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Review

A proteomic approach on the effects of TX527, a 1α,25-dihydroxyvitamin D_3 analog, in human T lymphocytes

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ABSTRACT

1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), and its analogs (i.e. 14,20-bis-epi-19-nor-23-yne-1α,25$(OH)_2D_3$ – TX527) have been shown to prevent autoimmunity and prolong islet graft survival in the non-obese diabetic (NOD) mouse. Their effects are mediated by their action on various immune cell types, such as dendritic cells (DC) and T cells. We have previously reported important direct effects of TX527 on human T cells, on their cytokine/chemokine profiles, T regulatory cell markers, homing characteristics and chemotaxis. In order to fully understand the molecular mechanisms underlying the beneficial properties of TX527 on human T cells, we applied here 2-dimensional difference gel electrophoresis (2-D DIGE) to analyze the global protein alterations induced by TX527 on human synchronized T cells. We detected differential expression of 64 protein spots upon TX527 treatment, of which 65.6% could be successfully identified using tandem mass spectrometry (MALDI-TOF/TOF). The identified proteins function in various processes, such as metabolism and energy pathways, cytoskeleton and protein metabolism. When comparing the proteomics data to our previously performed microarray data on the same set of cells, we found an overlap of 17 different mRNAs/proteins. For some of these (e.g. PSME2, HSPA8), the direction of regulation was not similar, hereby reinforcing the important role of post-transcriptional/translational processes in the functionality of proteins. In addition, although 2-D DIGE offers the possibility of picking up post-translational processes, it lacks the ability to detect molecules with extreme molecular weight (MW) and isoelectrical point (p*I*) values, or very low abundant/hydrophobic proteins. This study highlights therefore the importance of combining different experimental approaches to obtain a complete picture of the underlying mechanisms and general processes being affected in T cells upon TX527 treatment. These processes lead altogether to the generation of T cells with interesting immunomodulatory features for clinical applications in the treatment of autoimmune diseases or in the prevention of graft rejection.

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Contents

Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 2-D DIGE, 2-dimensional difference gel electrophoresis; DC, dendritic cell; p*I*, isoelectrical point; MW, molecular weight; NOD, non-obese diabetic; PTM, post-translational modification; TX527, 14,20-bis-epi-19-nor-23-yne-1α,25$(OH)_2D_3$.

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1. Introduction

Besides the well-established function of active vitamin D as a central regulator of mineral and bone homeostasis, $1\alpha,25(OH)_2D_3$ has been rediscovered as a major modulator of the immune system, playing an important physiological role in the regulation of innate and adaptive immune responses [1,2]. Receptors for $1\alpha,25(OH)_2D_3$ are indeed present in most cells of the immune system and $1\alpha,25(OH)_2D_3$ can be produced by macrophages and DCs under the control of immune signals [3,4]. In addition, we have previously reported important immunomodulatory effects of $1\alpha,25(OH)_2D_3$ and its analogs (i.e. 14,20-bis-epi-19-nor-23-yne-$1\alpha,25(OH)_2D_3$ – TX527) in the prevention of autoimmunity and prolongation of islet graft survival in the NOD mouse [5,6]. These effects were shown to be mainly mediated by their action on various immune cell types, where $1\alpha,25(OH)_2D_3$ and its analogs modulate monocyte/macrophage cytokine profile, generate tolerogenic DCs and promote regulatory T cells [7–19].

In regard to its action on T cells, we have previously reported important direct effects of TX527 on a model of synchronized human T cells, where the compound inhibited T cell effector function while promoting increased numbers of functional $CD4^+CD25^{high}CD127^{low}$ regulatory T cells [9]. In addition, TX527 imprinted T cells with a specific homing receptor signature, favoring their migration to sites of inflammation and towards chemokine receptor ligands. In order to fully understand the molecular mechanisms underlying the beneficial immunomodulatory properties of TX527 on human T cells, we applied 2-dimensional difference gel electrophoresis (2-D DIGE) as proteomics technique to analyze the global protein profile of TX527-treated human T cells. We show that TX527 affects the global cytoplasmic protein profile of human T lymphocytes inducing modest differences in protein expression (less than 2-fold up- or down-regulation as compared to controls, $p \leq 0.05$) and post-translational modifications (PTM) of different proteins. Comparing the changes in protein expression to our previously published microarray study on the same set of cells [9] revealed that only 17 genes responded to the TX527 treatment both at the mRNA and protein level. Interestingly, the effect of TX527 on translation did not always reflect the changes seen at the transcriptional level. This, together with the fact that 2-dimensional electrophoresis has limitations in the detection of proteins with extreme MW and p*I* values, or very low abundant/hydrophobic proteins, highlights the importance of combining different experimental approaches, such as microarrays, protein profiling and FACS analysis, in order to obtain a complete picture of the processes being affected in T cells upon TX527 treatment.

2. Materials and methods

2.1. T cell isolation and culture

Human $CD3^+$ T lymphocytes were obtained as previously described [9]. Briefly, $CD3^+$ T cells were cultured in the presence of plate-bound anti-CD3 (1 μg/mL), anti-CD28 (1 μg/mL) and IL-2 (12.5 ng/mL) for 2 days. TX527 (synthesized by M. Vandewalle and P. De Clercq (University of Ghent, Belgium) and obtained from Merck-Theramex, Germany) or ethanol were added at day 2 of the culture (10^{-8} M) and refreshed every 2 days. At day 10 of culture, cells were harvested and protein samples were prepared for 2-D DIGE analysis.

2.2. 2-D DIGE analysis

Six independent T cell culture experiments were performed, each originating from a different buffy coat of healthy donors. Protein extraction of control or TX527-treated samples followed by 2-D DIGE analysis were carried out as previously described [10,20]. Two pH ranges (pH 4–7 and pH 6–9) were used to separate protein samples during the first dimension, which was carried out on an Ettan IPGphor II manifold (GE Healthcare). Second dimension was carried out using an Ettan Dalt Six (GE Healthcare), as previously described [10]. The gels were then scanned on a Typhoon 9400 gel imager (GE Healthcare) and image analysis was performed using the Batch Processor module of the DeCyder™ V7.0 software (GE Healthcare), following the manufacturer's specifications.

2.3. Differentially expressed protein identification

Two preparative gels in each pH range for $CD3^+$ T cells, treated or not with TX527, containing 350 μg of protein were run for spot picking. Differentially expressed spots were picked after staining with Krypton Stain (Thermo Fisher, Geel, Belgium) according to the manufacturer's recommendations and picking with an Ettan Spot Picker (GE Healthcare). Spot manipulation and analysis on a MALDI-TOF/TOF 4800 instrument (Applied Biosystems, Foster City, USA) were made as previously described [10,20]. Data interpretation was carried out using the GPS Explorer software (V3.6), and database searching and peptide identification was carried out using Mascot (Version 2.2.00), with the same search parameters as described in [10]. Peptide identification scores were then calculated by Mascot based on the calculated probability that the observed match between the experimental data and the database sequence is a random event. Peptide scores greater than the given cutoff value for MS/MS fragmentation data were considered significant ($p < 0.05$). Moreover, individual peptides with a $p > 0.05$ were excluded, and the total score (Mascot score) was recalculated as the sum of the scores of the individual peptides with a $p < 0.05$.

2.4. Statistical analysis

The statistical differences between group average ratios were determined by the paired two-tailed Student's *t*-test, performed automatically by the BVA module of the DeCyder™ V7.0. $p \leq 0.05$ was considered statistically significant.

3. Results and discussion

The structural analog of vitamin D, TX527, has been put forward by our group as a potential tool for affecting the immune system *in vivo*, since it shares with $1\alpha,25(OH)_2D_3$ immunomodulatory properties on different immune cells (i.e. DCs, T cells), but has less calcemic side effects. In this regard, we have previously demonstrated that TX527 abrogates differentiation and maturation of DCs leading to the emergence of a tolerogenic cell type lacking typical DC features [12]. Moreover, TX527 was shown to promote regulatory $CD4^+CD25^{high}CD127^{low}$ T cells, with altered homing

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