

The Effects of CTGF Overexpression on β -Cell Regeneration and Survival

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KEYWORDS. β -cell regeneration, diabetes, CTGF

BRIEF. Overexpression of connective tissue growth factor enhances β -cell regeneration through increased proliferation but cannot protect from β -cell death.

ABSTRACT. Pancreatic β -cells secrete insulin to maintain blood-glucose homeostasis. Deficiency in β -cell number or function leads to diabetes. Connective tissue growth factor (CTGF) is essential for embryonic β -cell proliferation, and induced expression of CTGF in embryonic β -cells increases β -cell proliferation and β -cell mass. CTGF expression is silenced in β -cells soon after birth. Activation of CTGF in β -cells after 50% β -cell destruction enhanced β -cell regeneration through increased proliferation. It was hypothesized that CTGF overexpression prior to 50% β -cell ablation would stimulate regeneration but have no effect on β -cell survival. CTGF overexpression was mediated using a RIP-rtTA;TETO-CTGF model, in which CTGF is expressed in β -cells when mice are exposed to doxycycline. Female mice hemizygous for a β -cell specific diphtheria toxin receptor were used to ablate half of the β -cells. CTGF was overexpressed one week prior to and during ablation. While no change in β -cell mass was observed between “CTGF-treated” mice and controls, there was a statistically significant increase in proliferating β -cells in “CTGF-treated” mice. CTGF expression did not protect against β -cell apoptosis. This suggests that CTGF promotes β -cell regeneration but does not protect β -cells from destruction in this model. CTGF overexpression may aid in stem cell differentiation and remediation of Type 1 diabetes.

INTRODUCTION.

Diabetes is an imminent threat to American society. Roughly 8% of the American population has some form of diabetes and these individuals are more prone to serious conditions such as cardiovascular disease, stroke, blindness, and limb amputations [1]. Diabetics display functional impediments in endocrine hormone secretion.

The pancreatic endocrine system is composed of clusters of cells called Islets of Langerhans, which primarily consist of β -cells. In response to high blood-glucose levels, β -cells secrete insulin to signal for sugar to be taken from the blood stream and stored in the muscles and liver. When β -cell production of insulin is severely impaired, diabetes ensues. Type 1 diabetes (T1D) occurs when the immune system attacks and destroys β -cells. In Type 2 diabetes (T2D), peripheral tissues become resistant to insulin, resulting in increased insulin output from the β -cells, which ultimately become exhausted. Short-term solutions, such as insulin injections, do not replace the function of insulin-producing β -cells and are not capable of maintaining blood-glucose homeostasis alone.

Connective tissue growth factor (CTGF) is a secreted protein that is important in several developmental processes, such as angiogenesis, lung development, and bone formation. CTGF is expressed in the developing pancreatic ducts and blood vessels; this expression is maintained into adulthood [2]. However, β -cells only express CTGF during development and pregnancy [3]. CTGF-null mice display decreased β -cell proliferation during development. Conversely, overexpression of CTGF in insulin-producing cells during development results in increased β -cell proliferation and β -cell mass.

Since CTGF enhances proliferation and increases β -cell mass in embryonic β -cells, induced overexpression of CTGF could have an effect on a β -cell's response to injury. It was not known whether CTGF overexpression before partial β -cell destruction would promote β -cell survival and/or regeneration. We hypothesized that overexpression of CTGF before β -cell destruction would promote β -cell regeneration through proliferation but not β -cell survival.

β -cell regeneration is significant in diabetes research, because it provides researchers novel ways to combat failing β -cells in the diabetic disease state. CTGF shows promise as a therapy for diabetes by enhancing β -cell proliferation and differentiation from stem cells. The time optimization for CTGF treatment is essential to consider: if the treatment aids in β -cell regeneration, it is necessary to find out when it begins to take effect. Overall, diabetes remediation could be accomplished through the use of β -cell regeneration aids, instead of frequent insulin injections. Immune system attacks on β -cells would still occur in T1D patients, so improved proliferation is not a permanent solution.

MATERIALS AND METHODS.

Animals.

Genetic models were used to simulate injury and activate the production of CTGF. The CTGF overexpression mouse model was defined by co-activation of the transgenes RIP-rtTA and TetO-CTGF. The β -cell specific rat insulin promoter (RIP) drove expression of reverse tetracycline Trans-Activator (rtTA) [3]. Tet-Operon-CTGF (TetO-CTGF) promoted CTGF expression, but only in the presence of both doxycycline and rtTA [3]. Addition of doxycycline allowed rtTA to bind to the tet-operon and promote overexpression of CTGF only in the β -cells. Therefore, doxycycline administration allowed for temporal and spatial control of CTGF overexpression. For this project, CTGF was overexpressed for one week prior to the diphtheria-mediated β -cell ablation, during the five-day period of diphtheria toxin (DT) treatment, and for two days after the DT injections.

To simulate injury, a rat insulin promoter-diphtheria toxin receptor (RIP-DTR) mouse model was utilized [4]. RIP-DTR is β -cell specific through the RIP and drives expression of the DT receptor (DTR). The RIP-DTR transgene was inserted into the hprt locus on the X chromosome, allowing for gender-specific levels of ablation [4]. Upon three DT injections over five days, males and homozygous females display an almost absolute (99%) β -cell ablation, while hemizygous females display only 50% destruction. Some β -cell function was necessary for survival; consequently hemizygous female mice were used. In addition, developmental studies on CTGF observed β -cell mass expansion via increased β -replication, suggesting a “pool” of β -cells would be necessary for CTGF-mediated regeneration.

The four experimental groups tested were Control, CTGF Treated, Ablated, and CTGF Treated Ablated. Control and CTGF Treated β -cells contained the DTR, but did not receive DT and subsequently did not experience 50% β -cell destruction. All mice received doxycycline in the water supply and expressed the RIP-rtTA transgene, but only CTGF Treated and CTGF Treated Ablated β -cells overexpressed CTGF due to their expression of TetO-CTGF. The pancreata were harvested two days after the final DT injection.

Tissue Processing.

The pancreata were processed for analysis after harvesting and were subsequently fixed in 4% paraformaldehyde [2]. The wet weight of each pancreas was taken for later β -cell mass analyses. After dehydration through an increasing ethanol series and xylene, the pancreata were embedded in paraffin [2]. Samples were serially sectioned throughout the pancreas at 5 μ m.

Immunohistochemistry.

Every tenth slide was selected to stain for insulin to quantify β -cell mass, allowing for representative analysis of the whole pancreas. Insulin antibodies and

DAB (3,3'-diaminobenzidine) were used to immunolabel β -cells, while eosin stained the total pancreatic tissue according to Bio-Rad Laboratories instructions. A proportion of β -cell area to total pancreatic area was calculated. This ratio was then applied to the total mass of the pancreas to determine approximate β -cell mass.

Every twentieth slide was selected to analyze β -cell proliferation to get a holistic view of the pancreas. Co-immunolabeling of Ki67 (Invitrogen) and insulin (DAKO) was used to count proliferating β -cells. 4',6-Diamidino-2-phenylindole (DAPI) stained the nucleus of each individual cell. The total number of nuclei in the insulin-positive area was counted. An Aperio image scope was used to obtain scanned images and to extract the images of islets. Metamorph was employed to count the proliferating β -cells and total number of cells in the insulin area. Over 4,000 β -cells were counted for each specimen. A ratio of proliferating β -cells to total β -cells was calculated to determine the percentage of proliferating β -cells.

Every twentieth slide was selected to analyze β -cell death. TUNEL (Apo-Alert) co-immunolabeling with insulin (DAKO) was used. An Aperio image scope was used to extract the images, and Metamorph was used to count the dying β -cells and total number of cells in the insulin area. Over 4,000 β -cells were counted for each specimen. The percent of dying β -cells was calculated using the ratio of dying β -cells to the total β -cell count.

Glucose Tolerance Tests.

Mice were fasted overnight, and a baseline blood glucose reading was taken. Two mg/g of glucose was administered and blood glucose levels were recorded at 15, 30, 60, 90, and 120 minutes to assess glucose clearance and overall β -cell function.

Statistical Analysis.

Statistical analyses were generated through t-tests using SEM for β -cell mass, β -cell proliferation, and β -cell death. Glucose tolerance test time points were compared using one-way ANOVA.

RESULTS.

CTGF overexpression and 50% β -cell ablation do not affect glucose homeostasis.

β -cell function was assessed through glucose tolerance tests (GTTs), which measured blood glucose clearance over a two-hour period. The blood-glucose levels of all groups were restored to normal levels after 120 minutes, indicating that neither CTGF overexpression nor 50% β -cell ablation altered β -cell function (Figure S1).

CTGF overexpression does not affect β -cell mass two days after ablation.

After 50% β -cell ablation, β -cell mass significantly decreased as compared to uninjected controls ($p=0.008$). CTGF treatment did not promote β -cell mass expansion under normal condition or after β -cell ablation. The β -cell mass of ablated groups (0.7605 ± 0.0852 mg) was approximately half of control groups that did not experience any β -cell damage (1.361 ± 0.1434 mg) (Figure S2). Therefore, priming β -cells with CTGF does not appear to promote enhanced β -cell mass expansion.

CTGF overexpression enhances β -cell proliferation after ablation.

Control, CTGF Treated, and Ablated islets showed similar β -cell proliferation ($0.7841 \pm 0.1233\%$), while the CTGF Treated Ablated islets showed a significant increase in proliferating β -cells ($1.852 \pm 0.2179\%$) (Figure 1a). Despite the variability, every CTGF Treated Ablated islet showed a statistically significant increase in proliferation over control groups.

β -cell proliferation percentages at two days after ablation were significantly higher than at later time points in CTGF-mediated β -cell regeneration observed in an ongoing study. This could result from a larger amount of β -cells proliferating directly after the injury as opposed to at a later time. In addition, it may be that priming β -cells with CTGF prior to β -cell ablation results in enhanced β -cell proliferation and/or survival as compared to islets that only receive treatment after ablation. Therefore, all previously described

experiments were replicated with CTGF only expressed for the two-day period after ablation. In all cases, priming β -cells with CTGF did not enhance β -cell function, β -cell mass expansion, or β -cell proliferation as compared to the two-day treated animals (Figure 1b).

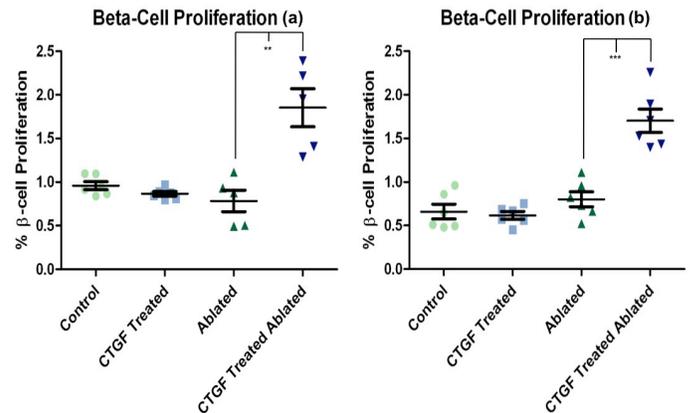


Figure 1. Figure 1: β -cell proliferation. 1a- CTGF treatment prior to, during, and two days after ablation. Co-immunolabeling of Ki67 and insulin was used to count proliferating β -cells; $p=0.0027$, $n>5$. 1b- CTGF treatment for two days after ablation. These repeated experiments without the week of priming verified that CTGF treatment prior to ablation does not promote increased β -cell proliferation; $p=0.0002$, $n=6$.

CTGF overexpression prior to β -cell ablation does not improve survival.

To assess whether priming β -cells with CTGF protected them from death, β -cell survival was quantified by measuring the apoptotic and necrotic β -cells. Ablated and CTGF Treated Ablated islets both showed higher percentages of dying β -cells ($3.372 \pm 0.3449\%$ and $2.767 \pm 0.4797\%$, respectively), but there was no significant difference between those two experimental groups ($p=0.3359$). The only factor that affected β -cell death was ablation, since control islets without DT showed β -cell death percentages of $0.2262 \pm 0.0327\%$ (Figure 2a).

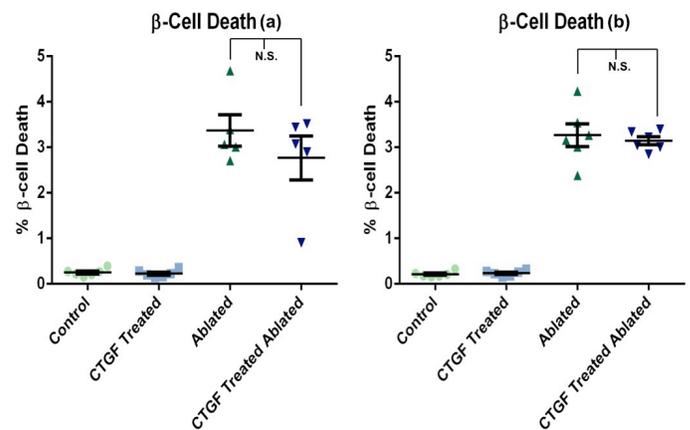


Figure 2. Figure 2: β -cell death. 2a- CTGF treatment prior to, during, and two days after ablation. TUNEL immunolabeling was used to show apoptotic and necrotic cells, while co-staining with insulin indicated dying β -cells; $p=0.3359$, $n>5$. 2b- CTGF treatment for two days after ablation. The percentage of dying β -cells was not significantly different from the CTGF treatment prior to, during, and two days after ablation islets; $p=0.6517$, $n=6$.

The β -cell survival of the islets that did not receive CTGF treatment prior to and during ablation was also quantified (Figure 2b). As before, we observed that the two-day overexpression time point did not alter the percentage of dying β -cells ($3.143 \pm 0.0872\%$) from the CTGF primed islets in this model ($2.767 \pm 0.4797\%$). Overall, these results suggest that CTGF expression prior to β -cell

ablation by DT cannot enhance β -cell survival. A visual representation of β -cell proliferation and death of all of the experimental groups is shown in Figure 3.

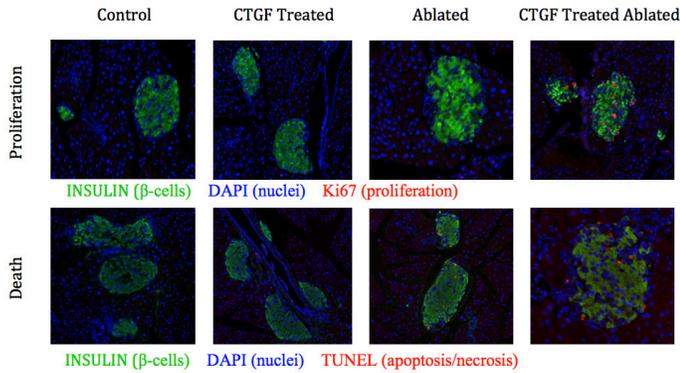


Figure 3: CTGF treatment with ablation promotes proliferation, but ablated islets show similar percentage of dying cells. β -cells are shown in green by insulin. Individual nuclei are shown in blue by DAPI. Ki67 marks proliferating cells in red for the top set of images, while the red cells in the bottom set are indicative of dying cells through TUNEL.

DISCUSSION.

CTGF overexpression and 50% β -cell ablation do not affect glucose homeostasis.

β -cell secretion of insulin is an essential function of the endocrine system and impairment in production lead to diabetes. Typically, $\sim 80\%$ of the β -cells are destroyed before insulin production and therefore blood sugar is affected. With impaired insulin secretion, high blood sugar ensues and promotes β -cell proliferation as well. With normal β -cell insulin secretion levels, proliferation changes must be due to the CTGF treatment and not from the innate response. As expected, 50% β -cell ablation and CTGF treatment did not change blood glucose, suggesting normal β -cell function. To truly mimic the β -cell injury that occurs in diabetes, a destruction model that reduces β -cell mass to less than 20% would be ideal, but this project was focused on β -cell regeneration.

CTGF overexpression does not affect β -cell mass two days after ablation.

β -cell mass is indicative of a combination of several possible factors, the most significant being β -cell proliferation, and was tested in order to determine any overall changes that resulted from ablation or CTGF treatment. Islets that received diphtheria toxin lost about 50% β -cell mass compared to the non-injected groups. The CTGF overexpression treatment after ablation did not have a noticeable effect on β -cell mass, most likely because the treatment was not long enough to induce significant changes. An ongoing study has shown that CTGF treatment for four weeks results in 50% regeneration of β -cell mass. Therefore, CTGF treatment eventually leads to increases in β -cell mass, but two days after ablation is not sufficient time. This study used the two-day time point in order to identify changes, if any, in β -cell death that occur during ablation.

CTGF overexpression enhances β -cell proliferation after ablation.

Islets have a long lifespan and do not readily create or destroy β -cells unless there is an increased need [5]. Proliferation was a measure of regeneration in this study; increased amounts of proliferating β -cells suggest more islet regeneration and a faster means of regaining the lost β -cell mass. In normal adult β -cells, CTGF is not expressed, and induced overexpression does not have an effect on β -cell growth. By itself, treatment did not induce the β -cells to divide; only in conjunction with destruction did β -cells proliferate (Figure 1a). This suggests that β -cells only respond to CTGF treatment during periods of increased need. Regeneration might occur more efficiently in patients with β -cell deficiency if CTGF overexpression is present.

An aim of this project was to determine if overexpression treatment is a preventative action and somehow aids regeneration or prevents β -cell death. The original experiments primed the islets by overexpressing CTGF for a week before ablation, as well as during and two days afterwards. It was not known whether

the increase in proliferating β -cells was due to the week of CTGF priming before ablation or whether the treatment only enhanced growth during and after destruction. The repeated experiments indicated that the week of CTGF priming did not have a noticeable effect on proliferating cells because the proliferation percentages were similar (Figure 1b). During destruction, changes in the islet microenvironment must allow β -cells to respond to CTGF, thus leading to more β -cell mass growth. Therefore, CTGF only enhances regeneration after injury and prior exposure does not prime the cells to respond more quickly. These results are significant because proliferation facilitates regeneration and increased β -cell growth is critical in diabetes remediation. Determining the specific mechanisms of this process would be a logical progression in this line of inquiry. Diabetes patients would not benefit from CTGF priming so the treatment would not be practical as a preventative measure.

CTGF overexpression prior to β -cell ablation does not improve survival.

β -cell death is less common than β -cell proliferation during normal adulthood [5]. This project quantified β -cell death to see if CTGF treatment had a preventative effect for this method of ablation. The method in which the immune system destroys β -cells in diabetes is difficult to model due to the lack of information about the specific mechanics of the process. The diphtheria toxin-mediated destruction model used in this research kills the cells through inhibiting translation. Since the ablated and CTGF treated ablated islets both showed similar percentages of dying cells, it can be reasoned that CTGF overexpression did not have an effect on β -cell death (Figure 2a). This was expected, as CTGF does not regulate protein translation. When the experiments were repeated with a shorter treatment time, the apoptosis and necrosis percentages did not change (Figure 2b). This confirms that the β -cells died in a similar quantity regardless if CTGF had not been expressed before ablation.

CONCLUSION.

The results of this research imply that CTGF treatment does not act as a preventative measure in this model, but does significantly aid in β -cell regeneration. It was necessary to establish this timeline of benefit to maximize the efficiency of the CTGF treatment, which has been confirmed by current studies as a viable method for β -cell regeneration. While there has been much research done on the effects of CTGF in embryonic development and during pregnancy, β -cell regeneration is a relatively popular and variable topic [6]. Regeneration can be achieved through many mediums, yet CTGF is a promising in that is a protein that can be easily administered to the patient. In addition to the regeneration of β -cells during diabetes, researchers have also attempted to alleviate metabolic stress on the small number of β -cells that are left [7]. By providing insulin in the early stages of diabetes, which is usually a last-resort, the remaining β -cells have time to regenerate more effectively without the excess insulin demand. This method shows promising results for diabetic patients, but the most effective treatment would involve the combination of regeneration and lower insulin demand. Future research could determine how CTGF might be reactivated or administered to promote β -cell proliferation in diabetes patients. This treatment alone would not be fully effective in T1D patients, due to chronic immune system attacks; β -cell protection from the immune system is also a highly discussed topic in the diabetes field. Overall, the effects of CTGF treatment after ablation are significant and highly useful to the endocrinology research pool and may eventually have larger implications in diabetes research.

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SUPPORTING INFORMATION.

Supplemental Methods.

Figure S1. Glucose tolerance test. To measure β -cell function in response to glucose, mice were fasted and then their blood glucose levels were monitored once 2mg/g of glucose was administered; $n > 5$.

Figure S2. β -cell mass. Insulin antibodies and DAB were used to immunolabel β -cells, while eosin stained the total pancreatic tissue. This ratio was then

applied to the total mass of the pancreas to get an approximate β -cell mass; $p=0.0078$, $n>5$.

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