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Chronological Change in Pancreatic Islet Injury
Induced by Streptozotocin
—An Immunocytochemical and Electron Microscopical Study—

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Abstract: Streptozotocin (STZ), drug used to treat for pancreatic endocrine tumors, is known to specifically impair B cells in the pancreatic islets of Langerhans (referred to here after as "islets") with a diabetogenic effect. In this study, a immunocytochemistry and electron microscopy were used to morphologically analyze the onset and progression of islet (mainly B cell) injury induced by STZ. Islet B cell injury could be detected by electron microscopical level two hours after administration of STZ, and by light microscopy after three hours. Positive findings using the TUNEL method began to appear six hours after, when the pancreatic islet injury induced by STZ appeared to involve both necrotic and apoptotic processes. At 72 hours, the number of endocrine cells in the islets was markedly increased, although these cells were immunocytochemically negative for any islet hormones, and appeared immature with only a few secretory granules detectable. After two weeks, the endocrine islets were completely recovered. Thus, regenerated B-cells appear to be derived from new endocrine cells, acting like an endocrine stem cell.

Key words: pancreatic isles injury, streptozotocin, necrosis, regeneration

Introduction

Streptozotocin (STZ) was developed from Streptomyces achromogenes against bacterial infection\(^1\), as an antitumor agent\(^2\), and particularly as a drug for leukemia\(^3,3\). In 1963, Rakieten et al. discovered that STZ also has a diabetogenic effect\(^4\). It has been reported to induce diabetes by selectively impairing islet B cells, without injuring islet A cells\(^4,6\). Since then, STZ has been used to treat malignant insulin-producing tumors, and as an in vitro agent for inducing diabetes\(^7,12\). The process by which STZ induces diabetes is similar to that for alloxan\(^13,15\). Despite this the onset of alloxan-induced diabetes can be prevented by nicotinamide\(^16,17\), glucose\(^18\), and nicotinic acid\(^18\), whilst STZ-induced diabetes is not impeded by either glucose or nicotinic acid\(^19\).

Alloxan is highly toxic, therefore STZ is usually chosen for experiment-based diabetes research. Most of the studies on diabetic rats using STZ, have involved observations made at 24-hour intervals during the 72 hours during which an abnormal blood sugar level is
present. There are few reports of shorter time intervals. This study has examined the morphology of cell impairment in pancreatic islets due to STZ over a period of short time intervals, up to the subacute stage, using light and electron microscopy, and immunohistochemistry.

Materials and Methods

Materials

A total of 33 male Wistar rats (6 weeks old, initial body weight 289.9±38.7g) were kept in a stable environment with free access to pellet feed (Oriental MF: Oriental Yeast Factory) and drinking water. They were divided into the following two groups:

Control group (n=3 rats)
These were untreated, normal rats kept for 10 months before being sacrificed for analysis.

STZ administration group (n=30 rats)
This group of rats received 50 mg/kg of STZ via the caudal vein. Replicates of four rats were sacrificed for analysis 6 hours, 12 hours, and 72 hours after STZ, whilst replicates of three rats were used for the 1 hour, 2 hours, 3 hours, 24 hours, 1 week, and 2 weeks time intervals. The method of STZ administration and the dose were decided in accordance with Hurukawa21 and Zhi Ming Wen28).

Methods

Light microscopy
Following collection of freshly dissected pancreas for the electron microscopy studies, sample for light microscopy were collected within the maximum cutting area. The tissue was for 24 hours in 10% neutral buffered formalin (In 0.1 M phosphate buffer, pH 7.4) prior to paraffin embedded, and the preparation of 3μm sections. Hematoxylin and eosin (H-E) staining, and Grimelius staining were performed. Immunohistochemical staining was conducted using anti-human glucagon (Nichirei), anti-human insulin (Nichirei), anti-human somatostatin (Nichirei), and anti-human pancreatic polypeptide (DAKO) antibodies. Specimens were also prepared according to the TUNEL method, and observed by light microscopy. The positivity (LI) was calculated for cells that were immunohistochemically positive and also positive by the TUNEL method.

Morphological measurements of the pancreatic islet area and the number of islet cells were made using a VQ-7000 high vision image analysis system (Keyence Corporation), at 500 X and 1000 X magnification.

Twenty pancreatic islets were measured at each time interval. Measurements were made, at each time interval, of islet surface area, the number of islet constituent endocrine cells, the number of insulin-producing (immuno positive) cells, and the number of glucagon-producing (positive) cells.

All values were expressed as mean ± SE, and the t-test was used to calculate statistical significance; P<0.05 was considered to indicate a significant difference.

Electron microscopy
Two tissue samples were rapidly excised from each of the pancreatic heads, bodies, and tails in the STZ administration group, then fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium oxide (mixed with equal parts of 0.2 M phosphate buffer, pH 7.4). Ultrathin sections were prepared using a diamond knife on a model MT2B microtome, and collected
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on 100 and 150 mesh. Copper grids sections were contrasted in uranium acetate and lead citrate, and observed using a transmission electron microscopes (Hitachi H7000, 75 kV acceleration voltage or Nihon Denshi JEM 1200 EX II, 80 kV acceleration voltage).

Results

One and two hours after STZ administration

There were no conspicuous changes on the outside of the islets, but the islets were somewhat tumescent. The islet constituent cells were also slightly swollen, indicating a static edema. The number of constituent cells and the surface area of the islets were significantly increased, as compared to the control group (Fig. 2, 3). By immunohistochemistry, no significant increases were noted in the number of insulin-positive or glucagon-positive cells over those in control islets. Immunonegative cells represented approximately 15% of the islets, and constituted either D cells or PP cells. Few cells were found to be positive by the TUNEL method, and no significant change was noted compared with the control group.

No conspicuous changes were defected by electron microscopy 1 hour after administration. After 2 hours, however, an accumulation of chromatin was noted on nuclear membranes. A comparatively large amount of endocrine granules were retained in the cytoplasm, and small vacuoles were conspicuous in the cytoplasm. Swelling was noted in the mitochondria, rER and other minute intracellular organcells.

Three hours after STZ administration

There was no conspicuous change in the islet constituent cells, but the cytoplasm was swollen in all cases. The inspissation inside the nuclei of the B cells was pronounced. Immunohistochemistry, revealed a relative increase in glucagon-positive cells, but otherwise no abnormality was observed in the distribution of islet constituent cells (Fig. 5).

No difference was found between treated and control rats using the TUNEL method at

![Fig. 1. Change in the number of pancreatic islets in the unit area](image)
As noted for the 2 hour time interval, mitochondria and rER showed significant swelling. Inspissated nuclei and an accumulation of chromatin on nuclear membranes, was also conspicuous.

*Six hours after STZ administration*

The islet constituent cells were significantly less in number by six hours of STZ administration.
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Fig. 4. Change in the insulin and glucagon producing cells in the pancreatic islets.

treatment. Inspissated nuclei were noted in some B cells, fragmentation was obvious, and a proportion of endocrine cells were enucleated. The cytoplasm of many of the B cells was homogenous in appearance, some had ruptured cell membranes, cellular boundaries were indistinct in a large number of cells, and the number of viable islet constituent cells had notably declined. In addition, the surface area of the islet constituent cells was significantly increased. The cytoplasm was uniformly immuno stained for insulin. It was observed that a relatively large number of A cells were retained, and glucagon immuno staining showed that the granular structures in the cytoplasm (Fig. 6). The appearance of cells testing positive by the TUNEL method was noted (Fig. 7).

By electron microscopy, the minute intracellular compartment appeared amorphous, and most of the endocrine granules had broken down or if they were present, were indistinct. Rupturing nuclear and cell membranes was observed, along with apoptotic bodies in some cells (Fig. 8).

Twelve hours after STZ administration

The surface area of the islet constituent cells had increased significantly (Fig. 3), and the number of islets had decreased (Fig. 1). B cell degradation was more pronounced than at earlier times, homogenous and fragmented nuclei were noted, and many enucleated or indistinct cells were observed. The A cells, however, were relatively intact. Immunostaining for insulin and glucagon showed amorphous islets and homogenous patterns of immunoreactivity.

In addition, the number of cells testing positive by the TUNEL method was clearly increased by the 6 hours time point.

By electron microscopy, diffuse cell breakdown was observed; in particular, the minute intracellular organs of the B cells were almost completely broken down, and there was some nuclear fragmentation.

Twenty four hours after STZ administration
Both the number of islet constituent cells and the islet surface area were significantly increased; although the number of islets was decreased (Fig. 1, 3). Although some swollen and denatured B cells were still visible at this time point, cells that were immuno positive for insulin and glucagon were few in number, and only non-B cells were seen in the emptied verge of the islet (Fig. 4). The surviving islet cells, mainly A cells, showed swollen cytoplasm, and vacuolated cells were commonly noted. Despite this, there was hardly any infiltration of inflammatory cells. TUNEL-positive cells were rarely noted by this time interval.

**Seventy two hours after STZ administration**

By this time point, the number of islet constituent cells and islet surface area were further increased over that seen at 24 hours whilst the number of islets showed a significant
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Fig. 9. Islet 72 hours after administration
a : anti-insulin positive cells, b : anti-glucagon positive cells, immunohistochemistry ×100 magnification
insulin positive cells had increased.
The number of glucagon positive cells also showed a significant increase.

Fig. 10. Islet 2 weeks after administration
a : anti-insulin positive cells, b : anti-glucagon positive cells, immunohistochemistry ×100 magnification
The number of insulin positive cells increased further and the ratio of the pancreatic endocrine cells was returned to normal levels

| Table 1. The effects of streptozotocin on pancreatic islet cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 2 hours         | 3 hours         | 6 hours         | 12 hours        | 24 hours        | 72 hours        |
| The number of   | no change       | no change       | decrement       | remarkable      | decrement       | slight decrement |
| cells           |                 |                 |                 | decrement       |                 |                 |
| H-E stain       | no change       | Inspsissated    | fragmentation   | enucleate       | distention of   | vacuolation     |
|                 |                 | nuclei          |                 |                 | the cytoplasm   |                 |
| Immunohistochemaly Staining | anti-insulin staining | no change | no change | levels | levels | remarkable | decrement |
|                 | anti-glucagon staining | no change | no change | partly levels | levels | increases relatively | increases relatively |
|                 | TUNEL           | negative        | negative        | positive        | positive        | negative        |

decrease (Fig. 2, 3). There was a declining trend in the number of islets per unit area of tissue. Cytoplasmic swelling and vacuolation was decreased over that seen at earlier times, and the number of insulin positive cells had increased. In relative terms, the number of glucagon positive cells also showed a significant increase (Fig. 4, 9). Cells that were not immunopositive for either insulin or glucagon represented at last 40% of all viable cells, as compared to approximately 15% in the control group, which were identified as pancreatic polypeptide-producing (PP) or somatostatin-producing cells. Thus, excluding these known cell types, over 30% of the cells were differentiated, suggesting the presence of cells other than PP and D cells. TUNEL-positive cells were not observed.

By electron microscopy, a relatively large number of cells showed hardly any endocrine granule structures; those that were defected were extremely small and difficult to identify.
One and two weeks after STZ administration

The number of islet constituent cells and the islet surface area showed a significant increase 1 week after STZ treatment. The number of insulin positive cells had also increased (Fig. 10). By electron microscopy, most of the immunonegative cells were oligo-granular cells with intracellular structures resembling endocrine granules, the cells were therefore thought to be endocrine cells.

Two weeks after the STZ administration, no significant differences were noted by light or electron microscopy or by immunohistochemistry, as compared to the control group, nor were there any cells testing positive by the TUNEL method.

Discussion

Streptozotocin (STZ) is known to specifically impair B cells in the pancreatic islets of Langerhans. Although the pharmacodynamic action of STZ was recently characterised, there is a lack of useful immunohistochemical and electron microscopic data. On the morphological effects of STZ with time. In addition, whereas much is known about the B cell injury process due to STZ, insufficient research has been conducted in to the restoration process. The present study addresses these issues.

It is established that the islet B cell changes due to STZ can be detected morphologically soon after administration. Using light microscopy the present study shows that chromatin accumulation under the nuclear membrane commenced within 2 hours of STZ administration. Pyknosis was observed in the islet constituent cells after 3 hours, along with slight cytoplasmic swelling. Static edema was detected as an interstitial tissue response, although the inflammatory cell response was not marked. This pattern of morphology is quite different from the images usually published. After 6 hours, the cataplasia observed 3 hours after administration had progressed further, most of the B cells had splintered nuclear fragments, the cytoplasm was significantly swollen, and obvious vacuolation had occurred. This pattern of morphological change was still more pronounced after 12 hours, by which time a large number of completely enucleated, denatured B cells were also observed. Doi et al. obtained the same results using light microscopy, although these authors did not concentrate on the phenomena of enucleation and nuclear fragmentation.

The early changes observed, following administration of STZ, such as the chromatin accumulation, pyknosis, and advanced enucleation, may indicate apoptosis rather than simply cataplasia leading to necrosis.

The TUNEL method was considered to be particularly useful here to morphologically ascertain the apoptotic. The presence of TUNEL-positive cells was most notable 6 and 12 hours after STZ administration. Electron microscopy was able to confirm the presence of apoptotic bodies and the accumulation of chromatin corresponding to this phase in time. Both the immunohistochemical and electron microscopic studies suggested the possibility that apoptosis contributed to the process of B cell enucleation seen with STZ administration.

Zhi Ming reported vacuolation and inflammatory cell invasion commencing at 24 hours after STZ administration. In the results of the present study, however, only acute circulatory impediments, such as static edema, were found in the mesenchyme, and almost no inflammatory cell reaction was noted. Nor do other studies in the literature show B cell impairment accompanied by inflammatory cell invasion. B cell enucleation not accompanying such inflammatory cell reactions are morphologically consistent with apoptosis.
As discussed, the target cells for STZ are B cells, and it is thought that other islet constituent cells, including A cells, are not impaired by the drug. In fact, Hara reported no changes in A cells. In the present study, however, several completely destroyed islets, including their A cells, were noted within 12 hours of STZ administration. The primary effect on B cells, which account for approximately 60% of the islets, may precede loss of the entire islet structure, with other constituent cells, being impaired and enucleated secondarily, as a result of acute circulatory impairment.

Twelve hours after STZ administration, the impaired B cells were rapidly eliminated, and after 24 hours, the remaining cells were predominantly A cells almost no B cells, denatured cells, or necrotic cells were observed. It may be that after 12 hours, impairment of the islets by STZ ceased, the impaired cells were eliminated, and the recovery preparation phase commenced. This study found that B cell impairment due to STZ peaked at 24 hours after administration, and thereafter, the islet recovery preparation phase commences. After 72 hours, a considerable number of endocrine cells showed recovery.

In should be noted, however, that most of the cells occupying recovering tissue showed no immunohistochemical response to any of the islet hormones, and endocrine granules were rarely seen. Typical endocrine granules were not observed, and few cells could be identified as any kind of islet constituent cell. It is proposed that endocrine cells displaying characteristics of immature cells, or stem cells, are the first to recover. The absolute number of islet constituent cells had increased within 1 week of STZ administration, and the cells were largely normal, and deemed to be endocrine. This morphological analysis suggests that recovery of islet endocrine cells following STZ treatment, beginning with B cells, may occur via stem cells differentiating into immature endocrine cells.

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