

Plasma Levels of Cell-Free Apoptotic DNA Ladders and Gamma-Glutamyltranspeptidase (GGT) in Diabetic Children

MARLYN P. LANGFORD,^{*,1} THOMAS B. REDENS,^{*} NORMAN R. HARRIS,[†] SEUNGJUN LEE,[†]
SUSHIL K. JAIN,[‡] SUDHA REDDY,[‡] AND ROBERT McVIE^{‡,1}

**Department of Ophthalmology, †Department of Molecular and Cellular Physiology, and ‡Department of Pediatrics, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130*

The plasma levels of apoptotic DNA ladders (i.e., apoptosemia) and γ -glutamyltranspeptidase (GGT) in diabetic outpatients and rats were investigated. Apoptotic DNA ladders were detected in plasma from 26.8% of type 1 (T1) and 18.5% of type 2 (T2) diabetic children 1–20 years of age, 25.7% of hospitalized children and 35.7% of adult RA outpatients, but in only 3.5% of adult pre-op patients. Plasma from 7.7% of young streptozotocin-induced diabetic but not control rats contained apoptotic DNA ladders. Apoptosemia was detected more often in male T1 (31%) and T2 (30.8%) diabetic outpatients than in female T1 (20.8%) and T2 (15.4%) diabetic outpatients. GGT in apoptosemic plasma was significantly higher than in nonapoptosemic plasma from T1 ($P = 0.001$) but not T2 diabetic children. The highest amounts of apoptotic DNA were detected most often in diabetic children ≥ 14 years of age. *In vitro* study results suggest that cell-free apoptotic DNA ladders appear prior to an increase in GGT activity in serum from human blood incubated at 37°C. The results suggest that 24.7% of plasma samples from diabetic children contained apoptotic DNA ladders, the incidence and amounts of apoptotic DNA ladders were higher in the older diabetic children, and GGT was elevated in apoptosemic T1 diabetic children ($P = 0.01$). The results indicate that “silent” apoptosemia occurs in T1 and T2 diabetic children and suggest elevated GGT in diabetic children could be due to release from apoptotic cells. *Exp Biol Med* 232:1160–1169, 2007

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¹ To whom correspondence should be addressed at Louisiana State University Health Sciences Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, LA 71130-3932. E-mail: mlangf@lsuhsc.edu or rmcvie@lsuhsc.edu

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Introduction

Nucleic acid levels are generally low in the plasma of healthy adults due to rapid clearance by the liver, kidneys, and lymphoid cells, but they increase with age (1–6). The elevation of cell-free nucleic acids and condensed nuclear fragments (nucleosomes) in plasma may be due to necrotic and apoptotic cells, release from nondividing cells, or inefficient clearance (7). Elevated levels of nucleic acids have been detected in plasma of patients with various primary and metastatic cancers (8) and following trauma (9), stroke (10), and myocardial infarction (11). Moreover, the detection of high plasma DNA levels has been associated with a poor prognosis in cancer, stroke, trauma, and intensive care patients (9–13), and reduced longevity in elderly individuals (6). The failure to clear apoptotic cell debris and DNA has been associated with an increased risk of autoimmune disease (14). Accordingly, elevated plasma DNA levels and antibodies to DNA have been detected in patients with rheumatoid arthritis (RA; Ref. 15), systemic lupus erythematosus (16), thyroiditis, and multiple sclerosis (17).

Apoptosis appears to play a role in the initiation of diabetes mellitus as well as the complications manifested in the vasculature, brain, heart, kidneys, joints, and eyes (17–20). Moreover, antibodies to DNA and cell components as well as apoptotic leukocytes, vascular endothelial cells, and renal cells have been reported in diabetic humans and rats (20–27). However, the presence and levels of DNA ladders characteristic of programmed cell death have not been investigated in plasma of diabetic children and rats. Our first objective was to analyze plasma from type 1 (T1) and type 2 (T2) diabetic children and streptozotocin (STZ)-induced diabetic rats for cell-free nuclear fragments discernible as DNA ladders indicative of caspase-mediated apoptosis (28, 29). Second, γ -glutamyltranspeptidase (GGT) levels in plasma were investigated, because GGT is elevated in diabetic and RA patients (30–33), is a risk factor for cataract formation (34), and is associated with

insulin resistance (35, 36). GGT is a multifunctional extrinsic enzyme located on leukocytes and vascular endothelial, liver, and luminal epithelial cells that converts glutathione, glutamine, and leukotrienes (37, 38) and induces the formation of osteoclasts (39). Third, plasma samples from hospitalized "ill" children (i.e., pediatric inpatients), adult RA outpatients, and "well" pre-op adult patients were screened as positive and negative patient control populations, respectively. The results suggest that apoptotic DNA ladders (i.e., apoptosemia) can be detected in plasma from T1 and T2 diabetic children, apoptosemia is more prevalent in older diabetic children, and there is a close association between the levels of apoptosemia and GGT in T1 diabetic children.

Materials and Methods

Human Samples. Plasma samples from 255 pediatric diabetic outpatients 1–20 years of age and 35 pediatric inpatients were obtained through the auspices of the Diabetes Clinic and Clinical Chemistry Lab (Louisiana State University Health Sciences Center [LSUHSC], Shreveport, LA) on an as-available basis. Standard phlebotomy techniques were employed to obtain plasma samples from pediatric diabetic outpatients during quarterly outpatient clinic visits and from pediatric inpatients. Each blood sample was collected by venipuncture into siliconized vacuum test tubes containing EDTA (Becton-Dickinson, Rutherford, NJ), and the plasma was collected as previously reported (40) and refrigerated at 4°C. Venous blood was drawn into EDTA to inhibit endonuclease activity. Note that the plasma samples from Diabetic Clinic outpatients and hospitalized inpatients were identified only by age and gender, and those analyzed represent about 40% of the plasma samples collected from the pediatric diabetic outpatient population attending the LSUHSC Diabetic Outpatient Clinic during the 2-year course of this study. The possibility that more than one plasma sample was taken from a pediatric outpatient during the first year of the study and that some of the pediatric inpatients and adult outpatients were diabetic cannot be ruled out. Also, a preoperation blood sample was obtained from 57 consenting adult patients undergoing cataract surgery (Eye Clinic, LSUHSC, Shreveport, LA) and 28 consenting adult RA outpatients (Center for Excellence in Arthritis & Rheumatology, LSUHSC, Shreveport, LA). The blood samples were centrifuged at 2000 *g* for 3 mins at room temperature, and the plasma was harvested. The plasma samples (0.5–1.8 ml) were pipetted into vials coded with respect to disease, gender, and age before storage at –80°C. To investigate apoptosis of blood cells and release of GGT, blood samples were drawn from three healthy adult volunteers, divided into 1-ml volumes, and placed in vials with or without EDTA (50 mM). These investigations were performed in accordance with the principles expressed in the Declaration of

Helsinki and were approved by the LSUHSC's Internal Review Committee for Human Research Subjects.

Detection of Apoptotic DNA Ladders in Plasma. DNA ladders are a hallmark of apoptosis (i.e., programmed cell death; Ref. 28). Thus, the detection of apoptotic DNA ladders in 0.3 ml plasma was used to assess systemic apoptosis (i.e., apoptosemia) in children and adults. Apoptotic DNA ladders were detected in plasma samples using the method reported previously (41). Briefly, 0.1 ml solubilization buffer (50 mM Tris-HCl, 40 mM EDTA, 1% [v/v] Triton X-100, and 1% [v/v] NP-40, pH 7.6; Sigma Biological, St. Louis, MO) was added to 0.3 ml plasma. The DNA was phenol extracted, ethanol precipitated at –80°C for 10 mins, and pelleted by centrifugation at 12,000 *g*. The pelleted DNA was washed with 70% and 100% ethanol in succession and air dried for 5 mins. The pelleted DNA was dissolved in RNase A digestion buffer (24 µl/sample; 50 mM Tris-HCl, 5 mM EDTA, 60 µg/ml RNase A, pH 7.5; Sigma) and incubated at 37°C for 60 mins to allow complete RNA digestion. Gel loading buffer (6 µl; Sigma) containing SYS Green 1 (Sigma; 10,000× in DMSO; diluted 1:1000 in loading buffer) was added to each sample and electrophoresed (60 V for 1.5 hrs) through 1% agarose gel containing ethidium bromide (100 ng/ml). DNA ladder standard (20 µl/lane; Invitrogen; Grand Island, NY; or 100 bp BMA DNA ladders; ISC BioExpress, Kaysville, UT) was included in each run. Green and/or orange fluorescing double-stranded DNA fragments were visualized under ultraviolet light. Black-and-white digital images were obtained using the Bio-Rad Gel Documentation System and Quantity One software (Bio-Rad, Hercules, CA). The relative levels of apoptotic DNA ladders per sample were estimated by side-by-side, semiquantitative analysis of the images by one viewer. The amount and profile of apoptotic DNA ladder in each sample was scored using a 0 to 3+ scale. That is, 0 represents no detectable ladder, 1+ represents detection of a discernible DNA ladder, 2+ represents detection of DNA ladders of intermediate intensity, and 3+ represents detection of a high-intensity DNA ladder.

Diabetic Rat Model. Diabetes (blood glucose > 200 mg/dl) was induced in 2- to 3-month-old male Wistar rats (Charles Rivers Laboratories Inc., Wilmington, MA) by injection of STZ (a model of type I diabetes) as previously described (42). A plasma sample (i.e., in 50 µM EDTA) was obtained 3–4 weeks after STZ injection (prior to initiating insulin treatment) from 26 diabetic and 24 age- and sex-matched control rats. The plasma samples from the diabetic and control rats were placed in vials and stored at –80°C. Detection of apoptotic DNA ladders was performed as described above for human plasma samples. The protocol was approved by the LSUHSC's Animal Resources Advisory Committee and performed in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>).

Table 1. Age and Sex Distribution of the Pediatric Study Populations

	<i>n</i>	Age (years) \pm SD		
		All	Males	Females
Diabetic children	255	12.2 \pm 4.3 ^a	12.2 \pm 3.9	12.2 \pm 4.5
T1 children	190	11.5 \pm 4.3 ^b	11.9 \pm 3.9 ^c	10.9 \pm 4.5 ^c
T2 children	65	14.4 \pm 2.9	15.6 \pm 2.6	14.2 \pm 3.0
Inpatient children	35	14.4 \pm 4.3	14.8 \pm 4.4	13.6 \pm 4.3

^a The diabetic children were younger than the inpatient children ($P = 0.01$).

^b The T1 diabetic children were younger than the T2 diabetic children ($P = 0.019$).

^c The T1 male and female children were younger than the T2 male and female children, respectively ($P < 0.001$).

GGT Activity and GGT Release. Quantification of GGT activity in serum and plasma fractions was determined spectrophotometrically using a standard assay (43). Briefly, 0.1 ml of each sample was pipetted into a test tube containing 1.0 ml GGT assay medium (GAM; 1.8 mM γ -glutamyl-*p*-nitroanalide and 20 nM glycyl-glycine in Tris-HCl buffer; pH 8.2) and incubated for 1–4 hrs at 37°C. The following procedures were used to investigate changes in GGT levels in the serum and plasma fractions of blood samples from three adult donors incubated at 37°C for up to 4 days without EDTA and up to 14 days with EDTA. The serum was harvested, and 0.3 ml packed blood cells was suspended in 1 ml distilled water and centrifuged at 5000 *g* for 3 min; the lysate was removed, and this lysis process was repeated four times. The wet weight of the cell membrane fraction of each sample was determined. As above, 1 ml GAM was added to serum and the isolated cell membrane fractions and incubated for 1–4 hrs. The GGT reaction was terminated by addition of an equal volume of 2 N acetic acid to the sample. The OD₄₁₂ of the GAM was determined spectrophotometrically, the nanomoles of para-nitroanalide converted were calculated in replicate samples, and the units of GGT activity (nanomoles converted)/hr/ml or /mg cell membrane fraction were determined. The increase in GGT activity in the serum fraction was expressed as a percent of the total GGT. That is, the difference between the GGT level (U/ml) in serum from the freshly drawn blood samples and the GGT level in respective serum harvested at the different incubation periods was divided by the GGT level in the respective cell fraction (U/mg) and multiplied by 100.

Glucose Levels. Glucose levels in plasma samples were determined by the OneTouch Ultra blood glucose monitoring system following the manufacturer's instructions (Lifescan Inc., Milpitas, CA).

Statistical Analysis. Means \pm standard deviations were calculated using Microsoft Excel software (Windows XP-2000 Professional; Seattle, WA). Differences between mean ages of males and females, T1 and T2 diabetic and inpatient groups, and between GGT levels in samples were compared by two-tailed Student's *t* test. P values ≤ 0.05 were considered statistically significant.

Results

Age Distribution of Pediatric and Adult Plasma Donors. The diabetic outpatients providing plasma samples were significantly younger (12.2 \pm 4.3 years; $n = 255$) than the inpatient children (14.4 \pm 4.3 years; $n = 35$; $P = 0.01$; Table 1). The mean age of the T1 diabetic outpatients was significantly lower than the mean age of the T2 diabetic outpatients (11.5 \pm 4.3 vs. 14.4 \pm 2.9 years; $P = 0.019$). However, the mean age of the diabetic males was similar to that of the diabetic females (12.2 \pm 3.9 and 12.2 \pm 4.5 years, respectively). T1 diabetic male (11.9 \pm 3.9 years) and female (10.9 \pm 4.5 years) outpatients were significantly younger ($P < 0.001$) than the T2 diabetic male (15.6 \pm 2.6 years) and female (14.2 \pm 3 years) outpatients. These data are consistent with the idea that this study population represents the normal age and sex distribution for diabetic children and are consistent with an early age of onset in T1 diabetes.

The mean age of the female inpatient children (13.6 \pm 4.3 years) was not significantly different ($P = 0.26$) from that of the male inpatient children (14.8 \pm 3.4 years). The mean ages of the 57 preoperative adult patients (before cataract extraction and lens implantation surgery) and 28 adult RA outpatients were 62.6 \pm 13.2 and 60 \pm 11 years, respectively.

Detection of Apoptosemia in Diabetic Children and STZ-Induced Diabetic Rats. Representative agarose gels showing the profiles of phenol-extracted DNA from plasma of diabetic outpatients and adult RA outpatients are presented in Figure 1. Apoptotic DNA ladders were detected in plasma samples from T1 and T2 diabetic outpatients (Fig. 1A), inpatient children, and adult RA outpatients (Fig. 1B). (Note that the 4-kbp band detected in several human plasma samples is a complex composed of DNA and an unidentified [based upon its unique mass spectrum] 39-kDa protein with DNA binding characteristics similar to serum amyloid P; Ref. 44). In comparison, apoptotic DNA ladders were detected (SYS Green positive) in 2 (7.7%) of 26 plasma samples from STZ-induced diabetic rats, but not in 24 control plasma samples from nondiabetic rats (data not shown). Taken together, the data indicate that apoptotic DNA ladders can be detected in DNA extracted from 0.3 ml

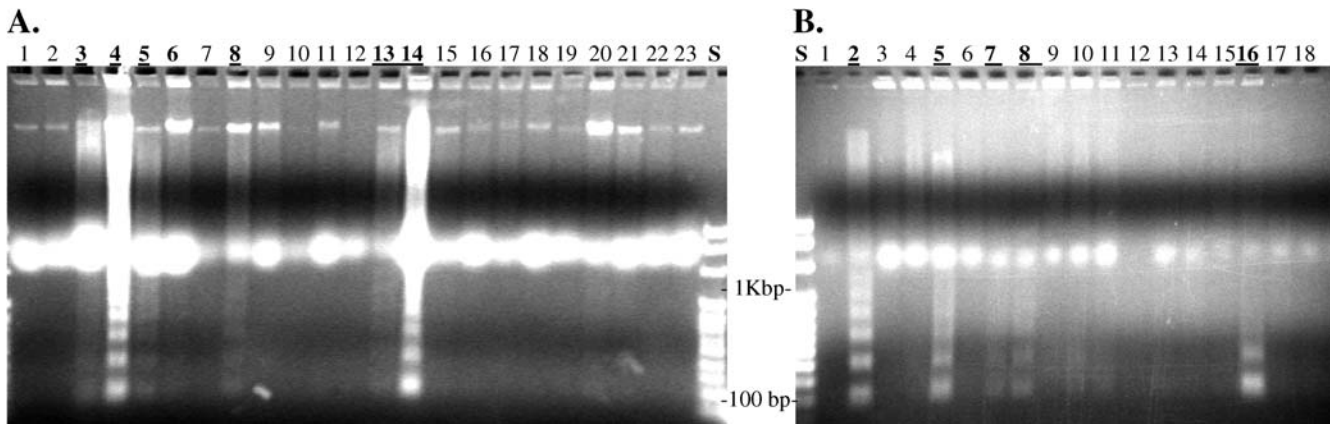


Figure 1. Representative gels demonstrating apoptotic DNA ladders in plasma from (A) diabetic children and (B) adult RA outpatients. Note 3+ apoptotic DNA ladders in diabetic plasma samples 4 and 14 and in RA plasma 2. A 2+ apoptotic DNA ladder was detected in RA samples 5 and 16 (B), and a 1+ apoptotic DNA ladder was detected in diabetic plasma samples 3, 5, 8, and 13 (A) and in RA plasma samples 7 and 8 (B).

plasma from diabetic children, hospitalized pediatric patients, and RA outpatients, as well as diabetic rats.

Age and Sex Distribution of Apoptotic DNA Ladder-Positive Diabetic Outpatients. The number of plasma samples from diabetic outpatients and inpatients and the number positive for apoptotic DNA ladders are presented for each age group in Table 2. Overall, 63 (24.7%) of the 255 plasma samples collected from diabetic outpatients and 9 (25.7%) of 35 plasma samples from pediatric inpatients contained detectable apoptotic DNA ladders. Interestingly, apoptotic DNA ladders were detected in 39 (30.8%) of 126 plasma samples from male and 24 (18.6%) of 129 plasma samples from female diabetic

outpatients. Plasma samples from 51 (26.8%) of 190 T1 and 12 (18.5%) of 65 T2 diabetic outpatients contained apoptotic DNA ladders. Concomitantly, plasma samples from 35 (31%) of 113 T1 males compared with 16 (20.8%) of 77 T1 females contained apoptotic DNA ladders. Apoptotic DNA ladders were detected in 12 (18.5%) of 65 plasma samples from T2 diabetic outpatients. Note that 4 (30.8%) of 13 plasma samples from T2 diabetic males contained apoptotic DNA, whereas 8 (15.4%) of 52 plasma samples from T2 diabetic females contained apoptotic DNA. Plasma samples from 9 (25.7%) of 35 pediatric inpatients contained detectable apoptotic DNA ladders (i.e., 4 [28.6%] of 14 males [ages 2–14 years] and 5 [23.8%] of

Table 2. Summary of the Distribution of Apoptosemic Plasma in the Pediatric Study Populations^a

Age, years	T1 males	T1 females	T1 totals	T2 males	T2 females	T2 totals	T1 and T2 males	T1 and T2 females	Total T1 and T2	Inpatient males	Inpatient females	Total inpatients
1	—	1	1	—	—	—	—	1	1	—	—	—
2	—	2	2	—	—	—	—	2	2	1 (1)	—	1
3	—	2	2	—	—	—	—	2	2	—	1	1
4	2 (1)	6 (1)	8 (2)	—	—	—	2 (1)	6 (1)	8 (2)	—	—	—
5	2 (1)	3	5 (1)	—	—	—	2 (1)	3	5 (1)	—	—	—
6	6 (1)	—	6 (1)	—	—	—	6 (1)	—	6 (1)	—	1	1
7	6	3 (1)	9 (1)	—	—	—	6	3 (1)	9 (1)	—	1	1
8	10 (3)	3 (2)	13 (5)	—	2 (1)	2 (1)	10 (3)	5 (3)	15 (6)	—	—	—
9	10 (1)	6 (1)	16 (2)	—	3	3	10 (1)	9 (1)	19 (2)	—	1 (1)	1 (1)
10	5 (3)	7 (1)	12 (4)	—	4	4	5 (3)	11 (1)	16 (4)	—	—	—
11	9 (2)	6 (2)	15 (4)	1	—	1	10 (2)	6 (2)	16 (4)	1 (1)	1	2 (1)
12	8 (2)	7 (1)	15 (3)	—	3	3	8 (2)	10 (1)	18 (3)	2 (1)	1	3 (3)
13	12 (3)	9 (3)	21 (6)	3	9 (3)	12 (3)	15 (3)	18 (6)	33 (9)	1	1	2
14	7 (4)	4 (2)	11 (6)	3	6	9	10 (4)	10 (2)	20 (6)	1 (1)	3 (1)	4 (2)
15	11 (3)	3	14 (3)	—	3	3	11 (3)	6	17 (3)	3	5 (1)	8 (1)
16	10 (5)	5	15 (5)	1 (1)	7 (1)	8 (2)	11 (6)	12 (1)	23 (7)	1	2 (1)	3 (2)
17	9 (2)	3 (1)	12 (3)	2 (2)	10 (2)	12 (4)	11 (4)	13 (3)	24 (7)	2	—	2 (1)
18	5 (3)	4 (1)	9 (4)	1 (1)	2	3 (1)	6 (4)	6 (1)	12 (5)	1	1	2
19	—	3	3	2	3 (1)	5 (1)	2	6 (1)	8 (1)	1	3 (1)	4 (1)
20	1 (1)	—	1 (1)	—	—	—	1 (1)	—	1 (1)	—	—	—
Total	113 (35)	77 (16)	190 (51)	13 (4)	52 (8)	65 (12)	126 (39)	129 (24)	255 (63)	14 (4)	21 (5)	35 (9)
%	31	20.8	26.8	30.8	15.4	18.5	30.9	18.6	24.7	28.6	23.8	25.7

^a Numbers in parentheses indicate the number of plasma samples positive for apoptotic DNA ladders.

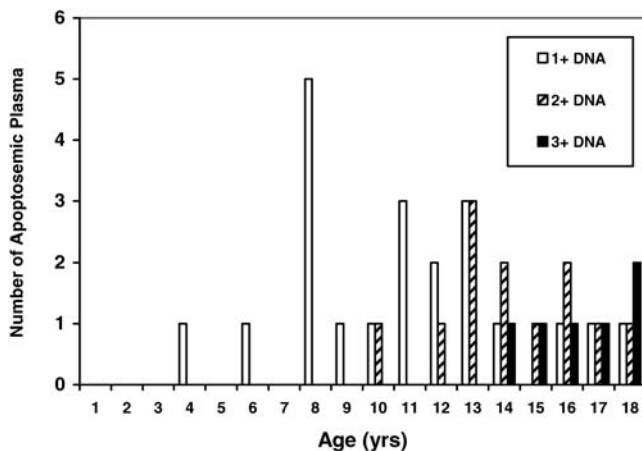


Figure 2. Apoptotic DNA ladder levels in apoptosemic diabetic outpatients. Note that the highest DNA levels were detected most often in the older children.

21 females [ages 9–19 years]). In parallel studies, apoptotic DNA ladders were detected in 10 (35.7%) of 28 plasma samples collected from adults with RA but in only 2 (3.5%) of 57 plasma samples collected from adult patients prior to cataractous lens extraction and lens implantation surgery. The results suggest that 25% of plasma samples collected from diabetic outpatient children, inpatient children, and adult RA outpatients contained detectable apoptotic DNA ladders. These results indicate that apoptosemia in the preoperative adult control patient group was 3- to 10-fold less than in diabetic outpatient children groups, inpatients, and adult RA patients.

Semiquantification of Apoptotic DNA Ladders in Plasma. Representative gels showing different levels of apoptotic DNA ladders in plasma are presented in Figure 1. The relative amount of apoptotic DNA ladder in the 63 apoptosemic plasma samples was semiquantified using a score of 1+ to 3+. The results indicate that 54% (34 plasma samples from outpatients 4–20 years of age) contained 1+ apoptotic DNA ladders, 29% (18 plasma samples from outpatients 9–18 years of age) contained 2+ apoptotic DNA ladders, and 17% (11 plasma samples from outpatients 12–18 years of age) contained 3+ apoptotic DNA ladders. Notably, 82% (9 of the 11) of the plasma samples containing 3+ apoptotic DNA ladders was obtained from 12- to 18-year-old T1 diabetic males. The two other plasma samples containing 3+ DNA ladders were from an 18-year-old T1 diabetic female and a 16-year-old T2 diabetic male. These results suggest that the highest levels of apoptosemia were detected most often in plasma from 12- to 18-year-old T1 diabetic males.

To analyze the perceived relationship between apoptotic DNA levels and age, the apoptotic DNA ladder scores of 39 plasma samples drawn from different apoptosemic children were plotted. Clearly, the highest levels of apoptotic DNA ladders were detected most often in plasma samples from older diabetic children (Fig. 2). Note that 1+

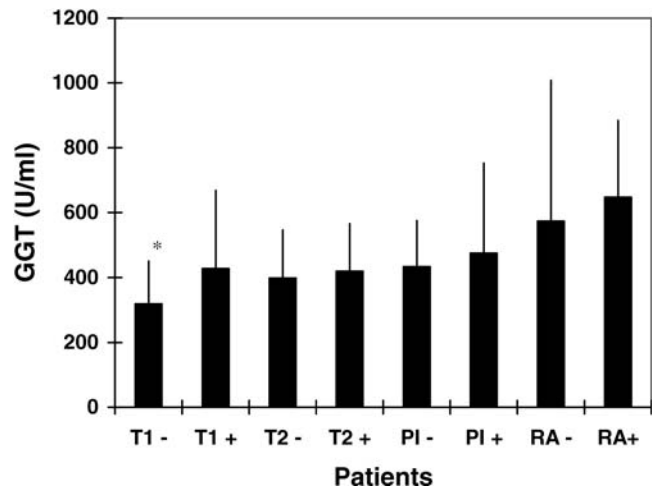


Figure 3. GGT activity in plasma from nonapoptosemic (-) and apoptosemic (+) T1 and T2 diabetic outpatients, pediatric inpatients (PI), and adult RA outpatients. * $P = 0.01$.

apoptotic DNA ladders were detected in 17 and 2+ apoptotic DNA ladders were detected in 5 of 22 plasma samples collected from diabetic children 4–13 years of age. Concomitantly, 1+ apoptotic DNA ladders were detected in 4, 2+ apoptotic DNA was detected in 7, and 3+ apoptotic DNA ladders were detected in 6 of 17 plasma samples collected from diabetic patients 14–18 years of age. In contrast, apoptosemic plasma from 3 of 5 hospitalized female children (ages 9, 15, and 16) contained 3+ apoptotic DNA, whereas 4 male apoptosemic inpatient plasma samples contained 1+ (ages 2 and 12) or 2+ (ages 11 and 14) apoptotic DNA. These data suggest that the highest levels of apoptotic DNA occur more often in the older diabetic children. With regard to the 10 apoptosemic RA patients, 4 plasma samples contained 1+ apoptotic DNA, 3 contained 2+ apoptotic DNA, and 3 contained 3+ apoptotic DNA. Further, only 1+ apoptotic DNA ladders were detected in preoperative adult plasma samples and young diabetic rats (not graphed).

Elevated GGT Activity in Plasma of Apoptosemic Children. GGT activities in plasma samples from nonapoptogenic T1 diabetic outpatients (319 ± 132 U/ml) were significantly lower ($P = 0.01$) than in apoptosemic T1 diabetic outpatients (428 ± 240 U/ml; Fig. 3). In contrast, no differences in GGT activity were detected in plasma from nonapoptosemic (399 ± 157 U/ml) and apoptosemic (420 ± 145 U/ml) T2 diabetic ($P = 0.674$) outpatients or between plasma samples from nonapoptosemic (400 ± 141 U/ml) and apoptosemic (434 ± 141 U/ml) inpatients ($P = 0.755$). The GGT levels in apoptosemic RA outpatients (647 ± 237 U/ml) were slightly but not significantly higher than in nonapoptosemic RA outpatients (573 ± 435 U/ml; $P = 0.644$). In comparison, the mean GGT activity in sera from 50 adult cataract patients was 722 ± 451 U/ml. The GGT activity in serum of STZ-induced diabetic rats (133 ± 71 U/ml) was 3.5-fold higher ($P = 0.01$) than in serum from

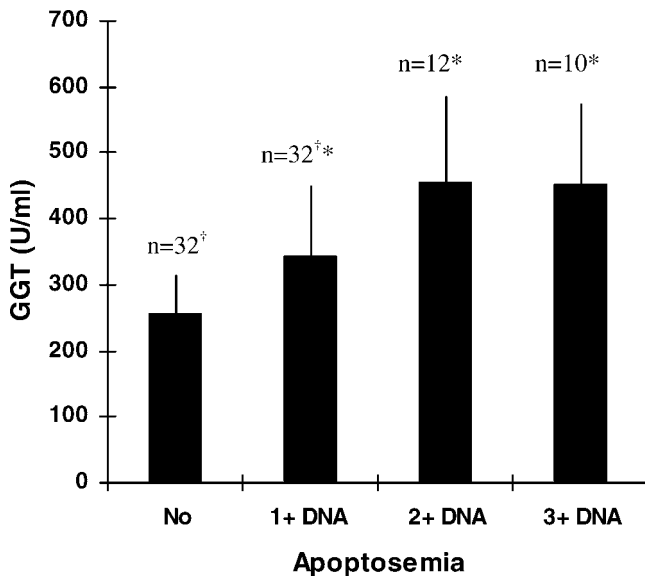


Figure 4. GGT activity in plasma from T1 and T2 diabetic outpatients without and with different amounts of apoptosemia. [†]Age and sex matched. *Significant at $P \leq 0.01$.

nondiabetic control rats (38 ± 17 U/ml) and was consistent with the high GGT levels previously reported in diabetic rats (30).

In an additional analytical study, the GGT activity in 32 plasma samples from T1 diabetic children that did not contain apoptotic DNA ladders was compared with 32 plasma samples from age- and sex-matched T1 diabetic children that contained 1+ DNA (Fig. 4). Also, the GGT activity in 12 plasma samples that contained 2+ and 10 plasma samples that contained 3+ apoptotic DNA was determined. The mean GGT activity in nonapoptosemic plasma (255 ± 58 U/ml) was significantly lower ($P = 0.001$) than the GGT activity in age- and sex-matched 1+ apoptosemic plasma (344 ± 104 U/ml). The mean GGT activity in nonapoptosemic plasma was significantly lower than the GGT activity in 2+ apoptosemic plasma (456 ± 128 U/ml; $P = 0.006$) and 3+ apoptosemic plasma (453 ± 121 U/ml; $P = 0.01$). These results suggest a close association between GGT and circulating apoptotic DNA ladder levels in T1 diabetic outpatients.

Detection of Apoptotic DNA Ladders and GGT Release. A relationship between apoptosis and elevated GGT was suggested by the close association of elevated GGT activity and high levels of apoptotic DNA in plasma of T1 diabetic outpatients. The following *in vitro* studies were performed to investigate the possibility that GGT may be released from apoptotic blood cells. First, 1.0-ml aliquots of venous blood samples from three consenting nondiabetic healthy volunteers were pipetted into six capped vials. EDTA (50 mM final concentration) was added to the blood samples, and after incubation for 0 or 6 hrs or 1, 2, 3, or 4 days at 37°C, the blood cells and plasma were separated by centrifugation at 2000 g for 3 mins. The DNA was extracted

from the 0.3 ml plasma and packed blood cells as described in *Materials and Methods*. Representative gels showing apoptotic DNA ladders in the cell, serum, and plasma are presented in Figure 5. Apoptotic DNA ladders were not detected in the serum of blood samples incubated for 6 or 24 hrs. Apoptotic DNA ladders (1+ to 3+) were detected in cell fractions of blood samples harvested after 6 hrs of incubation (Fig. 5A). Sera collected from blood incubated for 2, 3, and 4 days contained increasing levels of apoptotic DNA ladders. Concomitantly, the GGT activity increased progressively in the serum of the blood samples incubated through 4 days (Fig. 5B). That is, the mean percentage of increase in GGT activity was significantly higher ($P \leq 0.04$) in the serum of blood incubated for 3 days ($8.6\% \pm 4.3\%$) and 4 days ($12.8\% \pm 7.1\%$) than for 6 hrs ($0.97\% \pm 0.22\%$). The results suggest that the increases in GGT activity in the serum paralleled the increase in apoptotic DNA ladders in the blood samples. These results are consistent with the view that an increase in GGT activity in the serum occurred after the onset of apoptosis in the blood sample. Second, the appearance of apoptotic DNA ladders and GGT activity in the plasma (i.e., blood sample with EDTA) of blood samples from three consenting donors were determined as above after incubation at 37°C for 2, 6, 10, and 14 days. No apoptotic DNA ladders were detected in the plasma (Fig. 5C) or blood cell fractions of the EDTA-treated blood samples (not shown). At the same time, the GGT activities in the plasma from the three donors remained constant (i.e., $<0.5\%$ increase in GGT in the plasma fraction). This result suggests that the development of apoptotic DNA ladders and the increase in free GGT in the blood samples were inhibited by EDTA. Taken together, these results suggest GGT may be released from apoptotic blood cells and support the possibility that a fraction of the GGT activity in the blood of diabetic children may represent release from dead or dying GGT-positive cells.

Discussion

Approximately 25% of plasma samples from diabetic outpatient children contained detectable apoptotic DNA ladders. Apoptotic DNA ladders were detected in plasma samples from 27% of T1 and 19% of T2 diabetic children. Apoptosemia was detected more often in male T1 (31%) than in female T1 (20.8%) diabetic children, and more often in male T2 (30.8%) than in female T2 (15.4%) diabetic children. The incidence of apoptosemia in the diabetic outpatients appears to be significantly higher than in the general pediatric population. The idea that apoptosemia in diabetic children is higher is supported by our results indicating that apoptotic DNA is present in $\geq 25\%$ of plasma samples from hospitalized children and adult RA outpatients, but in only 3.5% of plasma samples from adult cataract patient controls. The assertion that the high incidence of apoptosemia in diabetic outpatients ≤ 18 years of age is abnormal is also supported by several reports that

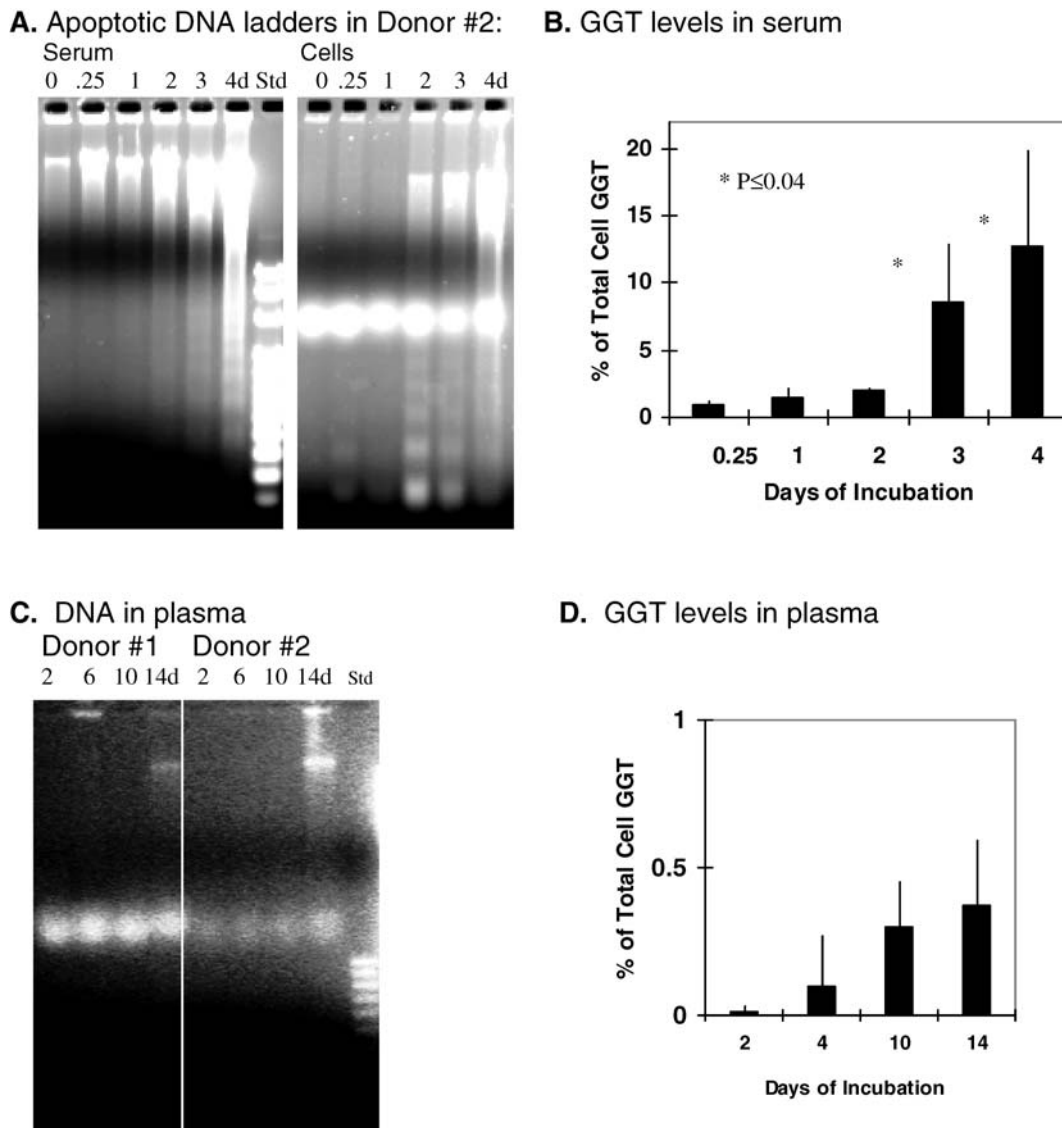


Figure 5. Detection of apoptotic DNA and GGT activity in human blood samples with and without EDTA. (A) Apoptotic DNA ladders were detectable at 2 days after incubation in serum and packed blood cells, (B) but they were not detectable in plasma (i.e., EDTA-treated blood). (C) The percent of the total GGT activity was significantly higher in sera fraction collected on Days 3 and 4 ($*P \leq 0.04$). (D) The percent of the total GGT activity in plasma fraction was unchanged through 14 days of incubation.

plasma from $\leq 1\%$ of healthy individuals contains apoptotic leukocytes and/or elevated plasma DNA levels (6, 22, 45–49). Further, the very high levels of apoptotic DNA in the plasma of older diabetic children, especially in males, argues against the view that pronounced apoptosemia (i.e., extremely high index of cell death) would be present in nondiabetic healthy cohorts. However, the opinion that circulating apoptotic DNA may be a normal occurrence in the pediatric population cannot be discounted completely until plasma from consenting “well” children can be analyzed. Overall, the results support the theory that a complication common to T1 and T2 diabetes in children is likely responsible for the apoptosemia.

The greater incidence and quantity of apoptotic DNA in plasma from older diabetic children suggests that the

apoptosemia may be associated with the disease duration, poor diabetic disease management, or inflammation. Accordingly, the detection of high levels of apoptotic DNA in older children appears to parallel the increase in secondary symptoms of diabetic disease and episodes of acidosis and ketosis reported previously in older children within this diabetic pediatric population (50). However, a direct association between the levels of apoptotic DNA ladders and levels of hemoglobin A_{1C} and ketone bodies in the plasma samples (suggestive of ketoacidosis) was not detected. The higher incidence of apoptotic DNA ladders in diabetic male (31%) than female (18.5%) outpatient children is consistent with poorer diabetic disease management observed more often in male diabetic children (50). Interferon activity (suggestive of infection/inflammation)

was not detected in apoptosemic plasma from inpatients or diabetic outpatients, but interferon was detected in 6 of the 10 apoptosemic adult RA plasma (data not shown). There was an inverse relationship between the glucose level and the amount of apoptotic DNA ladder in the apoptosemic plasma samples from the diabetic children, inpatients, and RA outpatients. The low glucose levels in these plasma samples were thought to be due to degradation by enzymes derived from apoptotic cells (data on the initial glucose level were not available). However, it was of interest to note the recent reports that blood glucose variability in nondiabetic hospitalized children is associated with increased morbidity and death (51) and that a decreased prolactin response to hypoglycemia correlates with RA disease activity (52). Additional studies are needed to assess the role of hyperglycemia, hypoglycemia, cytotoxic metabolites, autoimmune responses, and physical exertion (40, 53–58) in the development of the apoptosemia in diabetic children.

The highest levels of apoptosemia and GGT were detected in the older T1 diabetic male outpatients. The mechanism responsible for the close association between elevated GGT and high levels of apoptotic DNA in the T1 diabetic outpatients is unknown. However, it is plausible that some of the older T1 diabetic outpatients in our study group may have become insulin resistant, as suggested by increased GGT levels in plasma of apoptosemic versus nonapoptosemic T1 diabetic outpatients. This idea is supported by the reports that elevated GGT is associated with an increased risk for diabetes and insulin resistance in adults (30, 32–36). Moreover, the results of our *in vitro* studies support the idea that GGT may be released from apoptotic blood cells. Thus, the elevated GGT in sera from diabetic outpatients and rats may be due to a serum factor that induces apoptosis of a GGT-positive cell population(s), such as leukocytes, vascular endothelial cells, and/or hepatic cells (54, 58–62). The failure to detect a similar association between apoptotic DNA and GGT levels in T2 diabetic outpatients and inpatients is perplexing. The elevation of GGT in the absence of detectable apoptotic DNA may be due to several factors, such as differential clearance rates for apoptotic DNA and GGT, liver disease (61), renal disease (63), and/or shedding of GGT from T memory cells and neutrophils (60, 64). Further, the results of other investigations suggest the possibility that elevated GGT may exert untoward effects, in that GGT may activate bone resorption in RA patients (39).

Nuclear fragments released from apoptotic cells into cell culture media or into the blood are mainly mononucleosomes (i.e., apoptotic bodies) and may be detected within a few hours of experimental induction of apoptosis (29) or after a major bodily injury (65, 66). Thus, the factor(s) responsible for the very high levels of apoptotic DNA (suggestive of excessive cell death) in diabetic outpatients and inpatient children as well as the adult RA patients was likely occurring at the time the blood sample was drawn. This idea is consistent with the reports that

apoptotic lymphocytes and DNA fragments in plasma after exhaustive exercise (48) and hemodialysis (49) are cleared rapidly. However, the persistence of circulating apoptotic DNA in diabetic children cannot be ruled out at present, since circulating apoptotic DNA may persist for days in patients with multiple organ failure (65, 66). The detection of apoptosemia in T1 and T2 diabetic children suggests that cellular antigens are present and, if not cleared, could elicit tissue specific autoimmune responses. Further, the “silent” apoptosemia (i.e., plasma drawn during outpatient clinic visits) could exert deleterious effects due to increased viscosity of blood in the microcirculation (6), especially if the apoptosemia is chronic or recurrent, as suggested by the detection of anti-DNA antibody in other diabetic patients (21).

The detection of apoptotic DNA in 4 (17%) of 24 plasma from T1 diabetic children ≤ 6 years of age and in 7.7% of plasma samples from young diabetic rats (i.e., collected 3–4 weeks after STZ injection) supports the idea that apoptosemia can occur early in the course of natural and experimental diabetes. The detection of apoptotic DNA ladders in diabetic human and rat plasma is consistent with the detection of apoptotic lymphocytes in human diabetic patients and alloxan-induced diabetic rats (22, 23). The detection of apoptosemia in very young diabetic children and the detection of high levels of apoptotic DNA ladders and GGT in older diabetic children suggests a poor prognosis. This idea is supported by numerous reports that elevated DNA and GGT in plasma are poor prognostic signs (6–12, 34–37, 45–47, 65, 66).

In summary, we report the detection of apoptosemia (apoptotic DNA ladders in plasma) in T1 and T2 diabetic children. The apoptosemia was detected more often in male than in female diabetic children. The amounts and incidence of circulating apoptotic DNA and GGT were highest in the older T1 diabetic male children. It is also noteworthy that (i) detection of apoptotic DNA ladders may not be used to distinguish between T1 from T2 diabetes, (ii) preliminary analysis of multiple samples from some of the diabetic children suggests that the apoptosemia can be recurrent, and (iii) detection of 3+ apoptotic DNA in plasma in children and adults indicates an extremely high index of cell death. The source of the apoptosemia and GGT in the diabetic outpatients, inpatients, and RA adults is unknown, but the *in vitro* results suggest they may arise from apoptotic GGT-positive cells. The results support further etiologic and epidemiologic investigations of the pediatric population to identify the cause(s), classify the apoptosemic diabetic patients, and assess the usefulness of monitoring apoptosemia in predicting risk of disease progression and complications. Further, the results suggest that the use of therapeutic agents that exert anti-inflammatory/antiapoptogenic activities may be beneficial to the apoptosemic diabetic children.

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