• BASIC RESEARCH •

Establishment of an artificial β-cell line expressing insulin under the control of doxycycline

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Abstract

AIM: **Artificial β-cell lines may offer an abundant source of cells for the treatment of type I diabetes, but insulin secretion in β-cells is tightly regulated in physiological conditions. The Tet-On system is a "gene switch" system, which can induce gene expression by administration of tetracycline (Tet) derivatives such as doxycycline (Dox). Using this system, we established 293 cells to an artificial cell line secreting insulin in response to stimulation by Dox.**

METHODS: **The mutated proinsulin cDNA was obtained from plasmid pcDNA3.1/C-mINS by the polymerase chain reaction (PCR), and was inserted downstream from the promoter on the expression vector pTRE2, to construct a recombined expression vector pTRE2mINS. The promoter on pTRE2 consists of the tetracycline-response element and the CMV minimal promoter and is thus activated by the reverse tetracycline-controlled transactivator (rtTA) when Dox is administrated. pTRE2mINS and plasmid pTK-Hyg encoding hygromycin were co-transfected in the tet293 cells, which express rtTA stably. Following hygromycin screening, the survived cells expressing insulin were selected and enriched. Dox was used to control the expression of insulin in these cells. At the levels of mRNA and protein, the regulating effect of Dox in culture medium on the expression of proinsulin gene was estimated respectively with Northern blot, RT-PCR, and radioimmunoassay.**

RESULTS: **From the 28 hygromycin-resistant cell strains, we selected one cell strain (tet293/Ins6) secreting insulin not only automatically, but in response to stimulation by Dox. The amount on insulin secretion was dependent on the Dox dose (0,10,100,200,400,800 and 1000μg·L^{-1}), the level of insulin secreted by the cells treated with Dox (1000μg.L^{-1}) was 241.0pU·d^{-1}·cell^{-1}, which was 25-fold that of 9.7pU·d^{-1}·cell^{-1} without Dox treatment. Northern blot analyses and RT-PCR further confirmed that the transcription of insulin gene had already been up-regulated after exposing tet293/Ins6 cells to Dox for 15 minutes, and was also induced in a dose-dependent manner. However, the concentration of insulin in the media did not increase significantly until 5 hours following the addition of Dox.**

CONCLUSION: **Human proinsulin gene was transfected successfully and expressed efficiently in 293 cells, and the expression was modulated by tetracycline and its derivatives, improving the accuracy, safety, and reliability of gene therapy, suggesting that conditional establishment of artificial β-cells may be a useful approach to develop cellular therapy for diabetes mellitus.**

INTRODUCTION

Owing to the steady improvement in methodology for islet and whole pancreas transplantation over the past three decades, this approach remains as the only means which is likely to substitute completely the function of the impaired β-cells in typeIdiabetics patients[1]. However, the limited availability of human donors, the immunological rejection, the low survival percentage of grafts, and the high expense limit the broad-scale applicability of islet transplantation[2-4]. As a result, in recent years much effort is being made to improve insulin secretion in the patient's non-beta cells to avoid the immune destruction[5-9]. To successfully mimic beta cell function, it is necessary to overcome three major obstacles: proinsulin synthesis, proinsulin processing, and mature insulin storage and regulated secretion. Our previous study (unpublished) and studies by other authors had demonstrated that correct proinsulin processing and insulin storage can be obtained in somatic cells transduced with the mutated proinsulin gene[10-13], so the major problem to ectopic insulin expression is the difficulty of reconstructing in non-beta cells the highly regulated insulin secretion of the normal beta cells[14-19].In 1992, Gossen and Bugard[20] developed the tetracycline (or doxycycline)-induced gene expression (Tet-on) system, which is useful for controlling the expression of targeted genes in quantitative manner and for determining the roles of the gene products in cellular functions[21-26]. In the present study, using this system, we generated the first non-beta cell line with deoxycycline-regulated insulin secretion. The strategy described here will contribute to the development of the use of insulin gene therapy for patients with typeIdiabetes.

MATERIALS AND METHODS

Tet-on system

pTet-on regulator plasmid (pTet-on),pTRE2 response plasmid (pTRE2) and pTK-Hyg plasmid, which contain hygromycin-resistance gene, were purchased from Clontech. Using the polymerase chain reaction, the proinsulin cDNA was amplified from expression vector pcDNA3.1/C-mINS, which had been reconstructed from plasmids pcDNA3.1/C and pN-PEPCK-INS (the genomic sequence of human proinsulin, kindly provided by Professor LI Changchen, Dalian Medical University) in our lab. The primers used in the amplification created restriction sites *Bam*HI and *Hind*III which allowed to clone the human proinsulin cDNA into the expression vector pTRE2. The sequences of the forward and reverse promers were: 5'-cat*ggatcc*tgccatggccctgtggatg-3 and 5'-cag*aagctt*gcaggctgcgtctagttgc-3' respectively. After verification by

restriction and sequencing, the vector, pTRE2.mINS, for expressing human insulin was generated.

Cell culture and gene transfection

To simplify the transfection procedure, the tet-293 cell line (human embryonal cell), which had been transfected with the plasmid pTet-on and stably expressed reverse tetracycline-controlled transactivator (rtTA), was purchased from Clontech. So the first step of transfection and clone selection was left out. The culture medium was DMEM supplemented with 100mL·L^{-1} fetal bovine serum and G418 (100mg·L^{-1}). The tet-293 cells were maintained at 37℃ under a 50mL·L^{-1} CO$_2$ atmosphere until the cells were 80% confluent, then pTRE2.mINS was cotransfected with pTK-Hyg in a ratio of 20∶1, using Lipofectin reagent (Gibco-Brl). After selection in the culture medium containing 30mg·L^{-1} hygromycin (Sigma) for more than eight weeks, one clone (tet-293/Ins6) expressing insulin under the control of Dox was selected by screening from 28 clones.

Radioimmunoassay

Tet293/Ins6 cells (1.0×10^6) were seeded in 35-mm-diameter dishes, and treated with Dox at 0, 10, 100, 200, 400, 800 and 1000μg·L^{-1} for 24h, or at 1000μg·L^{-1} for 0, 15 and 30min, 1, 2.5, 5, 12 and 24h. The amounts of insulin secreted in the medium were measured with specific radioimmunoassay kit (Haikerei Co. Peking).

RT-PCR

Total RNA was extracted from cells with TRIzol (Gibco), and the first chain of cDNA was prepared by RT with Superscript Ⅱ reverse transcriptase (Gibco) using a oligo(dT)11 primer. The proinsulin gene was amplified with Taq polymerase (Progema). The PCR primers for proinsulin gene were: sense 5'-tgccatggccctgtggatg-3',and anti-sense 5'-gcaggctgcgtctagttgc-3'.

Northern blot analysis

For each Northern blot, 10μg total RNA was loaded per lane and separated, transferred onto nylon membrane (Schleicher and Schuell, Germany) and crossed-linked by UV irradiation. The hybridization probe specific for proinsulin, was labeled with (^{32}P)dCTP using a DNA labeling kit (Takera). Following hybridization at 68℃ for 2h, blots were washed twice for 30min at room temperature with solution 1 (2×SSC,0.5g·L^{-1}SDS) and solution 2 (0.1×SSC,1g·L^{-1}SDS), respectively, and exposed to a phosphorimaging screen overnight. The blots were then washed with 5g·L^{-1}SDS at 95℃ until complete removal of the probe and rehybridized with human β-actin cDNA (Clontech).

RESULTS

Establishment of a stable cell line excreting insulin controlled by Dox

After screening in the culture medium containing hygromycin for more than eight weeks, a total of 28 independent Hyg-resistant cell lines were created from the tet-293 cells which had been cotransfected with pTR2.mINS and pTK-Hyg. Of the 28 clones, one (tet-293/Ins6) was obviously regulated to secrete insulin by Dox. The amount of insulin in the medium secreted by the cells treated with Dox (1mg·L^{-1}) was 241.0pU·d^{-1}·cell^{-1}, which was 25-fold that of 9.7pU·d^{-1}·cell^{-1} released by the cells without Dox treatment.

Verify the regulation of Dox on insulin secretion in tet-293/Ins6 cells at the protein level

Tet-293/Ins6 cells were amplified and passaged (1.0×10^6 cells) into 16 35-mm dishes, and the concentrations of insulin in the culture media were measured with RIA. As shown in Figure 1, the level of

insulin secretion increased from 18.2 to 241.0pU·d^{-1}·cell^{-1} over a range of Dox concentrations from 10μg·L^{-1} to 1000μg·L^{-1}. Only a slightly increasing tendency of insulin concentration was observed at 2.5h following the addition of 1 mg·L^{-1} Dox to the culture medium. However, the amount of insulin increased significantly after exposing the cells to Dox for more than 5h (Figure 2).
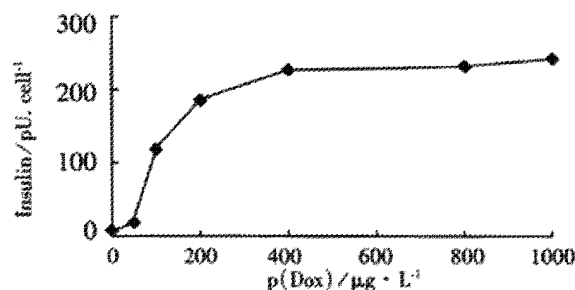

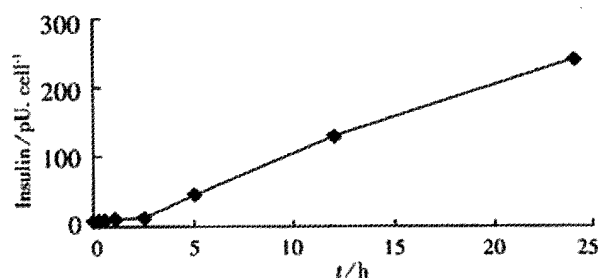**Figure 1** Insulin secretion from tet-293/Ins6 cells after treatment with Dox at different concentrations for 24h.

Figure 2 Time course of insulin secretion from tet-293/Ins6 cells after activation with Dox at 1000μg·L^{-1} in 24h.
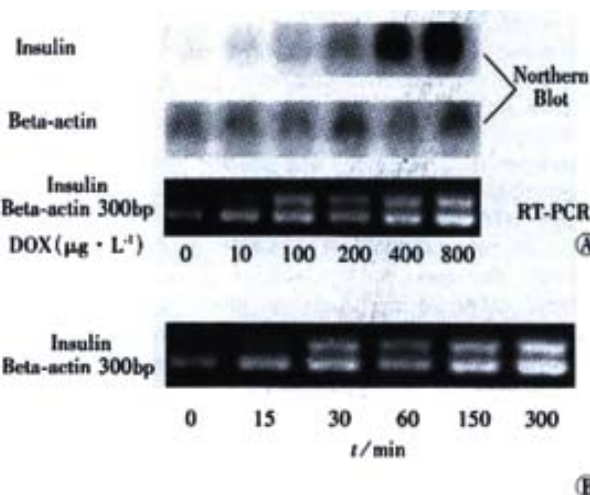

Figure 3 The conditional transcription of insulin gene by Dox in the tet-293/Ins6 cells using β-actin as loading control.

Verify the regulation of Dox on insulin expression in tet-293/Ins6 cells at the transcriptional level

Theoretically, the Tet-on system exerts its regulatory effect on target genes at the transcriptional level. So RT-PCR and Northern blot methods were used to further demonstrate the inducibility of insulin by Dox. After tet-293/Ins6 cells were cultured in the media containing different concentrations of Dox for 24h, it was found that 10μg·L^{-1} Dox could induce insulin expression obviously, and that the insulin mRNA level was up-regulated dramatically by Dox in a dose-dependent manner (Figure 3A). As shown in Figure 3B, the result of

RT-PCR indicated that the transcription of insulin gene ha d already been up-regulated significantly after exposing tet-293/Ins6 cells to 1mg·L^{-1} of Dox for 15 minutes.

DISCUSSION

Insulin-dependent diabetes mellitus (IDDM) results from the autoimmune destruction of the insulin-producing beta cells of the pancreas. As a consequence, diabetic patients experience profound metabolic derangements (hyperglycemia, ketosis and hyperlipemia) and develop vascular and neurologic chronic complications[27-29]. These alterations can be only partially controlled by the exogenous administration of insulin, which brings patients suffering and inconvenience[30]. In recent years, engineering insulin-secreting cell lines has received a great deal of interest and some exciting advances have been made. But the application of gene therapy to diabetes presents formidable challenges, one of which is how the level of insulin is restricted within a very narrow limit. Otherwise, if the engineered artificial β-cells were transplanted into the body, the excessive insulin for metabolism would cause hypoglycaemia[8], and even death. Several inducible promoters originating from eukaryotic genes have been used to deliver gene in a regulated manner. The substances inducing the promoters include steroid hormones, oxygen, heavy metals, or physical stimulus (such as radiation)[31, 32]. However, most of these promoters are not suitable for clinical application for various reasons: first, because these promoters are of mammalian origin, the exogenous regulation of transgene through such a promoter could at the same time affect the transcription of the host endogenous genes; and second, the inducers of these promoters are generally endogenous molecules (hormones, oxygen, etc.), the levels of which cannot be modulated significantly and safely. Other limitations include the potential toxicity or side effects of the inducer. Therefore, an inducer/promoter system, which regulates the transcription only of the transgene, without affecting endogenous genes, is acceptable for gene therapy in humans.

The Tet-on system, which utilizes an *E. coli* gene regulatory system[20], appears to fulfil the criteria for clinical application for several advantages[33-40]: (1)gene expression is easily regulated by administration of Dox; (2)Dox is minimally toxic; and (3)Dox acts specifically on the target gene, and does not activate other cellular genes. This expression system has two critical components, the regulatory plasmid and the response plasmid. The rtTA (reverse tetracycline-controlled transactivator) expressed by the regulated plasmid binds the TRE (tet-response element) in the response plasmid and activates the transcription of the target gene in the presence of Dox. Efrat *et al*[41] used the Tet-operon regulatory system to generate a β-cell line in transgenic mice, but there has been no report on the study of engineering non-β-cells for the treatment of diabetes in this regard. Efrat constructed a fusion protein, TETR-VP16 containing the tet repressor and the activating domain of the herpes simplex virus protein VP16,which converts the repressor into a transcriptional activator under the control of the insulin promoter. Then, the transgenic mice of the fusion protein were generated, whose β-cells could express the TETR-VP16 protein conditionally. In a separate lineage of transgenic mice, the simian virus 40 (SV40) large tumor (T) antigen (TAg) gene was introduced under the control of the tet operator sequences and a minimal promoter, which by itself is not sufficient for the expression of Tag gene. Mice from the two lineages were then crossed to generate double-transgenic mice. Expression of the TETR-VP16 protein in β-cells activated Tag transcription, resulting in the development of β-cells tumors. A stable β-cell line deriving from the tumor had the following characteristics: cell incubated in the absence of Tet proliferated normally; and cell incubation in the presence of Tet led to inhibition of proliferation. Thus, it is feasible that the expression of insulin gene can be regulated

under the control of the proliferation of these β-cells. In the present study, utilizing the Tet-on system, we have established a cell line tet-293/Ins6 in which insulin gene can be expressed conditionally by Dox treatment. Ten μg·L^{-1} Dox can activate the tet-response element and significantly enhance the expression of insulin gene in the cells in a dose-dependent manner. Additionally, the RT-PCR results showed that it is at 15min that 1mg·L^{-1} Dox could activate the transcription of insulin gene in the tet-293/Ins6 cells following the addition of Dox, and that the amount of insulin in the media did not increase until 5 hours after the treatment of Dox, suggesting that there is a periodic interval of time from the process of gene transcription, translation, and protein synthesis, processing to the final step, secreting the protein from the cells. The strategy used here, by which a stable cell line with low background and high Dox-induced expression of insulin was generated, is more efficient and rapid for regulating insulin gene expression as compared with Efrats' method[41], and will contribute not only to the development of artificial β-cells for the treatment of diabetes but to the generation of other condition-secreting cell lines for gene therapy of human diseases.

However, responding to the variation of blood glucose concentration, the expression level of insulin gene should be regulated strictly for gene therapy of diabetes[42-44]. The tet-293/Ins6 cells still have their imperfections for replacing islet transplantation in humans for the treatment of diabetes. Under normal conditions, increasing glucose concentration in blood stimulates β-cells to secrete insulin immediately; but the tet-293/Ins6 cells release insulin by control of Dox at the transcriptional level, the kinetics of feedback loops on transcriptional changes is much slower than that of the secretory response in β-cells. Therefore, based on the experiments we have carried out, we are making efforts to establish an artificial β-cell line in response to glucose stimulation with gene engineering.

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