Usefulness of photodynamic therapy using NPe6 for the prolongation of survival rate in BB/W rats with diabetes mellitus

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Abstract

Biobreeding/Worcester (BB/W) rats, a model of type 1 diabetes mellitus in which association with insulitis is a characteristic feature, were used to evaluate whether photodynamic therapy (PDT) can improve the survival rate of diabetic BB/W rats. Immunohistochemical examination of the BB/W rats immediately after the onset of diabetes mellitus showed accumulation of macrophages in the pancreas associated with insulitis. Rats were sacrificed and autopsied 24 hours after the administration of photosensitizer mono-L-aspartyl chlorin e6 (NPe6), a photosensitive substance, through the caudal vein. Examination of frozen sections of the pancreas revealed the existence of fluorescence originating from NPe6 in sections exposed to laser, implying that NPe6 was taken up by macrophages of the pancreas.

In another sets of BB/W rats of the same litter were divided into two groups; a laser-exposed group and a control group. In the laser exposed group, NPe6 (5 mg/kg) was injected into the caudal vein immediately after the onset of diabetes, and then, 24 hours after the injection, laser (100 mW/50 J/cm²) was applied externally from outside the peritoneal cavity. Control rats were also given NPe6 via the caudal vein, but they were not exposed to laser. The survival rate, when evaluated 70 days after the onset of diabetes, was higher in the laser-exposed group (36%) than in the control group (0%). The results suggested that the destruction of macrophages by oxygen radicals produced by laser exposed NPe6 containing macrophages ameliorated insulitis and contributed to the prolongation of survival rate in diabetic BB/W rats.

Introduction

Type 1 diabetes mellitus (type 1 DM) is a condition in which the destruction of pancreatic β-cells causes remarkably decreased insulin secretion, resulting in absolute insulin deficiency and elevated blood glucose level. This condition can be divided into two types depending on the etiology: the autoimmune type (type A) and the idiopathic type (type B). In type 1A DM, insulitis first develops in the pancreas, leading to the destruction of pancreatic β-cells, and resulting in marked decrease in insulin secretion and elevation of plasma glucose. Non-obese-diabetic mice (NOD mice) and Biobreeding/Worcester rats (BB/W rats) are known as animal models of type 1A DM.

Almost all (80-90%) of BB/W rats develop diabetes around 60-100 days after birth. The onset of the diabetes in these rats is preceded by insulitis due to the
macrophages and lymphocytes infiltration in the pancreas, and this insulitis is considered to be the initiation of pancreatic β-cell destruction\(^{2,3}\). Therefore, if accumulation of macrophages in the pancreas could be prevented, it might be possible to inhibit insulitis, and suppress the onset and progression of diabetes.

Mono-L-aspartyl chlorin e6 (NP6) is a photosensitizer, and when exposed to laser light, it releases oxygen radicals which can cause the destruction of NP6-containing tissue\(^{4,5}\). The substance can be taken up by various tissues in vivo and is retained for long periods specifically in cancer cells and macrophages\(^{6,7}\).

These characteristics could induce specific destruction of cancer cells and macrophages, and leave healthy tissue intact\(^{6,7}\). There is a possibility that oxygen radicals which are produced by laser exposure to NP6 containing macrophages destroy macrophages in the islet cells, and ameliorate insulitis which leads to the prolongation of survival rates in BB/W rats. The present study was undertaken to examine if the survival rate of diabetic BB/W rats can be prolonged by applying laser exposure to NP6 containing macrophages in islet cell in BB/W rats.

Materials and methods

BB/W rats were bred at the Animal Research Center of Tokyo Medical University. The animal experiments complied with the Guideline for the care and use of laboratory animals (NIH publication No 85–23, revised 1985).

1. Confirming the onset of diabetes mellitus

Beginning on the 40th day after birth, each BB/W rat received a daily urinary glucose test. In the rats with positive urinary glucose, blood glucose was measured by the glucose oxidase method (Sanwa Kagiku Kenkyusho Co., Nagoya Japan). Rats with blood glucose levels over 200 mg/dl were considered to be diabetic and were used in the present study.

2. Checking for the presence of macrophages in the pancreas of diabetic rats

Immediately after the onset of diabetes in each BB/W rat, immunohistochemical examination of the pancreas was performed to detect the presence of macrophages. When the blood glucose level exceeded 200 mg/dl, the animal were immediately sacrificed under ether anesthesia and autopsied. The pancreas was removed and a part of the pancreas was used for preparing frozen sections (5-μm-thick) which were stained immunohistochemically using macrophage-specific antibody Ki-M2R (BMA Co., August, Switzerland).

3. Confirmation of NP6 uptake by macrophages

In another experiment, diabetic BB/W rats received an injection of NP6 (5 mg/kg) into the caudal vein on the day of confirmation of the onset of diabetes. Twenty-four hours later, rats were sacrificed under ether anesthesia and autopsied, and frozen sections of the pancreas were prepared. The frozen sections were then exposed to laser light (633 nm) and examined under a fluorescence microscope. Emission of fluorescence at 672 nm was deemed to be indicative of the uptake of NP6 by macrophages.

4. Survival rate analysis

In third sets of experiments on the day of confirmation of the onset of diabetes mellitus, BB/W rats were divided into two groups, laser exposed group and laser unexposed (control) group. Laser exposed group (n=14) received NP6 (5 mg/kg) through the caudal vein, followed by application of Diode laser (100 mW/50 cm\(^2\), 633 nm) to the abdomen Diode Laser (Panasonic, Tokyo Japan) 24 hours later. Control group (n=10) also similarly received NP6 (5 mg/kg) through the caudal vein, but were not exposed to laser light. The number of surviving BB/W rats was counted until 70th days after the laser application, and the survival rate was calculated. The Kaplan-Meier method was used for inter-group comparisons of data. The significance of differences was tested using the log test. A value of P less than 0.05 was regarded as indicating a statistically significant difference.

Results

Immunostaining of the pancreas of diabetic BB/W rats with antibody specific for rat macrophages showed positively stained cells in the islets; this was an indication of accumulation of macrophages in the islets with insulitis. (Fig. 1)

Table 1. The experimented design using two groups of diabetic (BB/W) rats

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<tr>
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<th>NP6</th>
<th>Laser (50 J/cm(^2))</th>
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<tbody>
<tr>
<td>Control group</td>
<td>10</td>
<td>Injected into the caudal vein</td>
</tr>
<tr>
<td>Treatment group</td>
<td>14</td>
<td>Injected into the caudal vein</td>
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Fig. 2 shows fluorescence image of frozen section of the rat pancreas. In BB/W diabetic rats with NP6 injection and laser light exposure, red fluorescence from NP6 was observed at the sites in the pancreas where macrophages had been detected. Cells with strong red staining arrow were thought to be macrophages because NP6 was washed out from normal cells but kept in macrophages 6 hours after the administration of NP6.

Our group already reported that oxygen radical was produced only when cells were laser-exposed under the existence of NP6 in the cells\(^{8-10}\). Therefore, in this study, we compared the survival rate in the two groups when the existence of NP6; laser exposed and laser-exposed.
Fig. 1 Immunostaining of the pancreas with macrophage-specific antibody
Left: In rats without positive cells were absent insulitis, macrophage-specific antibody
Right: In rats with insulitis, macrophage-specific antibody positive cells were sporadic present.

Fig. 2 NPe6 was injected into the caudal vein immediately after onset of diabetes, and the animal was necropsied 24 hours later. Frozen section of the pancreas were prepared, and fluorescence was obtained when exposed to laser light. The white arrow indicate a cell displaying NPe6 fluorescence.

unexposed control groups. The survival rate was 36% (5/14) in laser-exposed group, and 0% (0/10) in control group. All the rats of the control group died within 45 days after the onset of diabetes, while 5 out of 14 laser-exposed rats survived until the end of the experiment (70 days). All rats showed blood sugar levels more than 400 mg/dl with the symptoms of dehydration at the time of death, indicating that the death resulted from ketoacidosis due to insulin deficiency.

Cumulative difference in the survival rate after the onset of diabetes is shown in Fig. 3, the survival rate in laser-exposed group showed significantly higher than in control group at the 15th day and thereafter. The mean survival period after the onset of diabetes mellitus was 44.7 days in laser-exposed group and 19.5 days in control group.

Discussion

First, this study clarified that NPe6 remained in macrophages, while it disappeared from normal cells 24
hours after the NP6 injection, since fluorescence was observed in macrophages but not in normal cells. Similar results were already reported obtained in the New Zealand white rabbit. In the atherosclerotic lesion in the NP6 was trapped by macrophages in the blood vessels of New Zealand white rabbits, as well as other cells but remained only in macrophages after 6 hours the administration. Furthermore, we have already reported that the laser irradiation caused the apoptosis of macrophages by oxygen radicals produced under the existence of NP6. Therefore, our present data suggest that oxygen radicals produced by laser irradiation in NP6 containing macrophages in islets caused the death of macrophages. In Type I diabetes animal models, BB/W rats and NOD mice develop insulin after 6 months. The majority of cells infiltrating the pancreatic islet are CD4-positive T lymphocytes, and T lymphocytes have been reported to be important in the development of insulin since administration of anti-CD4 positive T lymphocyte antibodies inhibited insulin. However, there have been a few reports addressing the questions of why macrophages are important for the development of type 1A diabetes. We hypothesize that oxygen radicals produced by laser irradiation in the NP6 containing macrophages in pancreatic islets destroy macrophages, and ameliorate insulinitis, which lead to the prolongation of survival rate in BB/W rats. To examine whether the removal of macrophages in diabetic BB/W rats immediately after the onset of diabetes can inhibit the development of diabetes and prolong the survival rate, we conducted the second study by photodynamic therapy (PDT) using NP6. Since oxygen radicals were only produced in the cells when the laser was irradiation under the existence of NP6. Our hypothesis was supported by the results that BB/W rats with laser exposure to NP6 showed the prolongation of survival rate. We presumed that the elimination of macrophages by PDT ameliorated the insulinitis in islet cells, and prolonged the survival rate in BB/W rats, although we did not conduct an immunohistochemical observation of pancreatic islets using insulin antibodies.

Oxygen radicals usually exert adverse effect in disease-affected animals, however, they exert favourable effects in some conditions. In this study, oxygen radicals exerted favourable effect through destroying macrophages, which led to ameliorate insulinitis in BB/W rats. When excitation is given in the wavelength NP6 absorption band, the energy level of the molecule is transposed from the ground state to the singlet-excited state. Fluorescence is generated when the energy is regained from the singlet-excited state to the ground state. We confirmed the NP6 existence which macrophages uptook by observing the fluorescence images. Most energy, which is not regained directly in the

![Energy transition of NP6 in Organella](image)

*Fig. 4* Represents oxygen activation by excitation of NP6

ground state, causes intersystem crossing, spreads to the triplet-excited state of oxygen, and activates oxygen with a singlet-oxygen. This singlet-oxygen may cause cytotoxicity for macrophages. Diabetes is thought to be manifested when more than 90% of the pancreatic islets are destroyed in type 1 DM. However, the presence of a small amount of insulin in the blood can inhibit development of ketoacidotic coma. In fact, the prolongation of survival rate in diabetic BB/W rats was presumed to be due to the prevention of the development of ketoacidotic coma in this study since all the rats showed blood glucose levels more than 400 mg/dl with symptom of dehydration at the time of death. If this therapy can be applied to humans, we could expect that blood glucose control in type 1 diabetic patient can be more easily achieved with the existence of a small number of intact pancreatic β-cells. However, there are still several problems for clinical use, such as the difficulty of identification of the specific gene expression related type 1 DM, and detection method of finding prestages of type 1 DM, and method of laser exposure to pancreatic islet cells in humans.

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**References**


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Npe6 を用いた光線療法の BB/W ラットの survival rate に及ぼす影響

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【要旨】Type 1A 糖尿病は胰島に T リンパ球、マクロファージといった単核細胞が浸潤する事により β 細胞のインスリン分泌が低下して発症する。Npe6 は光感受性物質でありその吸収帯のレーザーを照射すると光化学反応により活性酸素を生じ、細胞に障害を与えることができる。また Npe6 は正常組織からは投与後速やかに消失するが、マクロファージには長くとどまるという特徴を持っている。BB/Wrat に Npe6 を注入し、時間を経て一旦と筋肉にレーザーを照射する事で選択性にマクロファージを傷害する事が可能であるかどうか、またこのことにより insulitis の進行を抑制し、糖尿病の進展を遅らせ生命予後を改善できるかを検討した。Npe6 のマクロファージへの取り込みはマクロファージ特異抗体である KiM2R を用いた免疫染色で確認された。BB/Wrat は血糖値 200 mg/dl を超える日を糖尿病発症日とした。この発症日に尾静脈から Npe6 を注入し 24 時間後にレーザーを照射した群と照射しないコントロール群にわけた。照射は 100 mw/50 Jcm² で体外から行い、その後の survival rate を追跡した。照射群の発症後 70 日の生存率は 36% であり、コントロール群の 0% に比べ有意に高かった。Type 1A 糖尿病のモデル動物である BB/Wrat において Npe6 を用いた光線療法により survival rate の改善がみられた。Type 1A 糖尿病の進展にはマクロファージが関与する可能性が示唆され、マクロファージの排除が生命予後改善に貢献できる可能性が推測された。

＜Key words＞ BB/W ラット、insulitis、マクロファージ、Npe6、光線療法