Research Paper

Calcitonin gene-related peptide gene therapy suppresses reactive oxygen species in the pancreas and prevents mice from autoimmune diabetes

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Abstract: Reactive oxygen species (ROS) is involved in autoimmune destruction of islet β cells, which has been proven to be an important underlying pathogenesis for insulin dependent diabetes mellitus (IDDM). Calcitonin gene-related peptide (CGRP) is a widely distributed neuropeptide, which has been found to play an important role in protecting myocytes from ROS. We hypothesized that exogenous CGRP gene administration before the pathogenic stage of insulitis might suppress the production of ROS and provide a hopeful therapeutic intervention for autoimmune diabetes. We performed CGRP gene transfer by injecting naked plasmid directly into skeletal muscles of mice with electroporation enhancement to achieve a continuous expression of CGRP in skeletal muscles, and thereby its secretion into the circulation. The effect of CGRP gene transfer on the pathogenesis of diabetes was studied in autoimmune diabetic mice induced by multiple low dose streptozotocin (MLDS). The CGRP gene therapy decreased morbidity of autoimmune diabetes, and significantly ameliorated hyperglycemia in these mice. CGRP gene transfer inhibited the production of ROS and malondialdehyde (MDA). In addition, it enhanced the activity of catalase (CAT) and superoxide dismutase (SOD) significantly. The data suggest that intramuscular CGRP gene transfer ameliorates autoimmune destruction of islet β cells, resulting in significant reduction in diabetes incidence of MLDS diabetes mice. CGRP benefits might be mediated at least in part by inhibiting the oxidative stress in islet β cells of these mice.

Key words: insulin-dependent diabetes mellitus; calcitonin gene-related peptide; gene therapy; reactive oxygen species

降钙素基因相关肽转基因预防小鼠自身免疫性糖尿病发病及其抗氧化应激机制

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摘 要： 有证据表明，活性氧(reactive oxygen species，ROS)参与了胰岛β细胞自身免疫损伤，是自身免疫性糖尿病发病的重要原因之一。一氧化氮(NO)和过氧化氢(H_2O_2)介导了致炎性细胞因子对胰岛β细胞的损害，引起脂质过氧化反应，进而损伤胰岛细胞。降钙素基因相关肽(calcitonin gene-related peptide，CGRP)在心肌细胞中可抑制ROS生成而具有细胞保护作用。通过CGRP裸质粒肌肉注射体细胞电针强化转基因方法，使骨骼肌持续表达CGRP，观察其对小剂量多次注射链脲佐菌素(streptozotocin，STZ)造成的小鼠自身免疫性糖尿病发病的影响，进一步测定胰腺局部活性氧含量以及抗氧化酶的改变，探讨其抗氧化应激机制。结果发现，CGRP裸质粒直接注射入小鼠双后肢胫前肌，继以程控电针刺激导入(体内电穿孔法)，可使血浆和骨骼肌组织CGRP表达水平显著增高，且持续4周以

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上；注射 STZ 同时给予 CGRP 转基因治疗可减轻胰岛 β 细胞损伤，显著降低自身免疫性糖尿病的发病率及血糖水平；CGRP 转基因可显著抑制自身免疫性糖尿病小鼠胰腺局部活性氧和丙二醛的生成，增加过氧化氢酶(CAT)及超氧化物歧化酶(superoxide dismutase, SOD)的活性，结果提示，CGRP 裸质粒直接注射、电针辅助导入转基因可获得 CGRP 持续高水平的表达，能够预防小鼠自身免疫性糖尿病的发病，其机制之一可能为 CGRP 抑制了活性氧对胰岛 β 细胞的损伤。

关键词：自身免疫性糖尿病；降钙素基因相关肽；基因治疗；活性氧
中图分类号：Q343.1；R392.32

Insulin dependent diabetes mellitus (IDDM), a common autoimmune disease, is likely caused by autoimmune destruction of insulin-producing β cells in the islets. Many studies have demonstrated that T lymphocyte subsets play important roles in the pathogenesis of most autoimmune diseases[1-3]. The imbalance of Th1/Th2 subsets has been indicated to participate in the pathogenesis of β cell damage in type I diabetes[4,5]. The pro-inflammatory cytokines secreted from the Th1 subset, such as IL-2, IFN-γ or TNF-β, induced excessive production of reactive oxygen species (ROS) in β cells and macrophages in the islet, thus leading to destruction of β cells and reduction of insulin secretion[6-10].

Although many risk factors can trigger the development of IDDM, it is likely that ROS plays a central role in β-cell death and disease progression. Accumulating evidence indicated that enhanced antioxidant defense systems reduced the susceptibility to IDDM in animal models or in human studies[11,12]. Pancreas-specific ROS productions plays a critical role in signaling the cellular autoimmune/inflammatory response by activating the transcription factor, NF-kB[9,13]. This cascade results in a cyclic amplification of ROS and eventually leads to apoptosis and/or necrosis of β cells[14,15]. It is believed that antioxidants inhibit the hyperglycemic response through inhibiting NF-kB activation[9].

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide with multiple biological activities. CGRP is mainly synthesized and distributed in the nervous system, as the principal neurotransmitter in C fibers of sensory nerves in humans and mammals[16,17]. In 1997, Khachatryan and colleagues[18] established a model of CGRP transgenic nonobese diabetes (NOD) mice, in which CGRP was expressed under the control of insulin promoter to achieve a targeted expression of CGRP in the islets. A remarkable decrease of IDDM morbidity was observed in their study. However, the exact mechanism was not illustrated. As it is difficult to apply this approach to a clinical setting, it is necessary to further investigate the potential of CGRP therapy and develop more practical methods to continuously deliver CGRP for therapy.

Previous studies in our laboratory indicated that CGRP gene transfer selectively suppressed the pro-inflammatory Th1 subsets and promoted anti-inflammatory Th2 subsets, resulting in amelioration in the autoimmune destruction of islet β cells and significant reduction in diabetes occurrence in multiple low dose streptozotocin (MLDS)-induced diabetic mice[6].

It has been known that CGRP exhibits potent endogenous protective effects through suppressing the production of ROS (including nitrogen free radicals) and releasing oxidation stress in the cardiac myocytes and the kidney[19-25]. In light of its immune regulatory and anti-oxidative stress effects, we hypothesized that CGRP might inhibit pro-inflammatory cytokines secretion and subsequently reduce ROS production, which is responsible for its therapeutic benefits for autoimmune-mediated diabetes.

In the present study, CGRP gene therapy was performed by direct intramuscular injection of naked CGRP plasmid into a diabetic mouse model, MLDS-induced diabetic mice. To enhance gene transfer efficiency, this procedure was assisted with electric stimulation (electroporation) *in vivo*. The production of ROS and MDA, and the activity of CAT and SOD were investigated to elucidate the mechanisms of CGRP therapeutic effects.

1 MATERIALS AND METHODS

1.1 *Animals.* The treatment of laboratory animals and experimental protocols of the study conformed to the guidelines of Peking University Health Science Center and were approved by Institutional Authority for Laboratory Animal Care. All experiments were carried out in male C57BL mice aged 6 weeks, 18-20 g weight, obtained from Experimental Animal Laboratory of Health Science Center of Peking University (Beijing, China). Mice were housed at 22℃ with a 12 h light/dark cycle. To avoid disturbance of blood glucose measurement, the

food and water were given at 8 pm daily.

1.2 *Construction of CGRP gene expression vector.* PTT42 plasmid was kindly provided by Dr. Barry Nelkin (Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland). The plasmid contained 2, 3, 5, 6 exon sequences of human CT/CGRP gene. The plasmid was digested with Pst I to collect a CGRP fragment of 844 bps. The fragment was subcloned into pcDNA3.1(-) (Invitrogen Co., San Diego, CA, USA) and E. Coli. DH5α was transformed to produce large amounts of the plasmids. Plasmid was extracted and purified by Wizard Plus Maxipreps kit (Promega Co., Madison, WI Cat# 7270). A similarly purified pcDNA3.1(-) without inserted gene was used as vector control.

1.3 *Induction of IDDM mice by MLDS.* For induction of MLDS diabetes, 40 mg/kg body weight of streptozotocin (STZ, Sigma Co. St. Louis, MO USA), was dissolved in 0.01 mol/L sodium citrate buffer solution and was given intra peritoneally for 5 consecutive days [26]. Blood glucose level was determined using an instant glucose detector (GLUCOTREND™, Boehringer Mannheim Co., Germany). The mice were considered to develop diabetes when their blood glucose levels were elevated to higher than 12 mmol/L (the control C57BL mice had normal blood glucose of less than 6 mmol/L). A routine hematoxylin and eosin (H&E) staining was performed on the pancreas samples to identify the lymphocyte infiltration around the islets, which was used as evidence for autoimmune mediated insulitis.

1.4 *Intramuscular injection of naked CGRP DNA.* The gene transfer was performed as described previously[27]. CGRP plasmid or control plasmid was injected once at the first day of the consecutive STZ treatments. Plasmid DNA (CGRP or control vector) was diluted with sterile saline to 1 μg/μl. 100 μg of DNA was injected in multiple sites with a 100 μl syringe into two back sural muscles of each mouse. Transcutaneous electric pulses were applied immediately after the DNA injection with Electro Square Porator™ ECM 830 (a commercial *in vivo* gene delivery system, provided by BTX corporation, Hollisston, MA, USA), by two stainless steel needle electrodes placed 5 mm apart and 3 mm in depth at each side of the muscle. The electric pulse stimulation lasted 40 ms each time and administered 10 times. The electric field voltage was 200 V/cm, with a frequency of 1 Hz.

1.5 *Radioimmunoassay (RIA) of CGRP.* CGRP was measured with RIA as described[28]. First, the standards of synthetic hCGRP (Peninsula Laboratory, USA) or the samples were incubated with 100 μl of anti-CGRP antibody (Peninsula Laboratory, Belmont, CA, USA) for 24 h. Then the mixture was incubated for an additional 24 h with 100 μl of ^{125}I-labeled CGRP. Free and bound fractions were separated and the RIA test tubes were counted with a γ-counter for measurement of gamma radioactivity of ^{125}I remaining in the pellets.

1.6 *Detection of ROS by electron paramagnetic resonance (EPR).* The diabetes mice were sacrificed at d 0, 3, 7, 10, 14, 21, 28 after gene transfer and STZ injection. Their pancreases were removed into the EPR tubes and sealed in liquid nitrogen immediately. The EPR spectrum of pancreatic tissue at liquid nitrogen temperature (77 K) is shown in Fig. 1, where the signal heights of g // and g ⊥ correspond to the level of reactive oxygen radicals. Sometimes g// height (mm) was only used as the detection standard because the g ⊥ signal was overlapped with semiquinone (SQ) signal. EPR measurement parameter: the microwave power was 20 mV and the modulation frequency was 100 kHz with 10 guass modulation amplitude, the central field was 3400 guass, the sweep width was 400 guass at temperature of 77 K.

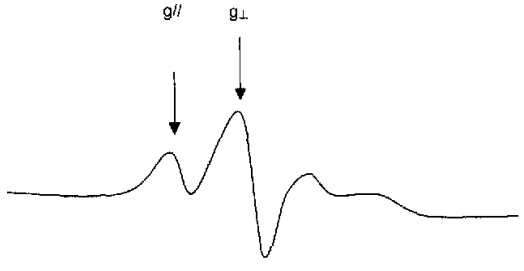

Fig. 1. The low temperature EPR spectrum of a pancreatic tissue at 77K.

1.7 *Determination of levels of malondialdehyde (MDA), activity of catalase (CAT) and superoxide dismutase (SOD).* The levels of MDA and activity of CAT, SOD in the pancreas of experimental animals were determined using test kits (Jian Cheng Biology Research Center, Nanjing, China), based on detection of the rate of their consumption. The levels of MDA was quantified with thiobarbituric acid reaction. SOD activity was assayed by the xanthine oxidase/cytochrome c method. CAT activity was assayed by Goth's colorimetric method, in which tissue was incubated in H_2O_2 substrate and the enzymatic reaction stopped by the addition of ammonium molybdate.

1.8 *Statistical analysis.* The data are expressed as

mean ± SE and analyzed by one-way ANOVA and further analyzed by Student-Newman-Keuls test for multiple comparisons between treatment groups, and by unpaired Student' t test for means between two groups or χ^2 test as indicated. $P<0.05$ indicates significant difference.

2 RESULTS

2.1 Identification of CGRP expression in transgenic skeletal muscles

We injected naked CGRP plasmid into the skeletal muscles with electroporation to achieve gene transfer. CGRP levels in serum and muscle homogenates were measured with CGRP specific RIA at different time intervals after plasmid injection. As shown in Table 1 and 2, in both the serum and the muscle tissue, CGRP levels were significantly increased in the CGRP plasmid injected group. The expressed CGRP maintained at high levels for at least 4 weeks after the gene transfer.

Table 1. Measurement of CGRP in serum with RIA

Days after CGRP transgene	Serum CGRP level (pg/ml)
Vector control + STZ	21.20 ± 2.75
3	76.30 ± 17.26*
7	66.88 ± 12.70*
21	51.32 ± 3.76*
28	52.21 ± 3.22*

* $P<0.05$ compared with the vector control + STZ. $n=6$.

Table 2. CGRP protein expression in transgenic skeletal muscle tissue

Days after CGRP transgene	Muscular CGRP level (ng/mg protein)
Vector control + STZ	0.66 ± 0.20
3	1.92 ± 0.26*
7	2.13 ± 0.21*
21	1.74 ± 0.28*
28	1.45 ± 0.14*
35	1.55 ± 0.24*

* $P<0.05$ compared with the vector control + STZ. $n=6$.

2.2 Effect of CGRP gene transfer on IDDM in MLDS diabetes mice

MLDS diabetes mouse has been frequently used as a model of IDDM to study the autoimmune processes associated with pancreatic β cell pathology and evaluate therapeutic interventions[26]. In MLDS-induced diabetic mice, blood glucose was increased within 1 week after STZ injection, reaching the level of above 15 mmol/L in 3 weeks, and remaining stable for 20 weeks. Diabetes was defined by increased blood glucose level higher than 12 mmol/L.

The morbidities of IDDM in CGRP plasmid treated and control groups were compared in a cohort of 60 mice (30 mice per group). Figure 2*A* showed that diabetes incidence was significantly reduced in the CGRP treated group. The incidence of IDDM was decreased from 80% in the control group to 30% in the treated mice at d 28. The average blood glucose level was also decreased by about 4 mmol/L in the CGRP group (Fig. 2*B*). The results indicate that CGRP gene transfer reduces the occurrence of IDDM and ameliorates hyperglycemia because of reducing the destruction of islet β cells.

Morphological evidence that CGRP gene therapy ameliorated the infiltration of inflammatory cells into islets

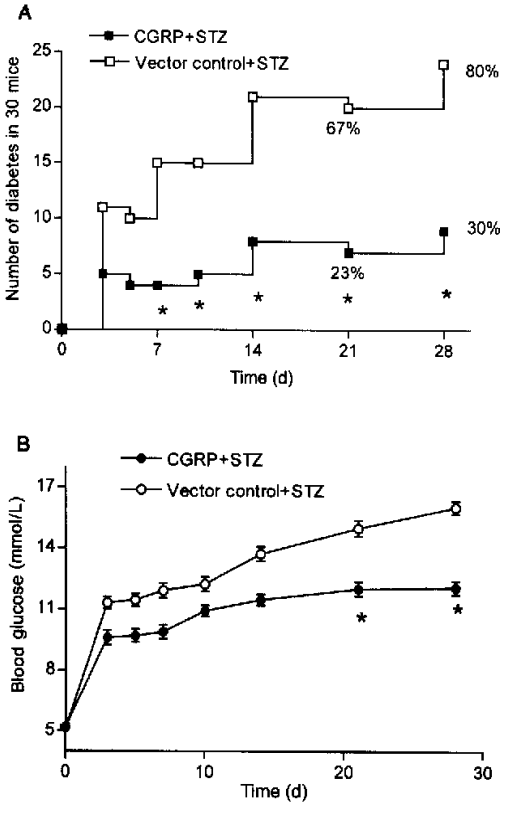

Fig. 2. The therapeutic effects of CGRP gene therapy on MLDS diabetic mice. *A*: CGRP gene therapy decreased the incidence of diabetes. The morbidity of IDDM was measured in a group of 30 mice. As analyzed with χ^2 test, a significant decrease in the morbidity was observed in the CGRP therapy group (*$P<0.05$). *B*: Changes in mean blood glucose in the CGRP therapeutic mice. The average blood glucose level was significantly decreased by about 4 mmol/L in the CGRP group (*$P<0.05$, compared with vector control + STZ, $n=30$).

during the diabetes onset (on d 7 after STZ administration) was shown in Fig. 3*B* (CGRP transgene group) when compared to that shown in Fig. 3*A* (vector control + STZ group), indicating that CGRP gene therapy inhibits the autoimmune insulitis induced by MLDS.

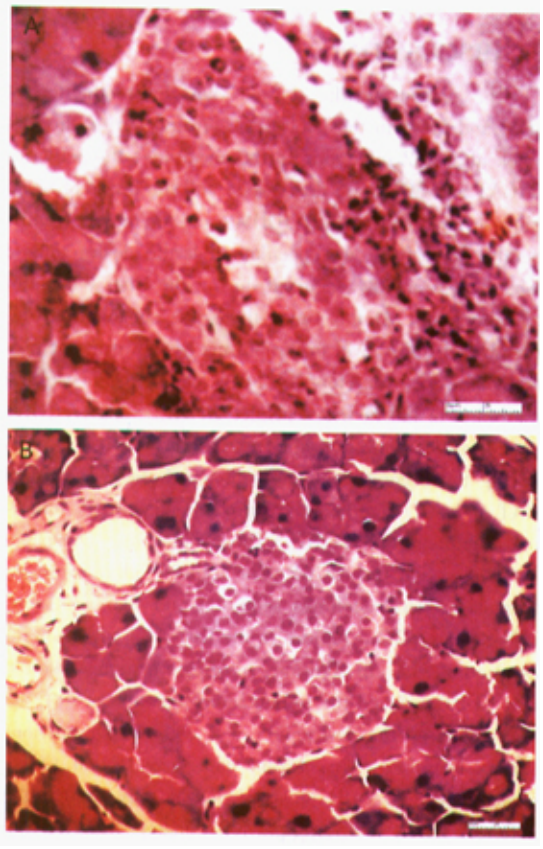

Fig. 3. Light microscopic photos of pancreatic tissue. They provided morphological evidence that CGRP gene therapy ameliorated the infiltration of lymphocytes (labled with arrow) in the islets during the onset of diabetes. *A*: Insulitis in the vector control diabetic mice. *B*: The infiltration of lymphocytes in CGRP gene-treated diabetic mice. The pancreatic tissues were obtained on d 7 after STZ administration and the tissue sections underwent a routine H & E staining. Reduced lymphocyte infiltration in the islets indicates that CGRP gene therapy inhibits the autoimmune insulitis induced with MLDS. Scale bar, 50 μm.

2.3 Effects of intramuscular CGRP gene transfer on production of ROS and MDA in pancreatic tissue

The effects of CGRP transgene therapy on production of ROS and MDA were determined. Pancreatic samples from CGRP gene treated mice were isolated at d 0, 3, 7, 10, 14, 21 and 28 after STZ administration, the production of ROS in pancreatic tissue was measured with EPR as described in Methods. As shown in Fig. 4, in the vector control + STZ group, the production of ROS was increased progressively and reached the peak at d 10, and then it was decreased gradually. In comparison, the ROS levels were significantly decreased in the CGRP treated mice at d 7, 10 and 14. Similarly, the production of MDA (a final production of lipid peroxidation) in vector control + STZ group was increased progressively and reached the peak at d 7. Then it was decreased gradually. In the CGRP transgene group, it was also sig-

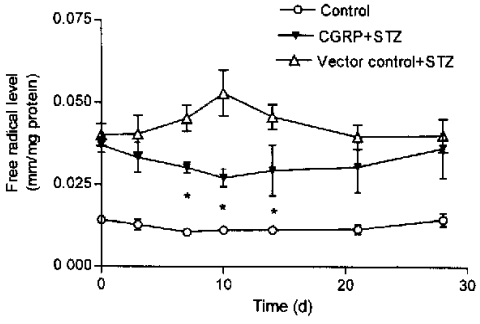

Fig. 4. Effects of intramuscular CGRP gene transfer on production of ROS in pancreatic tissue of diabetes mice. The production of ROS in pancreatic tissue was measured with EPR. The ROS levels were significantly decreased in the CGRP treated mice at d 7, 10 and 14 after STZ administration ($^*P < 0.05$, compared with vector control + STZ, $n = 3$).

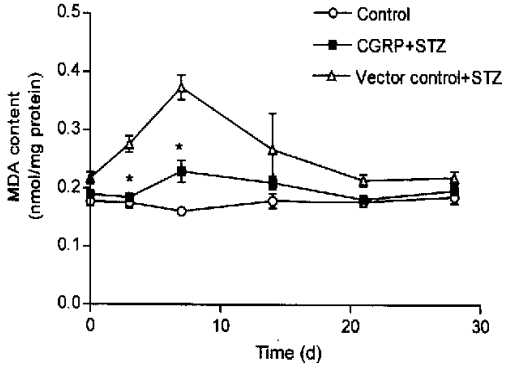

Fig. 5. Effects of intramuscular CGRP gene transfer on production of MDA in pancreatic tissue of diabetes mice. The levels of MDA were determined on the base of the rate of their consumption. The production of MDA in the CGRP transgene group was significantly lower than that in the vector control + STZ group at d 3 and 7 after STZ administration. ($^*P < 0.05$, CGRP + STZ *vs* vector control + STZ, $n = 4$).

nificantly lower than that of the vector control + STZ group at d 3 and 7 (Fig. 5).

2.4 Effects of intramuscular CGRP gene transfer on activity of SOD and CAT in pancreatic tissue

We also determined the activity of SOD and CAT, which have anti-oxidative effect, in pancreatic tissue of diabetes mice. The SOD activity in the vector control + STZ group was decreased and reached the lowest level at d 0 - 3 after STZ injection, and then it gradually returned. In the CGRP transgene group, it was significantly higher than that of the vector control + STZ group at d 0 - 3 after STZ injection (Fig. 6). In parallel with the results of SOD activity, the CAT activity in the vector control + STZ group was dramatically decreased at d 0 - 7 and was returned gradually. In the CGRP transgene group, it was remarkably higher than that of the vector control + STZ group at d 0 - 7 (Fig. 7).

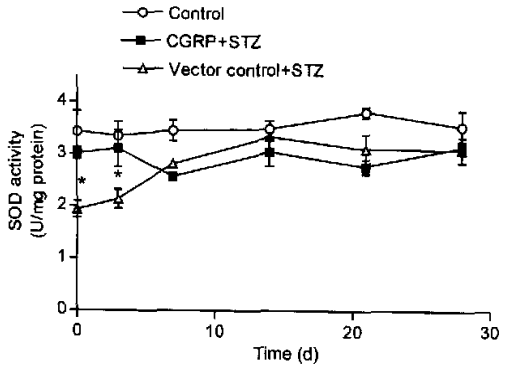

Fig. 6. Effects of intramuscular CGRP gene transfer on activity of SOD in pancreatic tissue of diabetes mice. The SOD was determined on the base of the rate of their consumption. The SOD activity in CGRP transgene group was significantly higher than those of the vector control + STZ group in d 0 and 3 after STZ injection. ($^*P < 0.05$, CGRP + STZ *vs* vector control + STZ, $n = 3$).

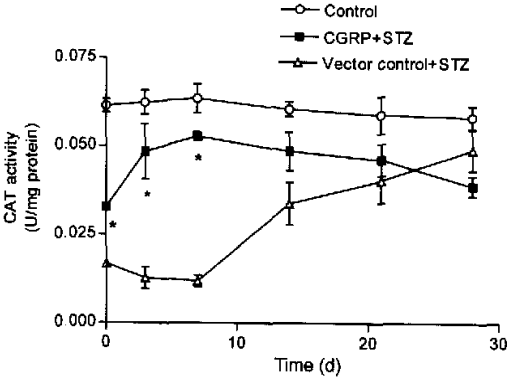

Fig. 7. Effects of intramuscular CGRP gene transfer on activity of CAT in pancreatic tissue of diabetes mice. The CAT was determined on the base of the rate of their consumption. The CAT activity in CGRP transgene group was significantly higher than those of the vector control + STZ group in d 0, 3 and 7 after STZ injection. ($^*P < 0.05$, CGRP + STZ *vs* vector control + STZ, $n = 3$).

3 DISCUSSION

The present results showed that the production of ROS and MDA in the islets was elevated during the first week after STZ injection. In the meantime, the activities of CAT and SOD were decreased, and the levels of blood glucose and morbidity of IDDM were increased significantly. Therefore, we speculate that the destruction of islet β cells mainly occurred during the first week after STZ injection. At this time, there was considerable decrease in insulin level in the plasma as shown in our previous study[6], therefore the levels of blood glucose and morbidity of IDDM were elevated in MLDS mice. CGRP gene transfer inhibited the production of ROS, MDA during the second week and enhanced the activities of CAT and SOD significantly during the first week following administration of STZ. These data were consistent with the notion that CGRP gene transfer inhibits pro-inflammatory Th1 cytokines secretion and increases anti-inflammatory Th2 cytokines in the first week after administration of STZ. In addition, histologic evidence showed that CGRP reduced the inflammatory reaction of the islet and decreased the destruction of β cells in our previous study. Therefore, our current data suggest that CGRP gene transfer in early stage of IDDM may reduce the pathogenesis by inhibiting pro-inflammatory cytokine and subsequently suppressing oxidative stress in islet β cells of diabetic mice.

Recent studies suggested that IDDM might arise, in part, from an increase in oxidative stress[11,12]. The concentration of MDA, a final production of lipid peroxidation, exhibits significant increase in diabetes. CAT, SOD and glutathione peroxidase (GPX) can eliminate ROS *in vivo*, thus play an important role in cell-protection. Some reports suggested that, islet β cells were sensitive to oxidative stress because they contained less CAT and GPX. Pathologic concentration of nitric oxide was believed to cause direct destruction of islet β cells, and this has been proven by a large body of evidence. H_2O_2-treated islets exhibited a marked decrease in the secretion of insulin[29]. Therefore, we conclude that ROS plays a very important role in the pathogenetic mecha-

nism of IDDM.

There is a close relationship between the destruction by autoimmune inflammation and oxidative stress in the pancreas in diabetes. A line of evidence indicated that, the disturbances in immunoregulatory circuits might lead to a dominance of Th1 cells and their cytokine products over Th2 cells and their cytokines. This might initiate autoimmune inflammation, thereby leading to initial inflammatory reaction and inflammatory cells infiltration in the islets of Langerhans[6, 7]. Inflammatory cells produce pro-inflammatory cytokines, such as IL-Iβ, TNF-α and IFN-γ. These cytokines induce excessive production of ROS, then lead to the destruction of insulin-producing β cells in the islets and the depression of insulin excretion. In order to provide a therapeutic intervention for IDDM, we concentrated on cytokine-induced oxidative stress from the pancreas of IDDM in the present study.

CGRP is a sensory neuropeptide and participates in the regulations of the functions of the cardiovascular, respiratory and digestive systems. Recently, CGRP has become a particular interesting candidate for neuroendocrine immune modulator. Khachatryan and his colleagues[18] have established CGRP transgene NOD mice to achieve the targeting expression of CGRP in β cells. They observed significant lightening of insulitis, which was characterized by reduced T lymphocyte infiltration. However, no systemic immune regulatory effect of CGRP was observed in their study. Therefore it was still not clear about how CGRP prevents the pathogenesis of IDDM. Our previous study demonstrated that CGRP gene transfer significantly inhibited T cell proliferation and Th1 pro-inflammatory cytokine IFN-γ secretion as well as increased Th2 anti-inflammatory cytokine IL-10 secretion, thereby resulting in amelioration of β cell destruction and reduction of IDDM occurrence in MLDS-induced diabetic mice[6]. Other studies demonstrated that CGRP had a protective effect on cardiac myocytes through suppression of ROS production[24]. In our present study we found for the first time that CGRP transgene could prevent IDDM through suppression of ROS and influence of peroxidase activity *in vivo*.

Intramuscular injection of naked plasmid is a novel approach to somatic gene transfer[27, 30, 31]. This method has been successfully applied to gene therapy with a number of cytokines, growth factors, hormones and other serum proteins[32, 33]. *In vivo* electroporation has been reported to markedly increase the gene transfer efficiency and prolong the duration of target DNA expression[27, 34]. In our previous work, CGRP mRNA was successfully detected by RT-PCR in local transgenic muscle tissue, and immunohistochemistry detected large amounts of CGRP immunoreactive substance in myofibers after the plasmid injection. As shown in the present study, CGRP protein expression was detected at d 3 and lasted for 4 weeks. This provides a convenient method for stable and long-lasting CGRP gene expression, avoiding repeated administrations of the expensive peptide due to its short half-life.

In conclusion, CGRP gene transfer by injection of naked plasmid may present an anti-oxidative stress effect and result in amelioration of β cell destruction and significant reduction of IDDM occurrence in MLDS diabetic mice. The results presented in our study may provide a base for a novel therapeutic strategy for IDDM.

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