Novel T-cell inhibiting peptides delay the onset of Type 1 diabetes in non-obese diabetic mice

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Abstract

The aim of this study was to investigate the effectiveness of immunomodulatory peptides in preventing the spontaneous onset of Type 1 diabetes in NOD mice. Two such peptides, CP and C1, were injected intraperitoneally in NOD mice three times a week starting at two different time points, nine weeks and 11 weeks of age, and blood sugar levels monitored for the development of diabetes. CP was shown to be effective in delaying the onset of diabetes compared to control (P=0.006). The timing of peptide administration was crucial since delay in treatment did not prevent the onset of diabetes (nine weeks versus 11 weeks of age). C1 was effective in delaying the onset of Type 1 diabetes with borderline significance when given at week 11 (P=0.05). These findings confirm the efficacy of these peptides in the prevention and possible treatment for Type 1 diabetes and thereby create new opportunities for genetic manipulation.

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1. Introduction

Type 1 diabetes (T1D) is an autoimmune T-cell mediated disease leading to elevated blood glucose levels (BGLs). T1D is a common disease affecting 87,100 individuals in Australia, who require daily insulin injections [1]. These individuals must also monitor their BGLs, diet and exercise regularly to ensure control of their diabetes. Unfortunately, many of these patients will subsequently develop secondary complications from their disease and place an enormous personal and financial burden on the community and government. In Australia, the total annual cost (healthcare costs, the cost of carers and Commonwealth government subsidies) is $570 million, with $4669 being the average annual cost per person [2]. For individuals that have not developed secondary complications, the average annual cost is $3468, however if secondary micro- and macro-vascular complications develop, the average annual cost rises to $16,698 [2].

Several potential treatment options for patients with end-stage diabetic renal failure include simultaneous pancreas and kidney transplantation; and more recently, to a subgroup of very brittle diabetics transplantation of islets [3,4]. The problem with both of these is rejection of the graft or islets, and the secondary complications arising from the use of immunosuppressive therapy, such as prednisone, tacrolimus and cyclosporin. Newer approaches and agents such as interleukin (IL-18 and IL-7Ra inhibitors, have been used and examined with limited success [5,6].

Core peptide (CP; GLRILLLKVV) is a novel immunosuppressive peptide that has the potential to treat T1D by inhibiting the activation of T-cells, and thus the T-cell mediated destruction of islets. CP consists of two basic positively charged and hydrophilic amino acids (arginine and lysine) separated by four neutral and hydrophobic amino acids [7]. The functional significance of this peptide was published in 1997 when it was shown that CP inhibited T-cell activation in vivo and in vitro [8]. In animal studies, CP given subcutaneously significantly reduced the induction of T-cell mediated inflammation in adjuvant induced arthritis (AIA), cyclophosphamide-induced diabetes, experimental allergic encephalomyelitis (EAE) and delayed type contact hypersensitivity [8,9].

C1 (GLDRLDLDLLKD) is a cyclic peptide similar in sequence to CP which has alternating D-, L- amino acids, increasing the peptide’s stability and resistance to proteolytic
2.3. C1 consists of two basic positively charged and hydrophilic amino acids (arginine and lysine) separated by five neutral and hydrophobic amino acids. C1 has the same ability to inhibit T-cell activation and IL-2 production as CP and in comparative studies, C1 was shown to be more effective than CP in its ability to inhibit T-cells \( P < 0.012 \) [12]. In animal models of asthma and arthritis, C1 has been shown to be effective in suppressing the immune response [12].

This study examined the effects of CP and C1 on the onset of diabetes in non-obese diabetic (NOD) mice. This model exploits the fact that approximately 60–80% of NOD mice spontaneously develop T1D by a similar mechanism seen in human T1D [13,14] and aims to address if CP and C1 can inhibit the onset and severity of diabetes.

2. Methods

2.1. Animals

All experimental protocols and methods were approved by the Western Sydney Local Health District (WSLHD) Animal Ethics Committee (AEC) and all experiments were performed in accordance with its guidelines. Female non-obese diabetic (NOD) mice were obtained at 8 weeks of age from the Animal Resources Centre (Murdoch, WA, Australia) and housed in the WSLHD Animal facility located within Westmead Hospital. Food and water was given ad libitum. The mice were randomly placed into a control group \( (n = 22) \) and treatment groups. In one group CP was given from nine weeks of age \( (n = 19) \); and in the second group CP was given from 11 weeks of age \( (n = 19) \). A third group was given C1 from 11 weeks \( (n = 14) \). Mice were deemed diabetic when the BGLs \( \geq 15 \) mM on two consecutive readings.

2.2. Treatment

Peptides were dissolved in milliQ water and were sonicated for 30 min. The solutions were filter sterilised and stored at \( 4^\circ \)C. CP was administered intraperitoneally (i.p.) at a concentration of 0.4 mg three times a week from either nine weeks or 11 weeks of age prior to the onset of diabetes. C1 was administered i.p. at a concentration of 0.1 mg three times a week from 11 weeks of age and the control group received no treatment. All treatments ceased at 22 weeks.

2.3. Monitoring BGLs

Mice in all groups were fasted for at least 2 h prior to the measurement of their BGLs three times a week.

2.4. Kaplan-Meier survival plot

A Kaplan-Meier survival plot was used to assess the percentage of NOD mice that developed diabetes over time. This statistical method is a common technique, performed to analyse survival-time data, when there is “censored” data. Censored data can describe a situation where the vital event has not occurred; in this case, a mouse that has not yet developed diabetes.

2.5. Insulitis scoring

Histological analysis was performed on pancreatic tissue collected from control and CP

\( W_9 \) mice at 22 weeks. A proportion of pancreatic tissue in the CP

\( W_9 \) group was collected three weeks after treatment ceased (at 25 weeks) and insulitis was scored separately from tissue collected at 22 weeks. The severity of insulitis was scored for at least 10 islets in each group according to Jimeno et al. [15]. When there was no lymphocytic infiltration, the sample was assigned “0, no-insulitis”. When lymphocytic infiltrations were seen around the islet and up to 30% of the islet, the sample was assigned as “1, peri-insulitis”. “2, moderate insulitis” was assigned to samples where there were lymphocytic infiltrations between 30% and 50% of the islet. Samples with lymphocytic infiltrations of more than 50% of the islet were assigned as “3, severe insulitis”. Kruskal-Wallis nonparametric analysis of variance and Mann-Whitney tests were used to test for differences in insulitis scores and for pair-wise comparisons between groups, respectively.

3. Results

As expected, 64% of the mice in the control group developed diabetes and only 36% of mice remained diabetes-free during a 13-week period of observation (week 9–22). The earliest onset
of diabetes in the control group was at 11.4 weeks of age. By contrast in the CP treated group commencing at nine weeks of age (CPW9) 79% of the mice remained diabetes-free and only 21% developed diabetes ($P = 0.006$). In this treatment group, the onset of diabetes was delayed by four weeks compared to the control group. In the CP treated group commencing at 11 weeks of age (CPW11) 63% of the mice remained diabetes-free and 37% of the mice developed diabetes, which was not significantly different from the control group. The earliest onset of diabetes noted in this treatment group was 11.4 weeks.

In mice given C1 from 11 weeks of age (C1W11), 71% of mice remained diabetes-free while 29% of the mice developed diabetes.

Fig. 2. Blood glucose levels of mice with or without immunosuppressive peptide treatment: a: untreated mice ($n = 22$); b: mice given CP from nine weeks of age (CPW9, $n = 19$); c: CP given from 11 weeks of age (CPW11, $n = 19$); d: mice given C1 from 11 weeks of age (C1W11, $n = 14$).
diabetes \((P = 0.05)\) during an 11-week period of observation. The earliest onset of diabetes in the C1 treatment group was delayed by one week to 12.4 weeks. These results suggest that prophylactically administered CP was able to prevent the onset of T1D in NOD mice for more than 90 days. Any delay in treatment was not effective.

The Kaplan-Meier survival plot (Fig. 1) shows the number of mice that developed diabetes after three months observation and the BGL curves for each group are shown in Fig. 2. Histology of pancreatic tissue obtained from mice at the end of the study showed that CP ameliorated the T-cell mediated destruction of islets (Fig. 3). The stacked bar curve (Fig. 4) shows the

![Image](a)

![Image](b)

![Image](c)

![Image](d)

Fig. 3. Immunohistochemical staining of pancreatic islets: a: haematoxylin and eosin stain of pancreatic tissue depicting an islet; b: insulin stain within an islet; c: haematoxylin and eosin stain of pancreatic tissue from a mouse treated with CP that did not develop diabetes, depicting an islet (lower arrow) surrounded by lymphocytes (upper arrow); d: haematoxylin and eosin stain of pancreatic tissue from a mouse treated with CP that did not develop diabetes. Despite the lymphocytic infiltration the islets are still functional as depicted by the insulin stain.

![Image](Fig_4)

Fig. 4. Stacked bar chart comparing the distribution of insulitis scores between control and CPW9 groups. The severity of insulitis was reduced \((P = 0.010)\) in islets from the CPW9 group \((n = 11)\) compared to islets in the control group \((n = 41)\). No difference in insulitis \((P = 0.076)\) was seen in islets from the CPW9 group regardless of whether the pancreas was collected immediately or three weeks after end of treatment \((n = 80)\).
distribution of insulitis scores between controls and the CPW9 pancreatic tissue collected three weeks apart. There was a statistical difference in insulitis scores between the three groups (P = 0.024). In particular, the insulitis scores in the control group were significantly higher than those in the CPW9 group (P = 0.01), indicating that treatment with CP reduced the severity of lymphocytic infiltration. Although it appeared that insulitis increased when CP was withheld for the final three weeks, there was no statistical difference between insulitis scores in the CPW9 (three weeks after end of Rx) group and the CPW9 group (P = 0.076).

4. Discussion

The ability to prevent the onset of diabetes and maintain euglycaemia would have dramatic and significant effects in any T1D patient if achieved. The Diabetes Control and Complications Trial and Follow-up Study (DCCT) found that maintaining normal blood glucose levels reduces the risk of secondary complications [16]. There was a 76% decreased risk of eye disease, 50% reduced risk of kidney disease and a 60% reduced risk of nerve disease. A continuation of the DCCT, the Epidemiology of Diabetes Interventions and Complications study (EDIC) found that there was a 42% decreased risk in cardiovascular disease occurrence and a 57% reduced risk of nonfatal heart attack, stroke or death from cardiovascular causes [17]. Therefore, there is a significant advantage that even delaying the onset of T1D by a few years can dramatically reduce the incidence of secondary complications.

In NOD mice, by the time mice are symptomatic at 12–14 weeks, 60–90% of the islet cell mass had already been destroyed [18]. By pre-treating NOD mice with CP from nine weeks of age, the onset of T1D could be delayed. As shown histologically, CP was able to influence the T-cell response with less cellular infiltrate and inflammation. CP given at 11 weeks of age did not delay the onset of disease. By contrast, C1 may be a more powerful immunosuppressive agent than CP in preventing the progression of T1D because when given at week 11 CP had no effect, however treatment with C1 was borderline significant compared to the control group (P = 0.05).

CP acts on T-cells at the transmembrane level to inhibit signal transduction following antigen recognition [7]. Published studies have indicated that CP interferes with the interactions between TCR-α, CD3ζ and ζ dimers [9,19]. Prevention of a completely assembled TCR-CD3-ζζ complex inhibits signal transduction and activation of T-cells. By giving CP i.p. to NOD mice at an early age, it was hypothesised that CP will inhibit antigen activation of islet-targeting T-cells. The mechanism of action for C1 has not been clearly defined but presumed to be similar to CP.

A limitation of the results could be the low animal numbers (14 mice in the C1 treated group) and increasing this number may possibly lead to a more significant difference compared to controls. Furthermore, treatment of mice with C1 from the earlier time point, nine weeks of age as in the CPW9 group, could influence the results. Administering treatment to coincide with the beginning of insulitis, which begins even earlier than nine weeks of age, at three to four weeks of age in NOD mice [14], could dramatically improve the results and will be further investigated. Although there was a statistical significance noted between the BGLs of CP-treated and non-treated mice, further studies and longer periods of observation are needed. Subsequent studies could also include the analysis of the T-cell phenotype in the spleen and pancreatic lymph nodes of protected mice, measuring the changes in cytokine production by T-cells in the presence of CP and C1 as well as trialling C1 as an oral agent.

Cyclosporin is also a cyclic peptide that is an immunosuppressant that has been used in the treatment of T1D [20,21]. Cyclosporin inhibits T-cell proliferation and the destruction of islets by blocking calcineurin and reducing IL-2 production [22]. Treatment with cyclosporin after clinical presentation of T1D has been shown to conserve insulin production, however the protective effects were not permanent [23,24]. Furthermore, the cyclosporin dose required to induce diabetes remission leads to nephrotoxicity and other adverse effects [21,25]. Development of new immunosuppressives, such as C1 and CP, could provide further drug choices in the treatment of T1D. Furthermore, the linear sequence of CP can be converted into cDNA, unlike cyclosporin, and may be used to induce tolerance when transduced into dendritic cells or provide a novel means of immunosuppression using gene therapy to transduce islets and prevent islet cell destruction.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data (French abstract) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.diabet.2014.01.007.

References


