Decreased Apoptosis as a Mechanism for Hepatomegaly in Streptozotocin-Induced Diabetic Rats

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Insulin-dependent diabetes mellitus in both humans and animals leads to structural and functional changes including hepatomegaly. This study examined hypertrophy, hyperplasia, and apoptosis, three basic aspects of tissue growth, in livers of Sprague-Dawley and Wistar rats made diabetic by iv injection of streptozotocin 8, 30, or 90 days previously. Immunohistochemical measurement of proliferating cell nuclear antigen revealed that hepatic DNA labeling indices were similar in normal control animals and diabetic rats 30 or 90 days post diabetic induction, but were reduced to 45 to 50% of control in insulin-treated diabetic animals, perhaps due to altered receptor activity or to partial insulin resistance, as reported previously. Flow cytometry indicated a 613% increase in diploid hepatocytes in the livers of diabetic rats 30 days after the onset of diabetes, compared to control. Diabetic livers contained 29% fewer tetraploid cells, 81% fewer octaploid cells, and 20% more binucleated hepatocytes than normal controls. At 90 days, the overall smaller size of hepatocytes in diabetic tissue was evidenced by more cells per area. Insulin treatment prevented some of these changes, but did not restore ploidy to a normal distribution. Mitosis, while 300% of normal at 8 days after streptozotocin injection, was reduced to 25% of normal after 90 days of diabetes. The morphological evidence of apoptosis was decreased by 23% to 76% in the diabetic liver, and was reversed but not normalized by insulin treatment. This study indicates that the hepatomegaly observed in streptozotocin-induced experimental diabetes may be due primarily to early hyperplasia, and later decreased apoptosis.

Key Words: apoptosis; mitotic index; hepatomegaly; flow cytometry; streptozotocin; proliferating cell nuclear antigen (PCNA); insulin-dependent diabetes.

Even with insulin treatment, diabetic patients show profound disturbances in tissue growth. Many of these problems have been linked to chronic hyperglycemia and the metabolic alterations that ensue (Porte and Schwartz, 1996). Clinically, increased liver size (hepatomegaly) is seen in both juvenile (Marangiello and Giorgetti, 1996) and adult (Chatila and West, 1996) diabetics, although the mechanism involved is not known.

An animal model of insulin-dependent diabetes mellitus can be experimentally produced in rats with the nitrosourea streptozotocin (STZ), which preferentially destroys β cells. Hepatomegaly is also associated with STZ-induced diabetes (Kume *et al.*, 1994; Watkins and Noda, 1986). However, the process by which diabetes causes this tissue change has yet to be ascertained.

An increase in tissue growth can be the result of alterations in cell number (hyperplasia), cell growth (hypertrophy), and/or cell death (apoptosis), all mechanisms that are at work in diabetes. For example, kidney hypertrophy is observed in both the glomerular basement membrane and capillaries of diabetics (Heidland *et al.*, 1996; Rabkin *et al.*, 1996; Sharma *et al.*, 1996) and may contribute to end-stage renal damage. Vascular hypertrophy may be one of the causes of diabetic hypertension (Hulthen *et al.*, 1996). Hypertrophy and hyperplasia in diabetic rat intestinal epithelium are linked to absorptive abnormalities (Younoszai *et al.*, 1993). Finally, diabetes and hyperglycemia have been linked to apoptosis through the activation of mitogen-activated protein kinase (Igarashi *et al.*, 1999).

The present study was designed to examine the role of altered cell growth, cell proliferation, and apoptosis in STZ-induced diabetic hepatomegaly in rats. Moreover, the effect of insulin replacement on these parameters was also determined.

MATERIALS AND METHODS

Induction of diabetes and experimental design. Female Sprague-Dawley rats (175–250 g) from Harlan Sprague Dawley Co. (Indianapolis, IN) were fed Purina Laboratory Rodent Chow (#5012, St. Louis, MO) and water *ad libitum*, and were maintained according to the *NIH Guide for the Care and Use of Laboratory Animals*. Diabetes was induced by injection of STZ (Sigma, St. Louis, MO; 50 mg/kg, dissolved in 10 mM sodium citrate, pH 4.5) into the saphenous vein. Serum glucose levels were measured using the hexokinase kit from Sigma Chemical Co. Seven days after the induction of diabetes (rats having serum glucose levels above 350 mg/dl), the insulin-treated group began receiving daily sc injections of 2–4 U of insulin [protamine zinc insulin (PZI), Eli Lilly, Indianapolis, IN]. Serum glucose was monitored periodically to assure control of hyperglycemia.

Between 9 and 11 A.M. on days 8, 30, or 90 after treatment with STZ, animals were anesthetized with diethyl ether. Livers were removed *in toto* and

Body Weight, Liver Weight, and Relative Liver Weight (Liver to Body Weight Ratio) following 8-, 30-, and 90-Day Periods of Diabetes in Sprague-Dawley Rats

	Days after STZ	Body weight (g)	Liver weight (g)	Relative liver weight
Normal (*n*, 6)	8	213 ± 2	8.9 ± 0.3	4.2 ± 0.1
Diabetic (*n*, 10)	8	196 ± 5[a]	9.2 ± 0.1	4.7 ± 0.1
Normal (*n*, 5)	30	234 ± 6	9.7 ± 0.9	4.1 ± 0.3
Diabetic (*n*, 5)	30	201 ± 10[a]	11.9 ± 0.9[a]	5.9 ± 0.3[a]
Insulin-treated (*n*, 5)	30	216 ± 10	9.4 ± 0.5[b]	4.3 ± 0.1[b]
Normal (*n*, 5)	90	252 ± 10	8.4 ± 0.5	3.3 ± 0.1
Diabetic (*n*, 5)	90	167 ± 12[a]	12.7 ± 0.8[a]	7.7 ± 0.6[a]
Insulin-treated (*n*, 5)	90	274 ± 7[a,b]	10.5 ± 1.1[a,b]	4.4 ± 0.3[a,b]

Note. Values represent mean ± standard deviation of body weight, liver weight, and relative liver weight [(liver weight/body weight) × 100] after 8, 30, and 90 days of diabetes.

[a] Significantly different from normal, $p < 0.05$.

[b] Significantly different from diabetic, $p < 0.05$.

weighed; samples from each lobe of liver were fixed in formalin for 2–3 days, embedded in paraffin, and sectioned (5 microns thick) for the following techniques: (a) stain with hematoxylin and eosin (H and E), or (b) immuno-histochemical analysis with proliferating cell nuclear antigen (PCNA) (below).

Apoptosis and mitosis. Light microscopic analysis of hematoxylin and eosin (H and E)-stained slides (Bursch *et al.*, 1985) at a magnification of 40× was used to quantitate the percent of mitotic and apoptotic hepatocytes for each lobe. Apoptotic hepatocytes were identified by morphologic criteria, which include increased eosinophilic cytoplasm, darkened nucleus, and pyknotic separation of cytoplasmic membrane from neighboring hepatocytes. Morpho-logically determined apoptotic indices were confirmed with a fluorescent microscopic technique (Stinchcombe *et al.*, 1995), which takes advantage of the degree of enhanced eosinophilia of the apoptotic hepatocyte under fluo-rescent light. Each group of apoptotic bodies was counted as one apoptotic event due to the short period of apoptosis and the possibility of missing a small, nearby body. Mitotic figures were recognized most often when the hepatocytes were in metaphase; hepatocytes in late prophase or anaphase were also counted.

From each lobe of each liver, at least 2000 contiguous hepatocytes were randomly examined to obtain a representative apoptotic or mitotic hepatocyte count. The general field size was 3.6×10^{-3} mm^2. The numbers of mitotic figures in all lobes of one rat liver were averaged to provide a single sample. For the apoptotic and mitotic indices, the total number of either category was divided by the number of hepatocytes counted and expressed as a percentage of hepatocytes undergoing mitosis or apoptosis in a given tissue sample.

Proliferating cell nuclear antigen. Immunohistochemistry was performed on liver sections using a 1:80 dilution of PC-10 (Biogenex, San Ramon, CA), a monoclonal antibody to proliferating cell nuclear antigen (PCNA), protein kinase C (PKC), and a key protein expressed in eukaryotic cells during DNA replication (Bravo, 1986; Celis *et al.*, 1987; Mathews, 1989). This was carried out according to previously published methods, with major modifications (Shi *et al.*, 1991). Paraffin-embedded sections of tissue were deparaffinized and cooled to room temperature. The slides were then incubated with anti-PCNA antibody (Biogenex) for 45 min at room temperature and 45 min at 37°C. The tissues were then incubated with biotinylated anti-mouse IgG for 20 min, followed by the addition of avidin-conjugated peroxidase (Biogenex). Anti-body binding was visualized with the chromogen 3-amino-9-ethyl-carbazole (Zymed, San Francisco, CA). Finally, the tissues were counterstained with hematoxylin. PCNA-positive hepatocytes, distinguished by the accumulation of red pigment in their nucleus, provide an indication of the number of hepatocytes engaged in replicative DNA synthesis. The labeling index was calculated in the same way as the apoptotic and mitotic indices above.

Flow cytometry. A separate group of Wistar rats (70–80 g) were rendered diabetic as described above with 100 mg/kg streptozotocin (STZ). An insulin-treated group was maintained on 2–5 U insulin/day. Thirty days after STZ treatment, rats were anesthetized with sodium pentobarbital (100 mg/kg; 4 ml/kg in water) and their livers were perfused under using the standard, two-step collagenase technique. The cells were washed 3× in Dulbecco's Minimal Essential Medium at 50 × g for 45 s, after filtration through 80 and 40 μm nylon mesh. Cell viability was determined by exclusion of 0.4% trypan blue. Cells were initially fixed in methanol, then DNA was stained with 60 μM propidium iodide. A FACS-Analyzer, with a 75 or 100 μm orifice, was used to quantitate DNA at 463–507 nm (excitation) and 532–658 nm (emission) in 10,000 events. The data were analyzed with a Becton-Dickinson Consort 30 program and were corrected for both aggregated and binucleated hepatocytes.

Statistics. Means and SE for all data were analyzed by ANOVA followed by Dunnett's post hoc test (Gad and Weil, 1986). The significance level was set at $p < 0.05$.

RESULTS

Liver and Body Weight

Although there was no difference in initial body weights among the groups, diabetes caused a failure to gain body weight, relative to normal, that became more pronounced from 8 to 30 to 90 days after STZ treatment (Table 1). Administration of insulin partially restored the body weight in 30- and 90-day diabetic rats as compared to normal controls. At 30 days after STZ, only the livers from diabetic rats increased in weight, whereas in the 90-day treatment groups, both the diabetic and insulin-treated diabetic rats had increased liver weight when compared to normal. The ratio of liver weight to body weight (relative liver weight) was 112% of normal in diabetic rats at 8 days, and 144% of normal at 30 days. Insulin treatment normalized the relative liver weight in 30-day dia-betic rats, but only attenuated the 2.3-fold increase in relative liver weight in 90-day diabetic rats.
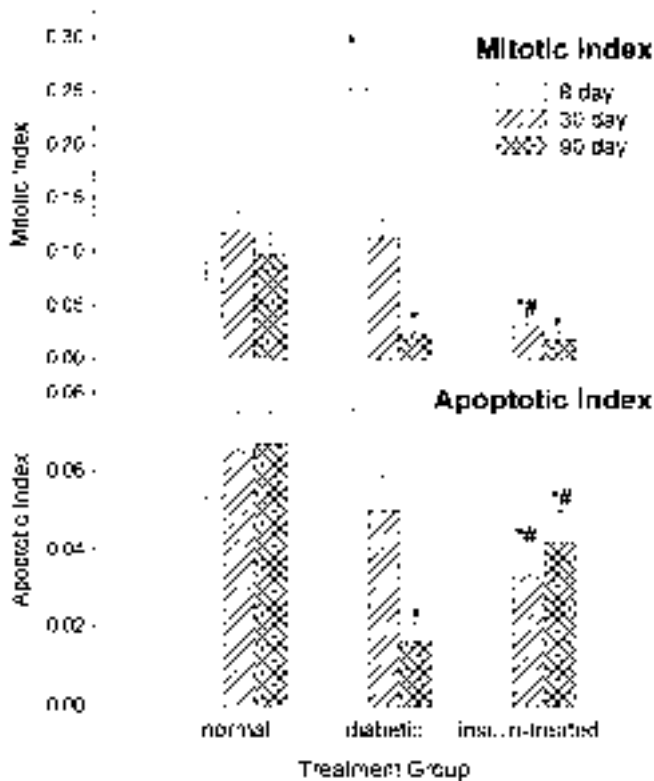
FIG. 1. Mitotic index and apoptotic index in livers of normal, diabetic, and insulin-treated diabetic rats at 8, 30, and 90 days after STZ treatment (see Materials and Methods). For the apoptotic and mitotic indices, the total number of either category was divided by the number of hepatocytes counted, then multiplied by 100 to express the percentage of cells undergoing mitosis or apoptosis in a given tissue sample. *Significantly different from normal, $p < 0.05$. #Significantly different from diabetic, $p < 0.05$.

Mitotic and Apoptotic Indices

The mitotic index (Fig. 1) in livers was increased to 300% of normal 8 days after STZ, was normal after 30 days of diabetes, and was 75% less than normal after 90 days. Insulin treatment led to reduced levels of mitosis when compared to the non-insulin treated STZ-induced diabetic rats at both 30 and 90 days. Trends in apoptotic indices (Fig. 1) paralleled those in mitotic indices, except that 8-day diabetic rats showed normal levels of apoptosis. The apoptotic index was significantly reduced in the 90-day diabetic rats when compared to normal. The apoptotic indices in both the 30- and 90-day insulin-treated groups were lower than normal controls. No consistent differences were observed among lobes in any livers from any group at any time point.

Cells per Field

The size of cells was quantitated by counting the number of hepatocytes per field (Fig. 2). Normal and diabetic rats, 8 days after STZ, had similar numbers of hepatocytes per field. By 30 days, both normals and diabetics had less than 50% as many

cells per field as 8-day animals. The 90-day diabetic rats had significantly more cells per field than 30-day diabetic or 90-day normal rats. Cells per field ratios of 30- and 90-day insulin-treated groups were intermediate between normals and diabetics.

PCNA Labeling Index

No changes in DNA replication were observed in rat livers after 30 and 90 days of diabetes (Fig. 2). However, both the 30- and 90-day insulin-treated rats showed significantly lower PCNA labeling indexes, reflecting fewer cells in S-phase. This parameter was not examined on the livers excised from rats 8 days after STZ.

Ploidy Distribution

The proportion of diploid hepatocytes decreased with age in normal, untreated rats (Table 2), while the proportion of tetraploid and binucleated hepatocytes increased with age. Diabetic rats, however, had 513% more diploid hepatocytes, 29% fewer tetraploid hepatocytes, 81% fewer octaploid hepatocytes, and 20% more binucleated hepatocytes than age-matched nor-
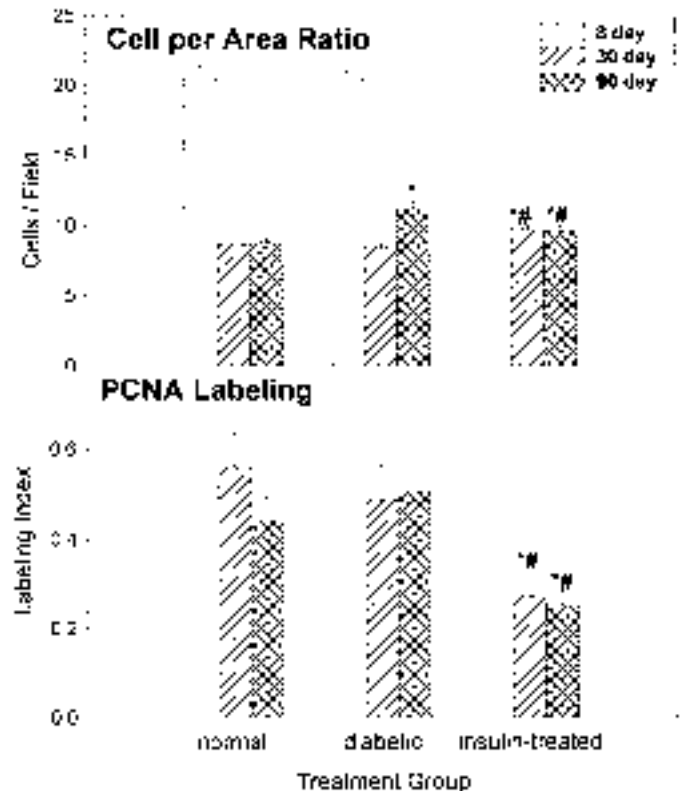

FIG. 2. Cell area ratio (cells counted per unit area of liver tissue) and labeling index as determined by PCNA labeling (number of PCNA labeled hepatocytes divided by total number of hepatocytes counted) in livers of normal, diabetic, and insulin-treated diabetic rats at 8, 30, and 90 days after STZ treatment (see Materials and Methods). *Significantly different from normal, $p < 0.05$. #Significantly different from diabetic, $p < 0.05$.

TABLE 2

Effect of Age (Days after Birth) on Hepatocyte Ploidy Distribution (% of Total Hepatocytes) in Untreated Normal Male Wistar Rats

	21 days	35 days	49 days
Diploid	60.0 ± 1.2	16.3 ± 1.0	6.8 ± 1.1
Tetraploid	40.0 ± 0.9	73.1 ± 0.9	77.3 ± 1.0
Octaploid	ND	10.6 ± 0.3	15.9 ± 1.9
Binucleated	17.9 ± 0.6	20.0 ± 1.7	21.8 ± 0.5

Note. Values represent mean $\pm$ standard error of % of total hepatocytes in 3–4 rats.

ND, not detectable.

mals (Table 3). Insulin attenuated the diabetes-induced differences in ploidy distribution, but did not restore ploidy to normal levels for any cell type.

DISCUSSION

This study examined several mechanisms possibly involved in the hepatomegaly that is observed in STZ-induced insulin-dependent diabetic rats, including increased hepatocyte proliferation, decreased apoptosis, and hypertrophy. Time-course studies have shown that the toxic effects of STZ, while still apparent after one week, disappear by 30 days (Watkins and Sanders, 1995), so that any abnormalities are attributable to the disease state.

Cell size is related to ploidy because a polyploid cell has more chromatin and thus a larger size than a diploid cell (Watanabe and Tanaka, 1982). In normal rats, the proportion of diploid cells sharply decreases and the polyploid population increases with increasing age (Table 2), as has been seen elsewhere in rats (Sanz *et al.*, 1996; 1997) and in humans (Kudryavtsev *et al.*, 1993). However, the high proportion of diploid cells actually observed in the liver of a 7-week-old, 30-day diabetic rat (Table 3) corresponds to that in a normal rat midway between 3 and 5 weeks of age (Table 2). Thus, the higher proportion of smaller cells would lead one to expect a smaller liver size, not larger as observed.

An increase in diploid:polyploid ratios is one criterion used to distinguish precancerous or cancerous liver tissue or other chronic liver diseases, including hepatitis and cirrhosis, from normal liver (Anti *et al.*, 1993). In addition, diploid hepatocytes are at higher risk for genetic mutation when compared to their polyploid counterparts (Schwarze *et al.*, 1984). This is borne out by clinical data from diabetic individuals indicating that the liver is the most likely tissue to become cancerous (La Vecchia *et al.*, 1994). In this study, however, no foci or lesions were observed in livers from diabetic animals.

Cellular proliferation characterizes potential hyperplastic tissue. The surge in mitotic activity in diabetic rats immediately after STZ treatment is normalized by 30 days, and even de-pressed by 90 days. However, cell-cycle indicators in insulin-treated diabetic controls were significantly different from both normals and diabetics. The decrease in PCNA labeling suggests a G_0- or G_1-phase block, similar to that seen with certain anticancer agents (Tamura *et al.*, 1992). This may indicate that insulin treatment itself actually decreases proliferative capacity as reflected in the mitotic index, resulting in delayed or inappropriate response to tissue injury.

Insulin treatment of induced diabetes has been shown to bring about disruptions in responses to insulin binding in the liver (Haft, 1968; Le Marchand *et al.*, 1977). Ordinarily, insulin, a proven mitogen, leads to activation of protein kinase and thus stimulates cellular proliferation (Cohen, 1992). However, in STZ-induced diabetes, decreased phosphorylation of the β subunit of the insulin receptor leads to decreased receptor kinase activity (Kadowaki *et al.*, 1984), which may explain why insulin treatment decreases cell proliferation as observed by decreased replicative DNA synthesis (Fig. 2) and mitotic indices (Fig. 1).

One factor that the untreated diabetic liver experiences, and that the insulin-treated rat liver does not, is hyperglycemia. In fact, elevated glucose concentrations are known to activate isoforms of protein kinase C (PKC) in several tissues (Porte and Schwartz, 1996). Furthermore, five PKC isoforms have been found in hepatocytes (Croquet *et al.*, 1996), at least 3 of which are increased in liver cells from STZ-induced diabetic rats (Tang *et al.*, 1993). In hepatocytes, PKC is an intermediate step in the insulin transduction pathway that activates mitogen-activated protein kinase (Adachi *et al.*, 1996). PKC also increases growth and proliferation of vascular smooth muscle cells when stimulated with glucose (Yasunari *et al.*, 1996). Thus, high levels of glucose may activate PKC, which then modulates activation of mitogen-activated protein kinase. This mechanism would not occur in insulin-treated diabetics, due to lowered glucose levels and possible inhibition of the insulin receptor by PKC isoforms.

In addition, decreased frequency of apoptosis may also help

TABLE 3

Effect of Streptozotocin-Induced Diabetes on Hepatocyte Ploidy Distribution (% of Total Hepatocytes) in 49-Day-old Wistar Rats

Hepatocyte type	Normal	30-day Diabetic	Insulin-treated
Diploid	6.8 ± 1.1	41.7 ± 9.0^a	$25.7 \pm 4.3^{a,b}$
Tetraploid	77.3 ± 1.0	55.3 ± 8.1^a	$68.5 \pm 3.0^{a,b}$
Octaploid	15.9 ± 1.9	3.0 ± 1.4^a	5.8 ± 1.3^b
Binucleated	21.8 ± 0.5	26.2 ± 0.9^a	$19.4 \pm 0.8^{a,b}$
Aggregates	2.5 ± 0.6	5.2 ± 1.3^a	5.2 ± 0.9^a

Note. Values represent mean $\pm$ standard error of % of total hepatocytes in 4 normal rats, 9 rats diabetic for 30 days, and 4 insulin-treated 30-day diabetic rats.

[a]Significantly different from normal, $p < 0.05$.
[b]Significantly different from diabetic, $p < 0.05$.

to produce the hepatomegaly of diabetic liver. Apoptosis plays a key role in eliminating potentially damaged cells and regulating overall cell numbers by active protein and RNA synthesis (Wyllie *et al.*, 1984) and in the absence of de novo protein synthesis (Martin *et al.*, 1994). For a tissue to maintain its size, the apoptotic rate must closely parallel the mitotic rate. In the present study, the control and insulin-treated groups showed this equilibrium. However, the diabetic liver showed a significant decrease in apoptosis.

The inhibition of protein synthesis may inhibit apoptosis. Thus, the overall decrease of liver protein synthesis, as previously observed in untreated insulin-dependent diabetes mellitus (Jefferson *et al.*, 1983), may contribute to the decreased hepatic apoptosis observed in this study. The decrease in apoptosis in the insulin-treated group, relative to controls, also may be due to the decrease in insulin responsiveness seen in induced diabetes mentioned above. Insulin has different effects on apoptosis, depending on the tissue type, and leads to both inhibition and stimulation (Tanaka *et al.*, 1995; Yang *et al.*, 1996). Thus, insulin may be able to modulate processes active in apoptosis in different tissue types, perhaps by alterations in tyrosine kinase activity.

This study evaluated several different parameters of liver growth and their role in the hepatomegaly that follows the induction of diabetes. Diabetic hepatocytes were predominantly in the diploid state. Diabetic livers showed high proliferation rates soon after STZ treatment, followed by decreases in mitotic rate, to the extremely low rate seen at 90 days after STZ. Mitogen-activated protein kinase stimulation by insulin and PKC was suggested as an explanation of this occurrence. Tissue apoptosis was decreased in the diabetic liver, but only by 90 days. Extracellular matrix expansion, which was not evaluated in this study, remains a plausible explanation as well. Thus, diabetic hepatomegaly appears to be due to hyperplasia and decreased apoptosis.

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