of amplicons have accumulated to be statistically different from the baseline.

Primers were designed using the software Primer Express® (Applied Biosystems). At least 3 couples of primers were tested for efficiency. Primers were selected only when their efficiency, calculated as described by the manufacturer, exceeded 95%. Samples of islet preparations in each condition were amplified in 96 well plates with Fas primers and β-actin and 18S as internal standards. As described by

Figure 1. Nitrite released by islet cells during 48 h of treatment was assessed by fluorometric assays as described in Materials and Methods. Values are expressed as percentage of control cells. Data are means ± SE of 11 different experiments. **p <0.01.



the manufacturer, quantification requires the determination of the mean Ct value of the replicate wells for each sample, the difference between the mean Ct values of the samples in the gene of interest and those of the internal standards (ΔCt), the difference between the mean ΔCt values of the treated samples and the mean ACt value of the non-treated (control) sample (ΔΔCt). The relative quantitation value is expressed as $2^{-\Delta\Delta Ct}$.

Expression profiling by cDNA microarrays

Glass microarray preparation. The oligonucleotides used in this study were 60 mers provided by Sigma Genosys. Each 60 mer probe was designed with a bias towards the 3′ end of the gene. The sequence of each probe was optimized using the search program Basic Local Alignment Search Tool (BLAST) by selecting the region of maximal specificity to the target gene while minimizing cross-hybridization to other genes.

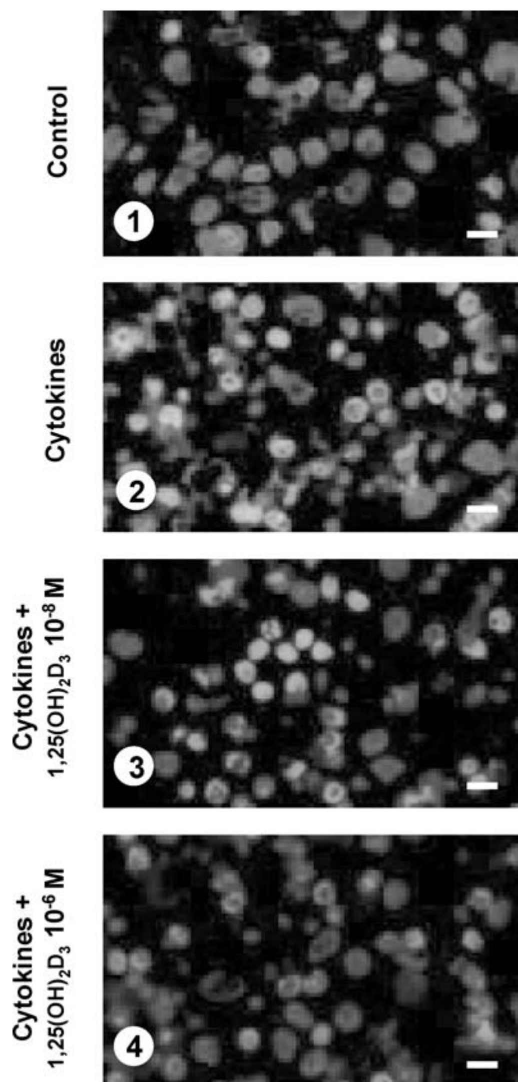
Each oligonucleotide consisted in a lyophilized powder (200 nmoles) in large enough quantities to print at least 1000 slides. Probes were printed on γ-aminopropyl silane-coated glass slides (Corning) with a VersArray ChipWriter Pro system arrayer (Bio-Rad, Marnes-la-Coquette, France). Each gene was spotted in triplicate (50 fmoles/spot) from a 50 μM solution in 3x saline sodium citrate buffer (SSC, 0.3 M sodium citrate, 3 M NaCl pH 7). There were 1973 elements on the apoptosis cDNA microarray including growth and transcription factors, cell death, cell cycle, oxidative stress and signal transduction proteins. Information about the pancreatic cDNA microarray is available at http://www.sigma-genosys.com/gea.asp

Preparation of RNA. Total RNA was extracted from control and treated (cytokines ± 1,25 $(OH)_2$ D_3) human pancreatic islets for 48 h. Cells were lysed in a 1% β-mercaptoethanol-containing buffer obtained from an RNA extraction kit (Macherey Nagel, Hoerdt, France); RNA was extracted as described by the manufacturer. The quality of total RNA was assessed with the Agilent RNA 6000 chips coupled with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California), by visualizing the 18S and 28S ribosomal ribonucleic acid (rRNA). RNA used in this study met the minimum requirement of at least a 1.8 ratio of 18S:28S rRNA. The RNA aliquots were stored at −80°C before use.

RNA labeling. The cDNAs were synthesized from 5 μg of DNA-free total RNA previously incubated for 10 min at 65°C with 1,5 μl hexanucleotide mix l0x (Roche Diagnostics). Reverse transcription (RT) was performed at 42°C for 1 h by adding 200 U reverse transcriptase (Superscript II, Invitrogen, Cergy-Pontoise, France) plus 2.5 mM of each dNTP (Amersham Pharmacia, Saint Quentin Yvelines, France). cDNAs were labeled by incorporation of 1.5 μl Cy-5-dUTP or Cy-3-dUTP (25 nM each, Amersham Pharmacia). At the end of the RT, 0.5 μl of reverse trancriptase was added for a new heating cycle of 45 min at 42°C. The final step was performed at 66°C for 30 min by adding 7.5 μl NaOH/EDTA (0,1 N/2 mM) that was finally inactivated with 7.5 μl HC10. IN.

Labeled cDNAs were purified through a centrifugal filter (Microcon YM-30, Millipore Ltd, Watford, U.K).

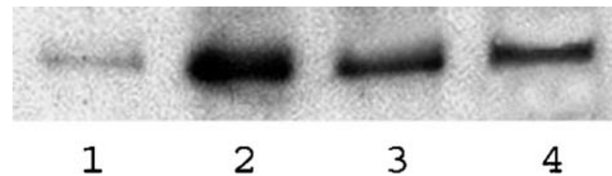
Figure 2. Micrographs showing the effects of 1,25 $(OH)_2$ D_3 on cytokine-treated human islet cells. Human islet cells that had been exposed to cytokines ± 1,25 $(OH)_2$ D_3 for 6 days were distended and Hoechst-stained as described in Materials and Methods. Apoptotic cells characterized by condensed or fragmented nuclei, were counted. Percentages of apoptotic cells are the means ± SE of 3 independent experiments assessed on 300–400 cells per condition : (1) control: 17 ± 4.2%; (2) cytokines : 48.1 ± 8.2%, $p<0.01$ versus control; (3) cytokines + 1,25$(OH)_2D_3(10^{-8}$ M): 31 ± 2.5%, $p<0.05$ versus cytokines; (4) cytokines + 1,25 $(OH)_2$ $D_3(10^{-6}$ M): 22.8±4.5%, $p<0.01$ versus cytokines. Scale bar : 15μm.



cDNAs were diluted in a final volume of 500 μ1 Tris/EDTA (TE) buffer. Samples were centrifugated for 12 min at 11000 rpm and washed 2 times in 450 μ1 TE buffer. The final elution step consisted in a centrifugation of 20 sec at 3000 rpm. Samples were stored at minus20°C before use.

Prehybridization. Microarrays were first incubated for 30 min at 42°C in the prehybridization solution (Chip Spread, Ventana Medical Systems Inc, Tucson, USA), rinsed several times

Figure 3. Analysis of Fas expression in human islet cells. Typical example of Western blot analysis performed on islet cell lysates from cells treated for 48 h with cytokines ± 1,25 $(OH)_2$ D_3 as described in Materials and Methods. Fas appears as a 45-kDa protein band. The figure is representative of 4 independent experiments. Band intensities of treated cells were corrected with respect to values of control cells (1) arbitrarily set at 1, (2) cytokines : 5.1 ± 0.65, $p < 0.01$ versus control; (3) cytokines +1,25 $(OH)_2$ D_3 $(10^{-8}$ M) : 3.2± 0.36, $p < 0.01$ versus cytokines, (4) cytokines + 1,25 $(OH)_2D_3(10^{-6}$ M) : 2.8 ± 0.06, $p<0.01$ versus cytokines.



at room temperature then incubated for 10 min at 70°C in Chip Prep 1 solution and for 30 min at room temperature in Chip Prep 2. Samples were laid on slides for a denaturation step of 2 min at 37°C followed by a second step of 2 min at 45°C. Slides were then ready for hybridization.

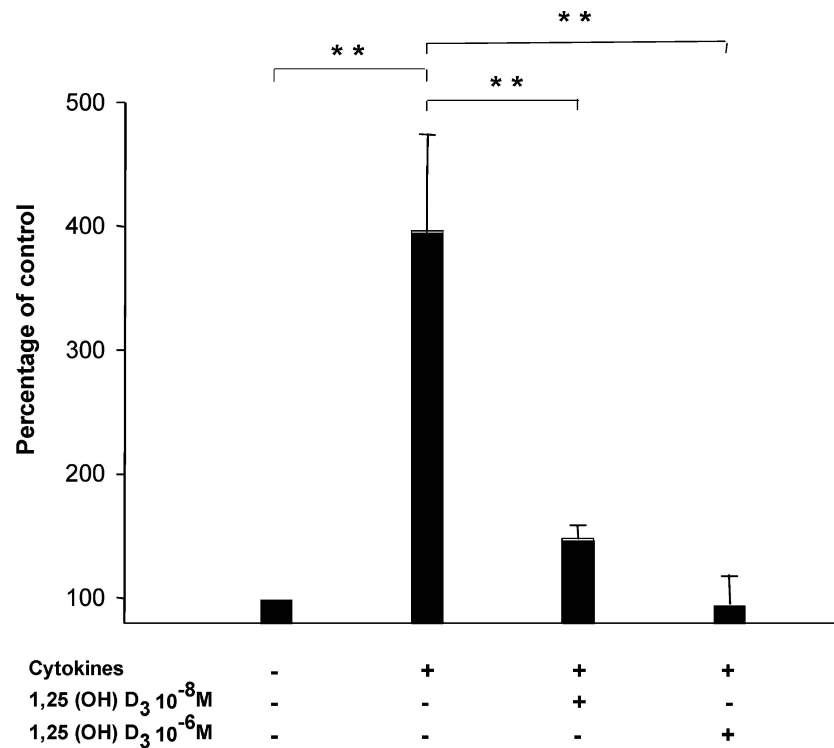
Hybridization. Before hybridization on pancreatic cDNA microarray, Cy-3- and Cy-5-labeled cDNA were combined with 200 μ1 hybridization solution (Ventana Medical Systems) and denatured for 5 min at 95°C.

Hybridization and cleaning were achieved according to an automated method using a Ventana Discovery ™ system (Ventana Medical Systems). Slides were hybridized for 8 h at 45°C then washed several times as described in the Ventana protocol which includes posthybridization washes at room temperature in 2x SSC. At the end of the hybridization step, slides were first manually washed at room temperature in the reaction buffer (l0x SSPE, saline sodium phosphate EDTA, buffer, Ventana Medical Systems) for 1 min, then for 1 min in deionized water and finally in ethanol 95° for 1 min. Slides were then dried by centrifugation in a 50 ml conical tube for 6 min at 3000 rpm in an Eppendorf Centrifuge 5810R.

Scanning and image analysis. Following hybridization and washings, slides were immediately scanned at a resolution of 20 μm per pixel using an Affymetrix 428 scanner (Affymetrix Ltd, High Wycombe, U.K). The resulting 16-bit TIFF images were analyzed with the Jaguar® software which is provided with the scanner.

Data processing and normalization. Comparative measurements of transcript abundance were performed. Time-course samples were analyzed by directly comparing the abundance of each gene's transcript relative to the control sample of the same pancreas. RNA samples were labeled with Cy-5, and RNA from the reference (Panc-1 cells, ATCC, Manassas, VA, USA) was labeled with Cy-3. Panc-1 is a cell line originated from a human pancreas carcinoma and was used for normalization between microarrays.

Figure 4. Analysis of Fas expression in human islet cells assessed by quantitative real-time RT-PCR after 48 h of stimulation with cytokines ± 1,25 $(OH)_2$ D_3 as described in Materials and Methods. Values of treated cells were corrected with respect to values of control cells arbitrarily set at 100, they are the means ±SE of 4 different experiments, **p < 0.01.



Statistical analysis

Data analyses were performed using Statview (Abacus Concepts Inc, Berkeley, CA, USA). Protocols were tested in islet cells from different donors. The stastitical significance between means was assessed by ANOVA followed by multiple t tests using the Fisher's least significant difference tests. Data were presented as means ± SE.

Results

Control of 1,25 $(OH)_2$ D_3 effect on nitrite secretion and apoptosis in cytokine-stimulated human islet cells

The efficiency of the different treatments was checked by their effects on nitrite secretions. The results depicted in Figure 1 confirmed our previous data[17] i.e. nitrite secretions increased significantly upon cytokine treatment but were reverted back towards normal values by 1,25 $(OH)_2$ D_3 cotreatment. Likewise as previously described,[16] cell Hoechst-staining confirmed the effect of cytokines on apoptosis i.e. chromatin condensation aggregating peripherally under the nuclear membrane while a cotreatment with 1,25 $(OH)_2D_3$ led to a return towards control features (Figure 2).

Effect of 1,25 $(OH)_2$ D_3 on cytokine-induced Fas expression

Fas protein expression: Western Blot analysis. Isolated human islets expressed barely detectable levels of Fas. However, clear immunoreactivity with anti-Fas antibody was observed after cytokine treatment, once again 1,25 $(OH)_2$ D_3 cotreatment resulted in marked attenuated effects (Figure 3).

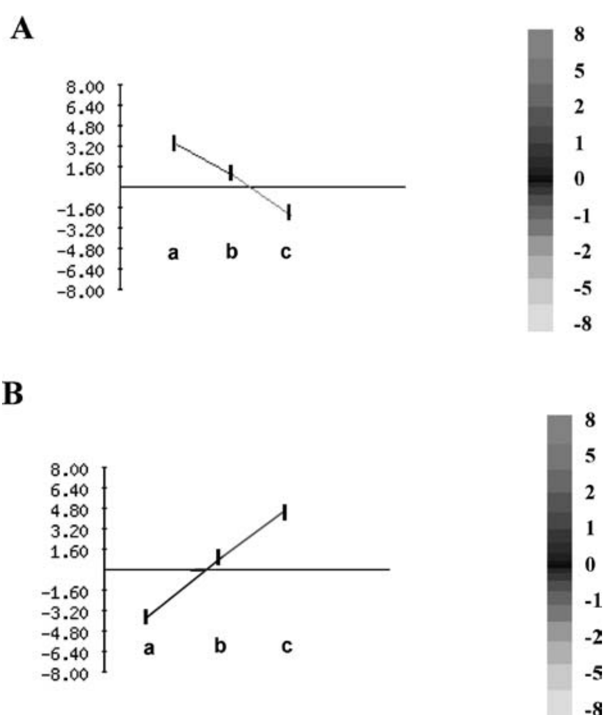
Fas gene expression: quantitative RT-PCR analysis. Quantitative real time gene expression analysis indicated that Fas gene expression was enhanced in human islet cells by cytokines. Addition of 1,25 $(OH)_2$ D_3 counteracted cytokine effects (Figure 4).

Effect of 1,25 $(OH)_2$ D_3 on cytokine-regulated gene expression

In order to place Fas in relation to its gene network, we extended the study with an analyse of the genes modulated by cytokine ± 1,25 $(OH)_2$ D_3 treatment on high-density oligonucleotide arrays.

Gene expression analysis. Our "apoptosis" array contained 1730 individual triple-spotted genes. The background comprised ≤1.2% of the total signal for each array whereas intensities for spot values covered a 50 fold range from 1.5 to 80%.

Figure 5. Genes regulated by cytokines and exhibiting a dose-dependent reverse expression by 1,25 $(OH)_2$ D_3 cotreatment as detected by cDNA microarrays. A: a cluster of 413 genes up-regulated by cytokines (a) and reverted by the addition of 1,25 $(OH)_2$ $D_3 10^{-8}$M (b) or 1,25 $(OH)_2$ $D_3 10^{-6}$M (c). B : a cluster of 131 genes down-regulated by cytokines (a) and reverted by the addition of 1,25 $(OH)_2$ D_3 10^{-8} M (b) or 1,25 $(OH)_2$ D_3 10^{-6} M (c). The data are expressed as the Log2 ratio (Cy5 intensity of treated cells/Cy5 intensity of control cells) of median intensity data for each condition. The list of genes of clusters A and B can be seen at http://www.univ-line2.fr/ilots/calcitriol%20study/calcitriol.htm.



Spot Cy5 intensities from islets cells treated or not with cytokine $\pm$ 1,25 $(OH)_2$ D_3 were normalized using the mean intensity of Cy3 labeled cDNA from a pool of Panc-1 cells that had been spotted on the same slides. The normalized values were used to calculate the ratios of the different points. The data were then preprocessed using GEPAS (Gene Expression Pattern Analysis Suite V 1.0) in order to handle replicates and perform log-transformation, filtering and normalization. The resulting datasets were then sent to the Epclust (@EBI). Among the 1730 genes, 413 were up-regulated by cytokines but reduced by the addition of 1,25 $(OH)_2$ D_3 in a dose-dependent fashion while 131 genes showed the opposite pattern i.e. a down regulation by cytokines and dose dependent increased expression by 1,25 $(OH)_2$ D_3 cotreatment (Figure 5).

The analysis of the cytokine-induced gene clusters confirms the stimulation of Fas expression (TNFRSF6) by cytokines (3.25 fold versus control) and the reverting effect of 1,25 $(OH)_2$ D_3 (Table 1). As for Fas, other significant genes were regulated including among the most relevant genes : Nuclear factor of kappa light polypeptide gene enhancer (NFkB, p49/pl00) and NF kappa-B transcription factor p65 DNA binding subunit, TRAF family member-associated NF-kappa activator (TANK), caspase 8 and caspase 8 associated protein 2, second mitochondria derived activator of caspase (SMAC), apoptosis-inducing factor (AIF), cell death-inducing DFFA-like effector B (CIDEB), DNA fragmentation factor 45 kD (DFFA), programmed cell death 2 (PDCD2), defender against cell death 1 (DAD1), dendritic cell immunoreceptor (DCIR), hypoxia-inducible factor 1 (HIF1).

Among the cluster of genes that are down-regulated by cytokines and restored by 1,25 $(OH)_2$ D_3 cotreatment were found apoptosis inhibitor 3 (API3), apoptosis-associated tyrosine kinase (AATK) and delta sleep inducing peptide immunoreactor (DSPI or GILZ) (Table 1). The characteristics of all the genes included in the two clusters are listed and can be seen at http://www.univ-lille2.ir/ilots/calcitriol%20study/calcitriol.htm.

Discussion

Beta cell destruction is a feature encountered at the onset of type 1 diabetes and in the post-transplant period of engrafted beta cells. In both processes, the loss of beta cells is mainly due to apoptosis.[2,11] Impaired functions and destruction of beta cells result from direct contact with islet-infiltrating macrophages and T lymphocytes and/or exposure to inflammatory products of the islet-infiltrating cells such as free radicals and cytokines. T-cell mediated cytotoxicity comprises two major pathways : the perforin-and Fas-dependent pathways[20]. The cell death receptor Fas (Apo-1, CD 95 or TNFRSF6) is able to signal apoptosis via an intracellular death domain. While beta cells do not normally express Fas, its expression may be induced in several situations making cells susceptible to apoptosis by interacting with Fas ligand (Fas-L)-expressing T cells and with neighboring beta cells which constitutively express Fas-L.[3,8–11] A clear correlation between Fas expression and insulitis indicates that inflammatory cytokines secreted by islet-infiltrating mononuclear cells are possible inducers of Fas expression in endocrine cells.[9] Consequently, Fas-Fas-L mediated apoptosis plays an important part in the destruction of pancreatic beta cells in insulitis-positive immune mediated type 1 diabetes.

Epidemilogical studies indicate a linkage between VDR, the receptor of 1,25 $(OH)_2$ D_3 and type 1 diabetes susceptibility. VDR gene polymorphisms have indeed been associated with type 1 diabetes in Indian Asian.,[21] German[22] and Taiwane[23] populations. Many pieces of evidence suggest that 1,25 $(OH)_2$ D_3 and its receptor may play a role in the pathogenesis of type 1 diabetes mellitus. 1,25 $(OH)_2$ D_3 has important immunomodulatory properties[24] and its administration prevents the development of type 1 diabetes as well as the associated autoimmune insulitis in NOD mice.[25] At physiological concentration 1,25 $(OH)_2$ D_3 has been found to protect cell proteins and membranes against oxidation

Table 1. List of selected genes modified by cytokines and normalized by 1,25 $(OH)_2$ D_3 cotreatment

Genbank accession number	Gene name	Cytokines	Cytokines + 1,25$(OH)_2D_3$ 10^{-8} M	Cytokines + 1,25$(OH)_2D_3$ 10^{-6} M
NM_000043	Homo sapiens tumor necrosis factor receptor superfamily, member 6 (TNFRSF6) mRNA	3,25	1,99	0,45
NM_001228	Homo sapiens caspase 8, apoptosis-related cysteine protease (CASP8) mRNA	4,55	0,81	0,75
NM_012115	Homo sapiens CASP8 associated protein 2 (CASP8AP2), mRNA	2,3	0,74	0,09
M62399	Human NF-kappa-B transcription factor p65 DNA binding subunit mRNA, complete cds	3,26	1,66	0,22
NM_002502	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) (NFKB2) mRNA	2,51	1,78	0,27
NM_004180	Homo sapiens TRAF family member-associated NFKB activator (TANK) mRNA	13,18	1,19	0,3
NM_004208	Homo sapiens apoptosis-inducing factor (AIF) mRNA	9,44	1,28	0,35
NM_0 19887	Homo sapiens second mitochondria-derived activator of caspase (SMAC), mRNA	8,21	2,16	0,74
NM_001229	Homo sapiens caspase 9, apoptosis-related cysteine protease (CASP9) mRNA	11,54	3,66	0,3
NM_014430	Homo sapiens cell death-inducing DFFA-like effector B (CIDEB), mRNA	5,62	2,3	0,34
NM_004401	Homo sapiens DNA fragmentation factor, 45 kD, alpha subunit (DFF A), mRNA	4,51	1,74	0,13
NM_001344	Homo sapiens defender against cell death 1 (DAD1), mRNA	4,39	3,11	0,79
NM_016184	Homo sapiens dendritic cell immunoreceptor (DCIR), mRNA	5,88	2,2	0,15
NM_002598	Homo sapiens programmed cell death 2 (PDCD2) mRNA	24,32	1,78	1,19
NM_001530	Homo sapiens hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1A) mRNA	4,77	0,72	0,54
NM_001167	Homo sapiens apoptosis inhibitor 3 (API3) mRNA	–0,37	2,69	3,38
NM_004089	Homo sapiens delta sleep inducing peptide, immunoreactor (DSIPI), mRNA	–0,25	1,52	3,89

Data are expressed as fold change versus control cells. ≪-≫ : decreased expression, no sign : increased expression

stress by inhibiting the peroxidative attack on membrane lipids.[26] 1,25 $(OH)_2$ D_3 has thus been documented as an effective antioxidant which increases hepatic glutathione and reduces glutathione reductase.[27] In most cancer cells 1,25 $(OH)_2$ D_3 induces apoptosis, but in normal cells, it stabilizes chromosomal structure and prevents double-strand break induced either by endogenous or exogenous factors.[26]

Cytotoxic in vitro challenge using cytokines has been shown to induce Fas in rat,[28] mouse[3] and human pancreatic islets.[7] Fas mediated destruction was primed by nitric oxide.[11] In human pancreatic islets we confirmed the induction of Fas by cytokines at the mRNA and protein levels. These up-regulations were accompanied by an increase in nitrite production, a reflection of nitric oxide synthesis. The addition of 1,25 $(OH)_2$ D_3 dose-dependently returned the data towards basal values. We have previously demonstrated[16] that the antiapoptotic effect of 1,25 $(OH)_2$ D_3 on cytokine-treated human pancreatic islets was mediated by the induction of the A20 anti-apoptotic protein known to exert a cytoprotective effect. The inhibitory effect of A20 is thought to be achieved by the transcriptional blockade of NO synthase by inhibiting the activation of NF-kappa-B at the level of TRAF6.[29–31] As described in rat beta cells and RINmSF cells,[32] cytokine-induced Fas gene expression requires the transcription of NF-kappa-B, a key "switch regulator" of gene networks controlling cytokine-induced beta cell dysfunction and death in rat pancreatic islets.[33] In an extended gene study using a human apoptosis expression microarray, we confirmed the up-regulation of NF-kappa-B p65 by cytokines in human islet cells. NF-kappa-B was part of a cluster of 413 genes that were up-regulated by cytokines and dose dependently reversed back to normal by 1,25 $(OH)_2$ D_3 cotreatment. Among these genes we particularly noted NF-kappa-B p65, but also TANK, a TRAF (TNF Receptor-Associated factor) family member-associated NF-kappa-B activator, identified as an intracellular protein mediating activation of NF-kappa-B. The protein encoded by TANK may interact with several members of the TRAF protein family. The interaction of TRAP with A20 inhibits the NF-kappa-B activation.[34]

Different routes to apoptosis initiated by the TNF and Fas death receptors have been evidenced.[35] In our model of

human islet cells stressed by cytokines with or without 1,25 $(OH)_2$ D_3, many of them might be implicated. Concomitantly with the induction of NF-kappa-B via the TRAF family member-associated NF-kappa-B activator, apoptosis induced by death receptors was also accompanied by activation of caspase 8 at the death inducing signaling complex. These ways lead to apoptosis by activating DNA fragmentation factor (DFFA), death-inducing DFFA-like effector B (CIDEB) and programmed cell death 2 (PDCD2). We also noticed the activation of factors implicated in a second route to apoptosis such as SMAC (second mitochondria-derived activator of caspase), a mitochondrial apoptotic signal that promotes apoptosis by inhibiting IAP (inhibitor of apoptosis). SMAC, normally located in the mitochondria, is released into the cytosol when cells undergo apoptosis. Many pro-apoptotic stimuli modify the permeability of the mitochondrial outer membrane resulting in the outflow of certain proteins such as AIF (apoptosis-inducing factor) and cytochrome C. AIF which was also up-regulated by cytokines and normalized by 1,25 $(OH)_2$ D_3, is thought to exert its effects in a caspase-independent manner by translocating to the nucleus. In contrast, cytochrome C exerts its effects by regulating the activities of Apaf-1, a molecule that promotes clustering of caspase-9 and its activation via a proximity-induced processing strategy which propagates the death signal by activating other caspases. As AIF, Caspase 9 was found in the cluster of genes that were up-regulated by cytokine treatment and normalized by 1,25 $(OH)_2$ D_3 cotreatment. We considered another cluster of 131 genes exhibiting the exact opposite pattern i.e. genes were down-regulated by cytokines and counteractively regulated by 1,25 $(OH)_2$ D_3 cotreatment. Among these genes we particularly noticed inhibitors of apoptosis such as IAP also called API3 which negatively regulate caspase activities[36] and DSIPI (Delta Sleep Inducing Peptide Immunoreactor) also called GILZ (Glucocorticoid-Induced Leucine Zipper) which inhibits NF-kappa-B nuclear translocation and DNA binding due to a direct protein-to-protein interaction with the NF-kappa-B subunits.[37] GILZ has been reported to inhibit activation-induced up-regulation of Fas and Fas-L expressions.[38]

Altogether, our data demonstrate a clear counteracting effect of 1,25 $(OH)_2$ D_3 on cytokine-induced Fas expression in human pancreatic islet cells. These results are reinforced by the finding of the parallel modulations of a cascade of death signals induced by Fas activation that, in different ways, participate in the molecular control of apoptosis.

Our results provide additional evidence that 1,25 $(OH)_2$ D_3 is an interesting molecule to help prevent the onset of type 1 diabetes and improve islet graft survival.

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