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Expression profile of a clonal insulin-expressing epithelial cell in the thymus

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ABSTRACT

Background: Type 1 diabetes is an autoimmune disease resulting from the destruction of pancreatic beta-cells. One of the main antigens targeted in this auto reactive response is insulin. It has been shown that insulin is expressed in small amounts in the thymus, and more specifically in the medullary thymic epithelial cells (mTECs), which also express a variety of other tissue-specific antigens. This thymic expression enables the maintenance of self-tolerance, and is essential in preventing auto-immune disease. Our laboratory has created a mouse mTEC clonal cell line specifically expressing insulin in order to better understand the regulatory mechanisms of this ectopic expression of insulin. In this study, we compared the insulin expressing cell line to an insulin non-expressing mTEC line by genome-wide expression profiling.

Results: The most important difference was overexpression of CD34 in the insulin expressing clone, confirmed by Real-time Rt-PCR and flow cytometry. Cells in the thymus expressing higher levels of CD34 were found to contain higher levels of insulin and, to a lesser extent, Aire, a master regulator of self-antigen expression in the thymus. The cells expressing CD34 were not enriched in CD80, a known mTEC maturity marker.

Conclusion: CD34 may be a specific marker for functionality, with some specificity for insulin.

1. Background

Type 1 diabetes (T1D) is caused by an autoimmune response against the beta cells of the pancreas, which results in the disruption of production of insulin in the body. This autoimmune response is thought to be directed against insulin, an important antigen in type 1 diabetes (Janeway et al., 2001). It has previously been shown that small amounts of insulin are expressed in the thymus (Chentoufi et al., 2004; Derbinski et al., 2001). This has also been found to be true for a large number and variety of other tissue-specific antigen (Derbinski et al., 2001). As the thymus, through the action of the medullary thymic epithelial cells (mTECs), is the site for negative selection of self-reactive T cells, this ectopic expression is believed to be essential in this purging process.

The level of insulin expression in the human thymus is inversely correlated to susceptibility to type 1 diabetes, as a genetic variant determining lower insulin expression in the thymus confers susceptibility (Vafiadis et al., 1997). Furthermore, the number of functional insulin gene copies in the mouse thymus, inversely correlates to levels of insulin auto-reactive T cells in circulation (Chentoufi and Polychronakos, 2002) and, against the NOD background, diabetes (Thebault-Baumont et al., 2003). The expression of insulin, as well as the level at which it is being expressed, are essential components to establishing and maintaining self-tolerance to this particular antigen. In particular, the lack of expression of insulin specifically and only in the thymus leads to an autoimmune response, and has been demonstrated in a mouse model (Fan et al., 2009). It is now known that the specific location of this distinct expression pattern in the thymus is the medullary epithelial cells (Derbinski et al., 2001). They express a variety of self antigens at low levels, representing many tissues. This unusual expression pattern has been termed “promiscuous” gene expression, in reference to the atypical pattern of genes expressed in this one cell type (Derbinski et al., 2001). Transcription of insulin in the pancreas is known to depend on the transcription factor Pdx1, while in the thymus it has no effect on insulin’s transcription (Palumbo et al., 2006; Danso-Abeam et al., 2013). This lack of dependency on a known regulator of insulin expression in the pancreas demonstrates a distinctive regulatory mechanism in the thymus for this gene.

This regulatory mechanisms that underlies “promiscuous” gene expression are only starting to be uncovered. The AIRE regulator is known to be essential for the expression of a number of self-antigens in the medullary thymic epithelial cells (mTECs) (Anderson et al., 2002). AIRE is mutated in the APECED

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syndrome (Ahonen, 1985; Nagamine et al., 1997), characterized by a multi-organ autoimmunity including T1D in 10–20% of cases. Additionally, mice lacking the Aire gene, show similar autoimmune responses in numerous organs, and have drastically decreased levels of expression of self antigens in the thymus, including insulin (Anderson et al., 2002). The precise molecular mechanism of Aire action on these genes in the mTECs is not yet entirely known. Recent studies have uncovered binding partners for Aire, enabling a broader understanding of the mechanism by which it induces tissue specific transcription in the thymus (Abramson et al., 2010). Furthermore, the transcription of tissue specific antigens in the thymus is regulated through different mechanisms than those used by the usual tissues where they are expressed (Villasenor et al., 2008). Therefore, uncovering these mechanisms and Aire binding partner function is key in understanding of this aberrant transcription of self-antigens.

Aire has also been shown to correlate with a known maturity marker, CD80, in mTECs (Derbinski et al., 2005). The epithelium of the thymic medulla is comprised of a gradient of maturity levels, whereby not all cells are in the same stage of development. mTECs that are more mature express higher levels of the co-stimulating molecule CD80, as well as Aire (Derbinski et al., 2005). This in turn, leads to higher levels, as well as a wider range, of antigen expression. Therefore, the maturity and development of mTECs have a strong impact on the final expression levels of self-antigens, and ultimately the preservation of self-tolerance.

Only a small proportion of all mTECs express insulin (1–3%, although a value as high as 15% has been reported by Villasenor et al., 2008) and it is thought that each antigen is represented by, on average, 0.5% mTECs (Anderson et al., 2005; Derbinski et al., 2001). In our laboratory, we have generated a clonal cell line from the medullary thymic epithelium, whereby we can assess insulin-expressing mTECs specifically, as opposed to thymic aggregates used in this field of study (Levi and Polychronakos, 2009; Palumbo et al., 2006). To better understand what makes these cells distinct, we profiled gene expression by microarray of an insulin-expressing clone and one insulin-non-expressing clone. While a few genes were found to be overexpressed in either clone, the outstanding gene that was found to be significantly higher in the insulin-expressing clone was CD34.

2. Methods

2.1. Microarray

The microarray was performed on RNA extracted (Rneasy Plus Kit, Qiagen, Netherlands) in triplicate from sets of cultured mTECs, either insulin expressing (INS(+)) or insulin non-expressing (INS(−)) clones described in (Palumbo et al., 2006). An Illumina microarray chip (Mouse WG-6 v2.0) was used for expression comparisons. We assessed expression in three biological replicates (separate cultures).

2.2. Microarray statistics

The data from the microarray chip were analyzed with FlexArray software (http://genomequebec.mcgill.ca/FlexArray/license.php). They were normalized using the lumi Bioconductor package for Illumina chips. The two clones were then compared by *t*-test, with false-discovery rate (FDR) correction. Of those that passed the FDR threshold of $q < 0.001$, only genes above a fold change of 1.15 in the INS(+) or INS(−) were kept for further analysis. The fold change cutoff of 1.15 was chosen based on literature indicating that thymic expression of tissue specific antigens is much more subtle than that in the tissue specifically expressing them (Vafiadis et al., 1997; Derbinski et al., 2001).

2.3. Chromosomal clustering

The chromosomal positions of significant ($q < 0.001$) tissue specific genes was determined and distances between the genes were calculated. The distribution of distances was evaluated for clustering, using the sum of the inverse of distances between consecutive genes on the same chromosome.

$$\sum_{i=1}^{n} \frac{1}{l_i}$$

where n is the number of pairs of consecutive genes on the same chromosome genome-wide and l_i the distance between the 5′ ends of the two genes of the ith pair. This metric is sensitive to two or more genes clustering together, additive between chromosomes and minimally penalized by the effect of long distances between clusters or isolated genes. Its statistical significance was tested against a distribution created by randomizing the gene positions on the Illumina array probe coordinates 10,000 times.

2.4. Tissue specificity

The significant genes above the 1.15-fold change were assessed for tissue specificity. The expression profile of the 74 genes was found on BioGPS (biogps.org) using the MOE430 data set. For each gene, tissue specificity was called if levels of expression of 10 times the median were found in less than 5 tissues, as has been defined in previous literature (Derbinski et al., 2005).

2.5. Primary mTEC extraction

Bl6 mice were purchased from Jackson Laboratories (Maine, USA) at 6 weeks of age. Ten mice were sacrificed per experiment and the thymus dissected and placed in PBS. The thymus was cut into quarters or smaller sections, and gently agitated in PBS with a magnetic stirrer to liberate the thymocytes. The PBS was decanted and the thymus sections were covered with 1 ml of Collagenase/Dispase and DnaseI (Roche, Mannheim, Germany) solution at a concentration of 125 μg/ml and 100 μg/ml, respectively. The digestion was completed after two sets of 30 min, removing the supernatant and adding fresh solution in between the sets. The digested thymus was then centrifuged on a Percoll density gradient at 3700 rpm for 30 min (Amersham Biosciences, Uppsala, Sweden), as described in (Chentoufi et al., 2004), and the mTEC fraction was collected and pelleted by centrifugation at 1800 rpm for 10 min. These studies were approved by the Animal Care Committee of McGill University.

2.6. Antibodies and sorting

All flow cytometry markers were purchased from eBioscience (San Diego, USA) with the exception of UEA-1, which was from Vector Laboratories (Burlingame, USA). The eBioscience Fixable Viability dye eFluor780 was used to assess cell death, and all dead cells were excluded from the pool to be sorted. mTECs were specifically sorted out by negative bone marrow cell markers and positive mTEC markers as described in (Derbinski et al., 2001): CD45lo/UEA-1+/EpCAM+/CD34hi and CD45lo/UEA-1+/EpCAM+/CD34lo separately. Thresholds for CD34 hi and lo was set with a fluorescence minus one (FMO) method, allowing to mark the appropriate gates for the population sort. This method eliminated any background in the positive population.