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Male and female NOD mice differentially express peroxisome proliferator-activated receptors and pathogenic cytokines

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ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) have been implicated in regulating the immune response. We determined the relative changes in the transcriptional expression of PPAR isoforms (α, γ1 and γ2) and cytokines involved in the pathogenesis of type 1 diabetes (T1D) in the immune cells of 5 weeks, 10 weeks and diabetic male non-obese diabetic (NOD) mice compared to those of female NOD mice from our previous studies, "normalized" against their respective non-obese diabetic resistant (NOR) mice controls. Overall PPARα was significantly more elevated in the macrophages of female NOD mice of all age groups whereas PPARγ, particularly the PPARγ2 isoform was more depressed in the macrophages and $CD4^+$ lymphocytes of female NOD mice compared to their male counterparts. The pro-inflammatory cytokines, IL-1 and TNFα, as well as the Th1 cytokines, IL-2 and IFNγ were more elevated in female NOD mice whereas the Th2 cytokine, IL-4, was more depressed in these mice compared to their male counterparts. These findings suggest that the preponderance of T1D in female NOD mice may be influenced by the more pronounced changes in the expression of PPAR isoforms and pathogenic cytokines compared to those in male NOD mice.

1. Introduction

Type 1 diabetes (T1D) is a cell-mediated autoimmune disease, characterized by an irreversible selective destruction of the insulin-producing β-cells involving pathogenic and pro-inflammatory cytokines and infiltration of specific T lymphocytes (Nerup et al., 1988). T1D is thought to be mediated and propagated by the effects of Th1 cytokines such as IL-2 and IFNγ, secreted by $CD4^+$ T cells, which induce inflammation and recruit other cell types that are the final effectors of β-islet cell destruction (Rabinovitch and Suarez-Pinzon, 1998; Raz et al., 2005).

The non-obese diabetic (NOD) mouse strain is a well known animal model for T1D with the observed pathogenesis similar to humans. NOD mice have been observed to have an age progressive accumulation of $CD4^+$ T cells expressing the Th1 cytokine profile in the infiltrated pancreas (Gregori et al., 2003). In addition, other cells such as $CD8^+$ T cells and macrophages have a role to play as they are also present during insulitis (Bendelac et al., 1987). However, the model differs from human T1D in that female mice are twice as prone to develop diabetes compared to males. We observed that at 30 weeks of age, the cumulative incidence of T1D in NOD mice was around 90% for females and 50% for males, which corroborated previous reports (see for example Anderson and Bluestone, 2005).

The peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors that belong to the nuclear hormone receptor superfamily (Isseman and Green, 1990). Three isoforms of PPARs (α, γ and β/δ) have been identified (Kliewer et al., 1994; Braissant et al., 1996). They are generally involved in lipid metabolism, adipogenesis and lipid oxidation (Braissant et al., 1996; Michalik et al., 2006). However, these isoforms (especially α and γ) have many other important functions including the regulation of inflammatory and immune responses (Daynes and Jones, 2002). PPARα has been reported to negatively regulate inflammation by repressing transcription factors such as NFκB and AP-1 (Jiang et al., 1998; Ricote et al., 1998). PPARγ has been shown to inhibit the production of inflammatory cytokines, the proliferation of mitogen-activated T cells (Clark et al., 2000; Yang et al., 2000; Wang et al., 2001; Cunard et al., 2002), the activation of macrophages (Jiang et al., 1998; Rotondo and Davidson, 2002) and the activation of inflammatory transcription factors like NF-κB, AP-1 and STAT1 (Yang et al., 2000; Wang et al., 2001).

Recently, Dunn et al. (2007) reported that PPARα expression in $CD4^+$ T lymphocytes is sensitive to androgen levels and that its expression in naïve T cells of male mice is higher than that in females. The higher expression of PPARα in male mice correlated with lower NFκB and c-jun activity with a concomitant elevated

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Th2 cytokine production of these cells. These findings may provide initial evidence for the preponderance of autoimmune diseases in females. However, the gender-associated involvement of other PPARs, especially the PPARγ1 and γ2 isoforms in the immune cells of NOD mice has not been reported.

The objective of this study is to evaluate the expression of PPAR isoforms and selected cytokines in the peritoneal macrophages, $CD4^+$ and $CD8^+$ lymphocytes of male NOD and NOR mice at different ages (5 weeks, 10 weeks and diabetic). The findings will be compared to those of our previous reports (Yaacob et al., 2004, 2009) by assessing the relative differences in the transcriptional expression of the genes mentioned above in the female NOD mice "normalized" to their respective controls. Direct comparisons between the male and female NOD mice were not performed since the experiments were not conducted at the same time and that there were slight differences in the preparation of the cells and method of analysis. However, this evaluation could shed some light on the influence of these genes in the preponderance of T1D in females compared to male NOD mice.

2. Materials and methods

2.1. Preparation of peritoneal macrophages and splenocytes

Male NOD and NOR mice (5- and 8-week-old) were purchased from Jackson Laboratory (Bar Harbor, USA) and maintained in a clean, light-controlled, air-conditioned room and given sterilized water and food *ad libitum*. The 5-week-old NOD and NOR mice were sacrificed within 1 week of arrival. The 8-week-old mice (maximum age supplied) were kept until some were sacrificed at the age of 10 weeks while others were continued to be maintained until the onset of diabetes which was defined as two consecutive blood glucose level of >12 mM.

The mice were sacrificed by cervical dislocation and the peritoneal washout was obtained for isolation of macrophages and the spleen collected for the isolation of splenocytes. The peritoneal washout was obtained by injecting 5 ml sterile RPMI-1640 medium into the mouse peritoneal cavity. The abdominal cavity was gently massaged and the cells were then retrieved using a syringe. The macrophages were isolated from the peritoneal washout by centrifugation and counted. The splenocytes were isolated by mechanical disruption and analyzed using the FACSCalibur flow cytometer (Becton–Dickinson) to enumerate the percentages of CD4/CD3-positive and CD8/CD3-positive lymphocytes. All animal experimentations were performed in accordance to the rules and regulations as approved by the Universiti Sains Malaysia Animal Ethics Committee.

$CD8^+$ cells were isolated first from the total splenocytes followed by $CD4^+$ cells, using antibody-coated magnetic microbeads (Miltenyi, Germany) according to the manufacturer's protocol. Briefly, total splenocytes were pelleted and resuspended at a ratio of 10 μl CD8 antibody-coated microbeads in 90 μl of PBS (1:10) per 10^7 splenocytes and incubated on ice. The splenocytes were then rinsed and pelleted. The pellet was resuspended with PBS and loaded into a MS isolating column placed on a magnetic stand and separator. The eluted splenocytes (depleted of $CD8^+$ cells) were collected in a tube and a small sample was taken for flow cytometric analysis. The whole process was repeated on the collected splenocytes with CD4 antibody-coated microbeads and a new sorting column to isolate $CD4^+$ cells. Finally, splenocytes that were depleted of $CD4^+$ and $CD8^+$ cells were also collected for flow cytometry analysis. Magnetically separated $CD8^+$ or $CD4^+$ positive cells were obtained by removing the column from the magnetic stand and flushing it twice with PBS buffer into a collection tube and stored on ice for RNA extraction.

2.2. RNA extraction and cDNA construction

Total RNA was extracted from the peritoneal macrophages, $CD4^+$ and $CD8^+$ T cells using the Tri-Reagent solution (Molecular Research Center, USA) according to the manufacturer's protocol. The RNA integrity was determined by gel electrophoresis and the quantity and purity were determined by spectrophotometry (A260/A280). Reverse transcription of total RNA to cDNA was performed using MMLV reverse-transcriptase with random hexamers according to the manufacturer's instructions (Fermentas, Lithuania).

2.3. Real-time PCR quantification

The expression of PPARα, γ_1 and γ_2 was quantified in an ABI 7000 real-time PCR apparatus (Applied Biosystems, USA) using fluorescent-labeled Taqman® hydrolysis probes in a 25 μl reaction consisting of 12.5 μl Taqman universal PCR master mix (Roche, Branchburg, USA), 1.25 μl probes (250 nM), forward and reverse primers (900 nM) and 20–40 ng cDNA sample in ddH_2O. The sequences for primers and probes were as follows: PPARα forward: CCT GAA CAT CGA GTG TCG AAT ATG, reverse: CGC CGA AAG AAG CCC TTA C, probe: CAG GGT ACC ACT ACG GAG TTC ACG CAT G. PPARγ1 forward: GCG GCT GAG AAA TCA CGT TC, reverse: TTA AAA ATG TCC TGA ATA TCA GTG GTT C, probe: GCT TCT TTC AAA TCT TGT CTG TCA CAC AGT. PPARγ2 forward: GGG TGA AAC TCT GGG AGA TTC TC, reverse: GTG GGC CAG AAT GGC ATC, probe: CAT CAG CGA AGG CAC CAT GCT CTG. The reaction condition was a two-step universal thermal cycling protocol which consists of a UNG carry-over decontamination (50 °C, 2 min), hot start *Taq* polymerase activation (95 °C, 10 min) followed by 45 cycles of denaturation (95 °C, 15 s), annealing and extension (60 °C, 1 min). All standard and sample reactions were performed in triplicates. PPAR homologous standards (Yaacob et al., 2009) (10-fold dilutions of 10^{-3} to 10^{-8} pmol) were reacted together with the samples to produce a standard curve.

2.4. Semi-quantitative PCR

Semi-quantitative PCR was used to determine the expression of pro-inflammatory (IL-1β and TNF-α), Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine genes. Each cytokine gene was amplified together with a housekeeping (GAPDH) gene (Maxim Biotech, USA) according to the manufacturer's instructions. The PCR products were subjected to electrophoresis and the bands were analyzed (densitometry) using the Genetools Version 3.03.03 image analysis software (Syngene, England). The band intensity was used to describe cytokine gene expression after normalization to the housekeeping gene.

2.5. Statistical analysis and comparison between male and female mice

Statistical calculations were performed using the SPSS Software (version 11.5). Mann–Whitney *U*-test was used to compare the expression of PPAR isoforms and cytokines between the male NOD and NOR groups at their respective ages.

The observed changes in male NOD mice compared to the male NOR mice were then compared to those observed in female NOD mice (compared to female NOR mice) from our previous studies (Yaacob et al., 2004, 2009).

3. Results and discussion

Table 1 summarizes the relative differences in the transcriptional expression of PPAR isoforms (α, γ1 and γ2) and selected