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Review Article

The CD39-Adenosinergic Axis in the Pathogenesis of Immune and Nonimmune Diabetes

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Diabetes mellitus encompasses two distinct disease processes: autoimmune Type 1 (T1D) and nonimmune Type 2 (T2D) diabetes. Despite the disparate aetiologies, the disease phenotype of hyperglycemia and the associated complications are similar. In this paper, we discuss the role of the CD39-adenosinergic axis in the pathogenesis of both T1D and T2D, with particular emphasis on the role of CD39 and CD73.

1. Introduction

Extracellular nucleotides, such as adenosine triphosphate (ATP), are important signalling molecules involved in many biological processes. Under basal conditions extracellular concentrations of ATP are maintained at low levels. Endogenous regulation of ATP concentration is mediated by ectoenzymes: the family of ectonucleotidases (E-NTPDases) and ecto-5′-nucelotidase (CD73; E.C. 3.1.3.5) located on the cell surface. Four plasma membrane-bound E-NTPDaess have been cloned: NTPDase1 (CD39; E.C.3.6.1.5), NTPDase2, NTPDase3, and NTPDase8 [1], each with distinct localization and biological properties. NTPDase1 hydrolyzes ATP and adenosine diphosphate (ADP) equally well; NTPDase2 preferentially hydrolyzes ADP; NTPDase3; NTPDase8 have intermediate hydrolysis profiles [2]. The hydrolysis of ATP and ADP generates adenosine monophosphate (AMP), which is then hydrolysed by CD73 to adenosine. CD39 is the rate-limiting enzyme [3] in this cascade and thus is the prime regulator of nucleotide and adenosine concentrations within the microenvironment.

Both CD39 and CD73 expressions are dynamic and change under pathophysiological conditions. Hypoxia upregulates both ectoenzymes—CD39 through Sp1-dependent pathways [4] and CD73 through binding of HIF-1 [5]. Further, within the CD73 gene, promoter region is a cAMP response element (CRE) which regulates transcription through cAMP-dependent CRE-binding protein (CREB). Activation of adenosine receptors increases cAMP and CREB suggesting that the enzymatic product of CD73 (adenosine) may transcriptionally regulate its expression (reviewed in [6]). Finally, the glucocorticoid dexamethasone increases AMP hydrolysis and CD73 expression which is mitigated by protein kinase C (PKC) inhibition [7]. PKC has been shown to activate the transcription of specific genes concluding CD73 [8].

Like ATP, adenosine is constitutively expressed at low levels with a dramatic increase during metabolic stress such as hypoxia and ischemia consequent to ATP hydrolysis. Adenosine is a biologically active molecule that signals through four G-protein-coupled receptors denoted A1, A2A, A2B, and A3. Activation of A1 and A3 inhibits adenylyl cyclase activity through coupling to G_i resulting in a decrease in intracellular cyclic AMP (cAMP), whereas A2A and A2B subtypes are coupled to G_s or G_o to stimulate adenylyl cyclase and lead to an increase of cAMP. A change in cAMP concentrations induces downstream signalling by phosphorylating key enzymes. Furthermore, the A2BR is also coupled to $G_{q/11}$ stimulating phospholipase C (PLC) reviewed in [9] and the A3R signals via PLC-β2/β3 [10]. Adenosine can also activate phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs) and extracellular receptor

signal-induced kinase (ERK). Additional effector mechanisms include activation of Akt to inhibit apoptosis by A3R, A1R activation which promotes the influx of Ca^{2+} and efflux of K^+ and the activation of the arrestin pathway by adenosine receptors (reviewed in [9]). The adenosine receptors are ubiquitously distributed in the body and the overriding effect of adenosine receptor activation in any one cell is dependent on the repertoire of receptors expressed. The main biological role of adenosine is to maintain vascular and immune homeostasis.

Diabetes, a disorder of glucose homeostasis, is an increasingly prevalent disease worldwide. Two distinct subtypes are recognised: autoimmune diabetes (Type 1 diabetes, T1D) typically afflicting the young and associated with destruction of β-cells and nonimmune diabetes (Type 2 diabetes, T2D) typically arising in those of older age, obese, and with the metabolic syndrome. Although the pathogenesis of the two disorders is distinct, central to both is that of pancreatic β-cell failure and hypoinsulinemia. Features unique to T2D include peripheral insulin resistance and failure of the incretin effect. Despite the disparate aetiologies, the sequelae hyperglycemia and its associated complications are common to both disorders. In this paper, the role of purinergic signalling via the CD39-adenosinergic axis will be discussed in the context of the pathogenesis of T1D and T2D.

2. Pancreatic Expression of Ectoenzymes

Insulin synthesis and secretion is tightly regulated in order to maintain stable blood glucose levels. When blood glucose levels increase, insulin secretion is augmented; conversely in the face of hypoglycaemia, insulin secretion is negligible. Hyperglycemia increases the metabolic demand of β-cells causing a rise in intracellular ATP concentrations [11] and ATP is released with insulin [12] reaching concentrations of 25 μm at the cell surface. Extracellular nucleotides activate P2 receptors—inotropic P2X and metabotropic P2Y. A number of P2 receptors have been implicated in nucleotide-mediated regulation of insulin including P2Y1, P2Y6, P2Y13, P2X3, and amongst others [12–15].

Ectonucleotidase expression has been defined within the mouse, rat, and human endocrine pancreas [16, 17]. Using immunohistochemical and enzyme histochemical techniques, NTPDase1/CD39 was expressed in all blood vessels and acinar tissue; NTPDase2 was localised in capillaries and in connective tissue surrounding islets and acini and NTPDase3 was expressed exclusively in Langerhan islet cells. NTPDase8 was not detected. Inhibition of NTPDase3 activity was shown to facilitate insulin release from rat β-cells. Similarly inhibition of ectonucleotidases with ARL 67156, an inhibitor of NTPDase1 and 3, augmented insulin secretion from human pancreas [13]. Intriguingly CD73 was expressed exclusively in rat islet cells [16] but not in human or mouse [16] (and Chia et al., submitted manuscript). Notably both CD39 and CD73 are secreted from acinar tissue together with ATP directly into the fluid controlling pancreatic exocrine function (reviewed in [18]).

3. Autoimmune Type 1 Diabetes (T1D)

In T1D islet destruction secondary to the autoimmune infiltration of $CD4^+$ T cells and macrophages results in the loss of insulin secretory capacity of β-cells. Treatment with multiple daily injections of insulin slows but does not prevent the development of complications. Transplantation of the whole pancreas or islets is a potential cure for the disease; however, there remains the risk of recurrent disease culminating in graft failure.

3.1. Mouse Models of T-Cell-Mediated Diabetes. The nonobese diabetic (NOD) mouse is the prototypical mouse model for T1D and shares a number of clinical, serological, and immunological features with the human condition. NOD mice spontaneously develop diabetes at ~25 weeks of age after progressing through a prediabetic stage correlating with increasing insulitis.

T-cell-mediated diabetes can also be induced chemically using multiple low dose streptozotocin (MLDS). Streptozotocin is a glucosamine-nitrosourea compound that enters the pancreatic β-cell through the specific glucose transporter 2 (GLUT2) expressed on its surface. Administered in high dose (250 mg/kg) streptozotocin is cytotoxic causing islet death. The onset of diabetes is immediate and there is an absolute lack of insulin. However, streptozotocin administered in low dose (50 mg/kg for 5 days) results in repetitive low-grade β-cell damage, which incites a local inflammatory response comprised principally of $CD4^+$ T cells that is maximal at 12–14 days [19]. The delay in the onset of hyperglycemia suggests immune-mediated damage to β-cells, rather than direct toxicity predominates. Further T cell depleted [20] or deficient mice [21] are resistant to MLDS-induced diabetes.

3.2. Role of E-NTPDase1/CD39 in T-Cell-Mediated Diabetes. $CD4^+$ regulatory T cells are integral to the maintenance of immune homeostasis and abnormalities in number and or function results in autoimmune disease. Indeed low numbers of resting regulatory T cells have been reported in NOD mice [22] and human patients with T1D [23]. CD39 is expressed on both murine [3, 24] and human [25, 26] $CD4^+$ regulatory T cells and is essential for the full suppressive activity of these cells in mice. Further, mice deficient in CD39 (CD39KO) develop an immune diathesis and spontaneous autoimmune alopecia [27]. As anticipated, these mice are highly susceptible to MLDS-induced diabetes with a rapid rate of onset of diabetes (within 10 days) and 100% incidence. Insulitis and reduction in insulin staining was evident at the onset of diabetes. When reconstituted with wild-type bone marrow comprising functional regulatory T cells, the kinetics and incidence are reduced to that of wild-type mice with the development of diabetes at day 42 and overall diabetes incidence of 57% (Chia et al., submitted manuscript). CD39KO mice also have evidence of hepatic insulin resistance [28], which will be discussed in detail below.

Mice have been genetically engineered to overexpress CD39 [29]. CD39 colocalises to β-cells without perturbing

glucose homeostasis [30] and these mice are resistant to MLDS-induced diabetes: minimal insulitis was evident and diabetes occurred in only 14% of animals. This robust protection persisted even following reconstitution with bone marrow from immunodeficient CD39KO mice (Chia et al., submitted manuscript) which may reflect enhanced cell regenerative capacity due to increased pancreatic NTPDase activity. Recent work by Andersson et al. [31] and Annes et al. [32] indicate a role for adenosine signalling in cell specific regeneration. In a zebrafish model, the nonselective agonist NECA did not alter protection against cell death but promoted cell regeneration by increasing the proportion of new cells that proliferate through A2A-dependent mechanisms. Interestingly, NECA did not significantly increase the number of cells in normal development. Further in mice treated with streptozotocin at 150 mg/kg for 2 days, BGL were 30% lower in mice concurrently treated with NECA and cell mass was 8 times larger.

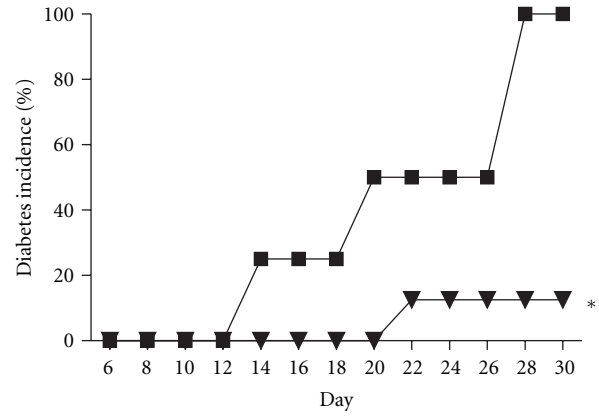

Figure 1: Mice deficient in CD73 are resistant to MLDS-induced diabetes. Diabetes incidence in C57BL/6 wild-type (WT) mice (black squares, $n = 4$) and CD73KO (black triangles, $n = 8$) mice following MLDS. $^{**}P < 0.01$ versus WT mice.

3.3. Role of Ecto-5′-nucelotidase/CD73 in T-Cell-Mediated Diabetes. Although CD73 is not expressed in mouse or human islets [16], it is widely expressed on leukocytes and plays an essential role in leukocyte trafficking. Further, like CD39, CD73 plays an integral role in providing immune competence. CD73 is expressed on $CD4^+$ regulatory T cells in mice [3, 33], but interestingly is not expressed by human $CD4^+$ regulatory T cells [26]. CD73KO mice have been generated [34] and extensively characterised. Markedly, reduced CD73 enzymatic activity [34] results in reduced levels of adenosine [35]. The biological relevance of CD73 had become evident from a number of small animal models: CD73 activity attenuates hypoxia-induced vascular leakage FMLP (formyl-Met-Leu-Phe-OH)-stimulated neutrophil adhesion to endothelial cells and neutrophil accumulation in tissues [34, 36, 37]. CD73KO mice have a proinflammatory phenotype with increased VCAM-1 expression on endothelial cells and heightened susceptibility to vascular inflammation and neointima formation [35]. These effects are a consequence of the loss of both enzymatic and nonenzymatic functions of CD73 [38]. Contrary to these reports, we have shown that CD73KO mice are protected in a model of renal ischemia-reperfusion injury [39–41]. Similarly, CD73KO mice are resistant to MLDS-induced diabetes (Figure 1), presumably a consequence of impaired leukocyte trafficking. In alloxan-induced diabetes in rats, a model which produces a pattern of T1D, both platelet-associated CD39 and CD73 activities are increased [42].

3.4. Adenosine Signalling in T-Cell-Mediated Diabetes. Adenosine signalling has emerged as a regulator of glucose homeostasis through modulating insulin and glucagon release. All four adenosine receptors are expressed in whole pancreas of CD-1 mice [43]; in isolated islets A1, A2A, and A2B receptors are expressed at the mRNA level (Chia et al., submitted manuscript). The A1 and A2A receptors have also been identified on α-cells [44]. Following MLDS, A1 receptor expression is downregulated, A2A expression is unchanged, and A2B receptor expression is augmented (Chia et al., submitted manuscript).

Basal levels of adenosine in isolated islets are in the micromolar range [45], which is sufficient to stimulate glucagon release [46] and inhibit insulin release [47] via the A1 receptor. Thus the peri-islet adenosine concentration is inversely related to extracellular glucose concentrations and may act as a paracrine or autocrine signal [45]. Using the β-cell line INS-1 cells *in vitro*, treatment with the nonspecific agonist NECA or A1, A2A, and A3 agonists reduced insulin secretion in a dose dependent manner. The effect of NECA was completely antagonised by A2B receptor inhibition [48].

In two mouse models of diabetes (cyclophosphamide treated NOD and MLDS), A1 receptor agonism mitigated diabetes but was less efficacious than the nonspecific agonist NECA [43]. In our hands, antagonism or agonism of the A1 receptor did not influence the rate of diabetes in C57BL/6 wild-type (WT) mice (Chia et al., submitted manuscript).

The A2A receptor is widely expressed on both tissues and circulating cells. Mice lacking the A2A receptor (A2ARKO) are highly susceptible to MLDS-induced diabetes with rapid onset (within 10 days) and 100% diabetes incidence. Like CD39KO mice, the A2ARKO mice are immunocompromised and have hyperproliferative T cells [3]. To delineate the site-specific importance of the A2A receptor, a series of adoptive transfer experiments were performed. Deletion of the A2A receptor either on the tissues or the circulating cells increased the susceptibility of these mice to the effects of MLDS (Chia et al., submitted manuscript). NECA ameliorated diabetes in A2ARKO mice and treatment with an A2AR agonist had no effect in wild-type mice following MLDS [43].

The prevention of MLDS-induced diabetes in CD-1 mice by NECA was reversed by pretreatment with a selective A2B receptor inhibitor [43]. Similarly, we have identified a role for the A2B receptor particularly in the early response to MLDS. The rise in blood glucose following MLDS in C57BL/6 wild-type mice was quicker, reaching hyperglycemia by 8–10 days,

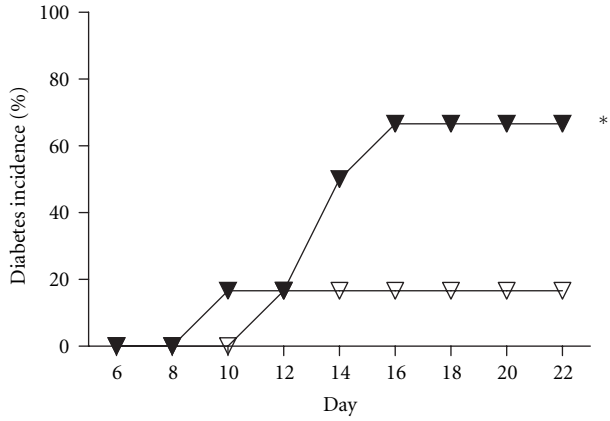

Figure 2: Inhibition of A2B receptor in CD73KO mice increases susceptibility to MLDS-induced diabetes. Diabetes incidence of CD73KO mice treated with either saline (open triangles, $n = 6$) or the A2BR inhibitor (dose: 0.5 μg/g body weight (BW), twice daily) (black triangles, $n = 6$). $^*P < 0.05$ versus saline-treated mice.

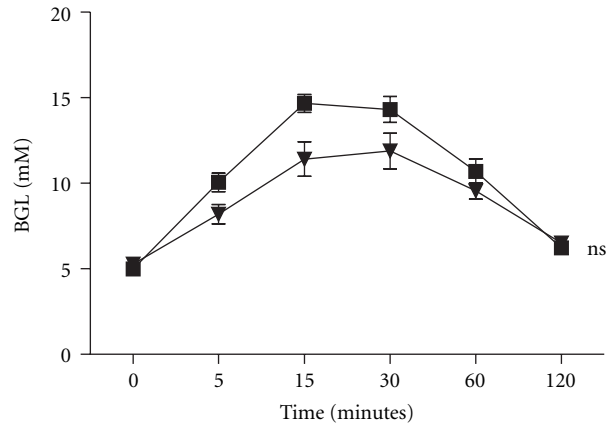

Figure 3: Normal glucose handling in CD73KO mice. Blood glucose levels following 1 mg/g BW of intraperitoneal glucose. WT mice (black squares, $n = 6$); CD73KO mice (black triangles, $n = 8$); ns—not significant versus WT mice.

although the overall rate of diabetes was unchanged (Chia et al., submitted manuscript).

The protection conferred by CD39 overexpression was mitigated by deletion of the A2A receptor or by pharmacological inhibition of the A2B receptor. Complete blockade of both receptors did not further exaggerate the diabetic phenotype (Chia et al., submitted manuscript). Involvement of more than one adenosine receptor parallels the effects of adenosine in renal IRI, where A2A receptor signaling predominates on circulating $CD4^+$ T cells [49] and macrophages [50], while A2B receptor signaling within the renal parenchyma is also important [51]. Intriguingly, CD73KO mice coadministered with an A2B receptor inhibitor became susceptible to the effects of MLDS, with an onset of diabetes at day 10 and a diabetes incidence of 66% (Figure 2).

4. Nonimmune Type 2 Diabetes (T2D)

Insulin resistance characterises T2D, however, β-cell dysfunction must coexist for hyperglycemia to occur. Indeed it is progressive β-cell dysfunction that underpins the progression from normoglycemia to impaired glucose tolerance to overt diabetes.

4.1. Role of CD39 and CD73 in T2D. Mice deficient in CD39 demonstrate impaired glucose tolerance following oral glucose tolerance testing a consequence of hepatic insulin resistance rather than peripheral muscle resistance. There was an associated increased level of hepatocyte c-Jun NH_2-terminal kinase (c-JNK) in response to extracellular nucleotides and aberrant insulin receptor substrate (IRS)—2 phosphorylation in the liver of these mice [28]. There was no abnormality in glucose handling following an intraperitoneal glucose load in mice overexpressing CD39 [30] nor intriguingly in CD73KO mice (Figure 3). In human T2D, CD39 expression has been determined in peripheral blood mononuclear cells. Poor glycemic control was associate with proportions of CD39+ cells particularly within the CD19+ subset. Further an increase in CD39 enzymatic activity was observed in this patient cohort [52]. Further, platelet-associated CD39 enzymatic activity was increased in patients with T2D, hypertension, and coexisting T2D and hypertension. Platelet-associated CD73 enzymatic activity was only increased in patients with hypertension or coexisting hypertension and T2D and not T2D alone [53]. CD39 expression also influences the susceptibility to diabetes-induced renal disease in both mice [54] and humans [55]. In African Americans, a common ENTPD1 (CD39) two-single nucleotide polymorphism haplotype was associated with an increased risk for end stage renal disease secondary to T2D.

4.2. Adenosine Signalling in T2D. All adenosine receptors are expressed at the mRNA level in skeletal muscle of mice [56] and the role of adenosine receptor blockade in reversing insulin resistance in skeletal muscle from diabetic rats has been realised for some time [57, 58]. In keeping with this treatment of wild-type C57BL/6 mice with NECA promoted impaired glucose tolerance by inhibiting glucose disposal [59]. Although initially thought to be mediated by the A1 receptor, studies with A1RKO [56] and A2RKO [59] mice show that these receptors have a minimal effect on skeletal muscle uptake of glucose. Rather it appears that activation of A2B receptor promotes peripheral insulin resistance and blockade of the receptor in diabetic KKA^Y mice enhances glucose disposal into skeletal muscle and adipose tissue as well as reducing hepatic glucose production [59]. Further, in Goto-Kakizaki rats, which resemble T2D, insulin levels were increased temporarily following A2B receptor inhibition, although without effecting blood glucose level [48]. There may however be a role for A1 receptor activation through the suppression of lipolysis and free fatty acid levels (FFA) [60] both of which are involved in the pathogenesis of

T2D. Indeed, mice overexpressing the A1 receptor in diet-induced insulin resistant mice have lower FFA levels and insulin resistance compared to controls [61]. The effect of the null mutation of A1R on glucose homeostasis following a high fat diet is controversial: Faulhaber-Walter et al. [62] demonstrated decreased glucose tolerance with increased BGL and insulin levels in A1RKO mice (C57BL/6 and Swiss compared to controls) as early as 5 weeks following a high fat diet. Yang et al. [63], however, reported A1RKO mice (C57BL/6) clear blood glucose more efficiently, however, following a high fat diet both WT and A1RKO mice develop glucose intolerance.

4.3. Adenosine and the Incretin Effect. The incretin hormones glucagon-like peptides-1 (GLP-1) and glucagon intestinal peptide (GIP) are released from the gastrointestinal tract in response to food and promote insulin secretion in a glucose concentration-dependent manner in β-cells and inhibit glucagon secretion. The incretins are rapidly metabolised by dipeptidyl peptidase-4 (DPP-4) and drugs that inhibit this enzyme are very effective in the treatment of T2D. DDP-4, also known as CD26 or adenosine deaminase (ADA), enzymatically and irreversibly converts adenosine to inosine. ADA activity has been found in most organs but is notably high in adipose tissue, liver, skeletal muscle and heart. An increase in ADA activity has been reported in patients with T2D and a relationship with insulin resistance has been postulated [64]. High ADA activity is associated with low adenosine levels; however a direct relationship between adenosine and the incretin effect in T2D has not yet been defined.

5. Concluding Remarks

The CD39-adenosinergic axis is involved in the pathophysiology of pancreatic dysfunction and thus drug development targeting different components of the pathway may be of relevance in the treatment of both type 1 and type 2 diabetes. There remain a number of unanswered questions including the source of CD73 enzymatic activity given the lack of expression within the pancreas; the mechanisms behind protection observed with CD73 deletion in MLDS- induced diabetes and the role of purinergic signalling in the incretin effect, which is of particular importance in the pathogenesis of T2D.

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