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Original Research Communication

Effect of Hyperketonemia on Blood Monocytes in Type-I Diabetic Patients and Apoptosis in Cultured U937 Monocytes

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ABSTRACT

Recent studies have proposed a role for a reduced number of circulating monocytes in the development of atherosclerosis and circulatory diseases in diabetes. Ketosis is frequently encountered in type-I diabetics. This study was undertaken to test the hypothesis that hyperketonemia can lower blood monocyte count in type-I diabetic patients. Blood monocyte count was significantly lower ($p < 0.05$) in diabetics ($n = 27$) compared with age-matched normal siblings ($n = 22$). Blood levels of acetoacetate (AA) and triglycerides were significantly higher in diabetics compared with normals. To examine whether hyperketonemia can directly alter the monocyte count in diabetics, we studied the effect of ketone bodies AA and β-hydroxybutyrate (BHB) on U937 cells, a human-derived promonocytic cell line as an *in vitro* model. The cell culture studies showed a dose-dependent growth inhibition and induction of apoptosis as a result of treatment with AA in U937 cells. Furthermore, there was a significant decrease in GSH and increase in lipid peroxidation products in AA-treated U937 cells. Taken together, this study suggests that elevated levels of ketone body AA can result in oxidative damage, accelerated apoptosis, and inhibition of cell growth in monocytes, which in turn can lower monocyte count in the blood of type-I diabetic patients. Antiox. Redox Signal. 1, 211–220, 1999.

INTRODUCTION

THE BLOOD MONOCYTES have the propensity for adherence to the vascular endothelium and release mediators, such as, tumor necrosis factor-α, interleukin-1, and reactive oxygen species (Larrick and Wright, 1990; Quillet-Mary *et al.*, 1997). One of the earliest abnormalities seen in atherogenesis is enhanced monocyte adherence to the endothelium. Oxidative stress can increase adhesion of monocytes to endothelium and induce the transendothelial migration of monocytes, a crucial event in the diapedesis of leukocytes during the pathophysiology of vascular disease (Rattan *et al.*, 1997a,b). The peripheral blood monocyte count is lower in type-I diabetes (Perrson *et al.*, 1998). This lower monocyte count can result in the hypercholesterolemia and hypertriglyceridemia because of their decreased uptake and metabolism by fewer monocyte-derived macrophages (Huang *et al.*, 1996; Persson *et al.*, 1998; Luscher, 1993). It has been proposed that lower level of peripheral

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monocytes may be an important pathological event in the development of vascular disease in diabetes (Luscher, 1993; Hegele, 1996; Huang *et al.*, 1996; Rattan *et al.*, 1997a,b; Persson *et al.*, 1998).

Poorly controlled type-I diabetics frequently encounter elevated levels of ketone bodies—acetoacetate (AA) and β-hydroxybutyrate (BHB)—in the blood (hyperketonemia) because body fuel is mainly derived from fat (Vignati *et al.*, 1985). Recent studies have shown that ketosis can generate oxygen radicals, increase cellular lipid peroxidation, and reduce cell survival in cultured human endothelial cells (Jain *et al.*, 1998). Whether ketosis has any direct role in the survival and lower level of monocytes in type-I diabetes is not known. The present study was undertaken to test the hypothesis that ketosis can lower monocyte count in the blood of diabetic patients. Specific objectives of this study were to determine blood ketone bodies and circulating monocyte levels in type-I diabetic children, and to examine the effect of ketone bodies AA and BHB on apoptosis and survival of cultured U937 monocytes.

MATERIALS AND METHODS

Diabetic children and their healthy siblings

Informed written consent of all patients and control subjects was obtained in accordance with the protocol approved by the Institutional Review Board (IRB) on Human Experiments. Diabetics complicated with any other diseases were excluded from this study. All patients included in this study were insulin-dependent diabetics (type-I). Diabetic patients and healthy siblings were on overnight fasting and were not taking insulin at the time of their arrival to the clinic. They were told to bring their insulin and syringes for use after drawing blood. Peripheral blood was collected before 9:00 A.M. into tubes containing EDTA and without EDTA. Necessary patient care was given in the clinic to all the patients included in the study. A portion of the EDTA blood collected from patients and control subjects was centrifuged, and the clear plasma was labeled appropriately and saved for future analysis. The remaining blood was sent to the clinical lab for the glycated hemoglobin (HbA_1) and complete blood count analyses. All biochemical analyses were performed immediately after blood collection.

Cell culture studies

The U937 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was maintained at 37°C in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine (GIBCO-BRL, Grand Island, NY) in a humidified atmosphere containing 5% (vol/vol) CO_2. For treatment with ketone bodies, cells were washed in plain RPMI 1640 before being suspended in fresh medium (complete) containing serum and other supplements. Stock solutions of 1 mmol/ml AA and 1 mmol/ml BHB were prepared fresh every 2 weeks in sterile deionized water. All other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned.

AlamarBlue cell proliferation assay

The alamarBlue fluorometric/colorimetric growth indicator (alamarBlue biosciences, Sacramento, CA) was used to measure cell proliferation and cytotoxicity due to AA and BHB on U937 cells, as outlined before (Jain *et al.*, 1998). In brief, U937 cells grown in logarithmic phase were spun down and washed in plain RPMI 1640 medium and finally resuspended in complete RPMI 1640 medium. After counting cells in a hemocytometer, cell populations were adjusted to 2×10^5 cells/ml. Two milliliters of this cell suspension was transferred to each well in a 24-well culture plate. These cells were then exposed to media only, AA or BHB at the following concentrations: 0, 5, 10, and 20 μmol/ml. Using a multichannel pipettor, 100 μl of cells (20,000 cells/well) were transferred to six replicate wells of a 96-well flat-bottomed microtiter tissue culture plate. A water-soluble indicator dye, alamarBlue dye (alamar biosciences, Sacramento, CA), was added to an amount equal to 10% (vol/vol) of the culture volume 4 hr before the end of the incubation. The relative number of proliferating cells was

determined at the end of the incubation period by colorimetry using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices Ltd., Sunnyvale, CA). The optical density readout at 570–600 ($\lambda 1$–$\lambda 2$) measures the reduction of alamarBlue dye by the cells during a 4-hr assay period. In each case, the experiments were performed in six replicates, and the relative number of proliferating cells was determined as the mean absorbance scanning result for each culture condition, less the mean obtained for three wells with media alone (background). These experiments were repeated at least six times with similar results.

In situ *detection of apoptosis by TUNEL assay*

The number of cells dying of apoptosis in the culture was measured by the TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling] method, which allows detection of apoptosis at the single-cell level (Gavrieli *et al.*, 1992). The *in situ* cell death detection kit (Boehringer-Mannheim, Indianapolis, IN) allows the detection of apoptosis quantitatively by flow cytometry. It is based on the detection of single- and double-stranded DNA breaks occurring at early stages of apoptosis. This method, in particular uses TdT to label the 3′-OH ends of DNA fragments generated during the process of apoptosis. In brief, U937 cells were cultured in a six-well tissue culture plate in the presence or absence of AA at various concentrations for 0–72 hr. Each well contained 0.5 million cells/ml for 24 hr of incubation and 0.1 million cells/ml for a 3-day incubation. The total volume of the culture well in each case was 5 ml. This initial difference in cell seeding was essential to address technical difficulties such as overpopulation in control wells during a 3-day culture period, which resulted in lower pH, cell death, and cell debris. Cultures incubated for 24 hr received a single treatment with AA added at the initiation of the culture. Three-day cultures received fresh AA treatments every 24 hr. This was done every 24 hr at a specified time by removing 500 μl of supernatant for biochemical analysis and replacing it with 500 μl of fresh medium and AA as before.

At the specified time intervals, cells were gently harvested, washed in phosphate-buffered saline–bovine serum albumin (PBS-BSA) (1%) and fixed in 100 μl of 4% paraformaldehyde for 20 min on ice. Subsequently, cells were permeabilized with 100 μl of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After extensive washing with PBS-BSA buffer, these cells were incubated in the presence of 50 μl of freshly prepared TUNEL reaction mixture containing TdT and fluorescein-dUTP for 60 min at 37°C as per the manufacturer's guidelines. After two washes in wash buffer, the label incorporated at the damaged sites of DNA in the intact cell was quantified by flow cytometry. In each experiment, unstained control cells, cells stained with dUTP-fluorescein isothiocyanate (FITC) (negative control), and 50 ng/ml of Fas-treated cells (24 hr; positive control) were included.

Flow cytometry analysis was carried out in the core facility for flow cytometry at LSU Medical Center. In each circumstance, at least 10,000 cells were analyzed in a fluorescence-activated cell sorting (FACS) Vantage flow cytometer (Becton-Dickinson) equipped to perform five-color analysis. Quality control parameters and appropriate negative gating were set before the start of each analysis. Forward- and right-angle scatter gating were set to include unstained cells and cells treated with dUTP-FITC alone, and to eliminate debris and dead cells. Basal data for control cells were obtained with TdT enzyme + dUTP-FITC as outlined by the manufacturers of the kit. The channel number corresponding to mean fluorescence intensity was determined by positive cells using an arbitrary scale of 1–5,000.

GSH, lipid peroxidation, AA, and BHB determinations

Levels of GSH in U937 cells were determined after their high-performance liquid chromatography (HPLC) separation as described by Reed *et al.* (1980). Membrane lipid peroxidation was determined by the HPLC of malondialdehyde (MDA, an end product of fatty acid peroxidation) and of the thiobarbituric acid complex using ion-exclusion and a reverse-phase Shodex KC-811 column (Waters

TABLE 1. AGES, DURATION OF DIABETES, AND BLOOD KETONE BODIES AND GLYCATED HEMOGLOBIN LEVELS IN TYPE-I DIABETICS AND NORMAL CHILDREN

	Normals	*Diabetics*	p *value*
n	22	27	
Age (yrs)	10.9 ± 0.9	12.9 ± 0.7	NS
Duration of diabetes (yrs)	—	5.2 ± 0.8	—
AA (m*M*)	0.19 ± 0.02	0.26 ± 0.03	<0.04
BHB (m*M*)	0.21 ± 0.06	0.32 ± 0.10	NS
HbA_1 (%)	5.9 ± 0.1	12.4 ± 0.6	<0.01

Values are mean ± SE. *n* is the number of subjects in each group. NS, Not significant.

Corporation, Milford, MA) with the detector set st 532 nm (Esterbauer *et al.*, 1984). Levels of AA were determined by the method of Artuch *et al.* (1995) and of BHB by the method of Koch and Feldbrugegge (1987). Data were analyzed using the nonpaired Student's *t*-test, the Sum Test, and Pearson Correlation with the Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A *p* value of less than 0.05 was considered significant.

RESULTS

Table 1 shows age, gender ratio, AA, BHB, and HbA_1 levels of type-I diabetics and normal siblings. As compared to normal subjects, diabetics have significantly higher levels of AA and HbA_1. The data presented in Fig. 1 illustrates significantly lower monocyte count in diabetics as compared to normals. However, levels of other white cells—neutrophils, lymphocytes, eosinophils, and basophils—were comparable between diabetics and normals (data not given).

Figure 2 illustrates a dose-dependent growth inhibition of U937 cells by AA. At 10 and 20 μmol/ml concentrations, AA caused a statistically significant ($p < 0.01$) reduction in cell proliferation. At the lower concentration (2.5 μmol/ml), the growth inhibition was reduced but was not statistically significant ($p = 0.08$). MDA levels (0.30 ± 0.03 versus 0.13 ± 0.02 μmol/gram of protein, $p < 0.01$) were significantly higher and GSH levels (22.8 ± 2.1 versus 31.8 ± 3.7 μmol/gram of protein, $p < 0.01$)

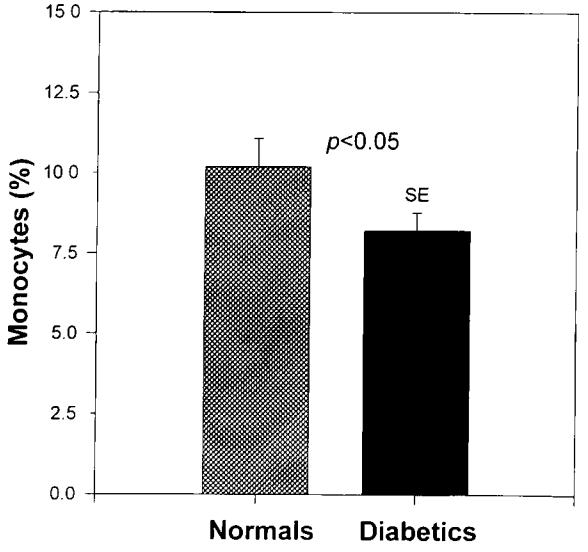


FIG. 1. Blood monocyte count in type-I diabetic children and age-matched normal siblings.

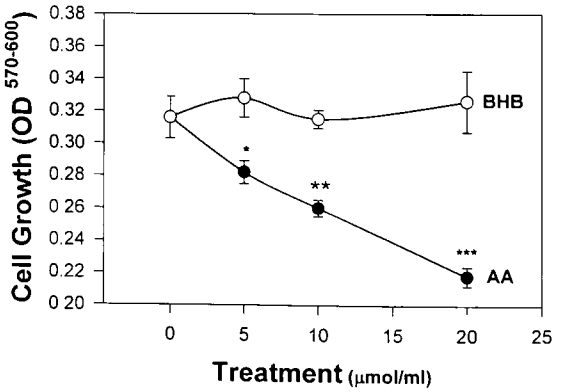
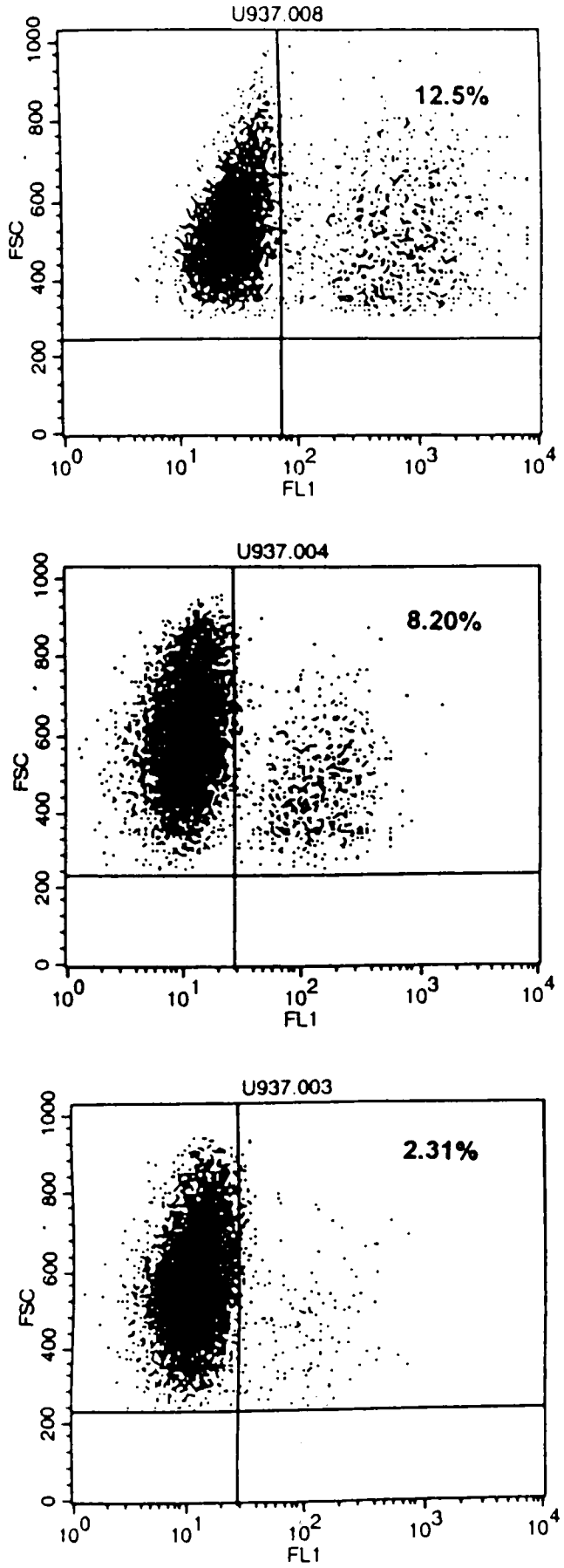


FIG. 2. Effect of AA and BHB on the cell growth of U937 cells. Cells were cultured in 96-well microtiter plates (20,000 cells/ml) for 24 hr. AA or BHB was added at the beginning of the culture. Alamar blue indicator dye was added 4 hr prior to reading the plate in an ELISA reader, as outlined in the Materials and Methods. Values are means ± SEM of three experiments. $*p < 0.08$, $**p < 0.01$, and $***p < 0.0001$ in comparison to untreated controls.



were significantly lower in AA (20 μmol/ml) treated versus untreated cultured monocytes at 24 hr. Unlike AA-treated cells, MDA (0.15 ± 0.02 μmol/gm protein) and GSH (28.5 ± 4.0 μmol/gm protein) levels in BHB-treated cells were similar to those in untreated cells. Similarly, BHB did not cause any marked effect on cell growth.

Microscopic examination of cells incubated with AA exhibited morphological features typical of apoptotic cells, such as membrane blebbing and disintegration of cells into small vesicle-type bodies (Gardner *et al.*, 1997). To confirm this, the TUNEL assay for apoptosis was performed. Figure 3 illustrates an increase in apoptotic cell death (12.5% versus 2.3% in controls) in 20 μmol/ml treated cells after 3 days. Morphologically, AA-treated cells appeared swollen with increasing doses of AA. At concentrations of 30 μmol/ml and 40 μmol/ml, the number of cells dying of apoptosis was 20% and 26%, respectively. Similar to the increase in percent apoptotic cells, mean fluorescence intensity (MFI) was also higher in AA-treated cells. A marked dose-dependent increase in MFI was observed at concentrations 20 μmol/ml and above. There were 10-, 32-, and 44-fold increases in MFI in 20, 30, and 40 μmol/ml AA-treated cells, respectively. There

FIG. 3. Histogram showing AA-induced apoptotic cell death in a 120-hr culture. U937 cells at a density of 5×10^5/ml (24-hr culture) or 1×10^5 cells/ml (72-hr culture) was incubated in the presence of 0, 5, 10, and 20 μmol/ml of AA for 72 hr. At every 24-hr period, 0.5 ml of supernatant was removed for biochemical analysis. This volume was replaced with fresh media and AA was added at the concentrations indicated earlier for each culture. The total volume of the culture was maintained at 5 ml per treatment. As a positive control, anti-Fas monoclonal antibody (50 ng/ml) was added to one of the culture wells during the last 24-hr period before termination of the culture. Apoptotic cell death was scored using the TUNEL assay as described in Materials and Methods. In each circumstance, at least 10,000 cells were analyzed by flow cytometry. The data presented in the above flow cytometry histogram show the results obtained from one of three experiments, all with similar results. Data on media control, positive control (anti-Fas), and cells treated with 20 μmol/ml AA alone are presented in the histogram. The upper right-hand corner of each histogram shows the percent apoptotic cell population scored in each treatment condition **Top panel:** AA (20 μmol/ml). **Middle panel:** Anti-Fas control (positive). **Bottom panel:** Control.

was no significant difference between controls and experimental cells treated with 2.5-10 μmol/ml AA.

Figure 4 shows an inverse relationship ($r = -0.41$, $p < 0.04$) between monocyte count and plasma MDA levels in type-I diabetic patients. However, the relationship between plasma acetoacetate level and monocyte count was not statistically significant ($r = -0.24$, data not included).

DISCUSSION

Epidemiological studies have established that diabetes is an independent risk factor in the development of atherosclerosis (Hegele, 1996). The pathogenesis of atherosclerosis involves monocyte–endothelial cell interaction, inflammatory infiltration in the vessel wall, hyperlipidemia, cellular proliferation, fibrous plaque formation and ultimate plaque rupture and occlusive thrombosis. The mechanisms by which diabetes enhances monocyte–endothelial cell interactions are not clear (Hegele, 1996).

In severe diabetes, the level of ketone bodies begins to rise in the blood (hyperketonemia) because body fuel is mainly derived from fat (Vignati *et al.*, 1985). The urinary excretion of ketone bodies may be as high as 5,000 mg/24 hr, and the blood concentration may reach 10 m*M* in diabetics with severe ketosis versus less than 0.5 m*M* in normals (Vignati *et al.*, 1985; Champe and Harvey, 1994). Ketosis accounts for more than half of the deaths of diabetic children (Krane, 1987; Quillet-Mary *et al.*, 1997). This study has examined whether hyperketonemia plays any role in the monocyte physiology, which in turn may influence monocyte–endothelial interactions and in particular the peripheral blood monocyte count in type-I diabetes.

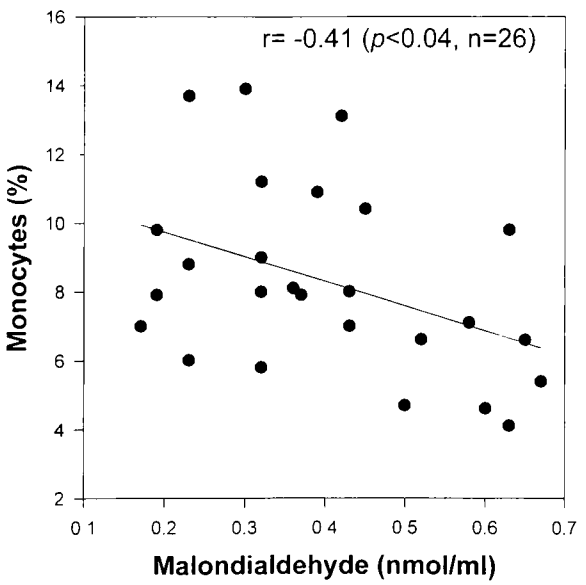


FIG. 4. Relationship of lipid peroxidation and monocyte levels in blood of type-I diabetic patients. Note a decrease in blood monocytes count with increasing level of oxidative stress in diabetic patients.

Numerous studies have reported increased production of MDA, a marker of lipid peroxidation in red cells and other cells of diabetic animals and patients (Jain *et al.*, 1989a,b, 1991; Rajeswari *et al.*, 1991; Giugliano *et al.*, 1996). The levels of lipid peroxidation products in the blood are higher in diabetic patients with vascular disease (Satoh *et al.*, 1979; Ozben *et al.*, 1995; Sundram *et al.*, 1996). The elevated level of circulating lipid peroxides can cause cross-linking of membrane lipids and proteins, phosphatidylserine (PS) externalization in the membrane bilayer, reduced cell survival, and cellular dysfunction (Jain, 1989, 1985; Wali *et al.*, 1987; Holvoet *et al.*, 1995). Various studies showing a decrease in the occurrence of complications after supplementation with different antioxidants in diabetic animal model provide evidence for some role of lipid peroxidation damage in the development of diabetic complications (Ross *et al.*, 1982; Aoki *et al.*, 1992; Bravenboer *et al.*, 1992; Cameron *et al.*, 1993; Kaul *et al.*, 1995; Karasu *et al.*, 1995; Hida *et al.*, 1996).

Monocytes as circulating white blood cells are easily affected by the environment in which they thrive. In diabetic patients, these cells are exposed to ketone bodies for a prolonged period of time, thus becoming one of the target cells to an oxidized environment similar to endothelial cells of the blood vessels. Because monocytes and endothelial cells interact at sites of vascular injury during inflammatory processes, such interactions result in the modulation of several biological functions of the two cell types, including activation, adhesion, and release of proinflammatory cytokines. Re-

cently, peroxisome proliferator-activated receptors (PPARs) have been implicated in the pathogenesis of diabetes. In particular, PPAR activators induce macrophage apoptosis by negatively interfering with anti-apoptotic nuclear factor-κB (NF-κB) signaling pathway (Chinetti *et al.*, 1998). Therefore, it is plausible to suggest that ketosis may initiate some of these pathological events. Under *in vivo* conditions, it is hard to dissect out the primary versus secondary events mediated by ketone bodies on monocytes. Therefore, to understand better the deleterious effects mediated by ketone bodies on the circulating peripheral blood monocytes, we used U937 cell line as an *in vitro* model.

This study reports for the first time a reduction in glutathione (GSH) and increases in lipid peroxidation, and thus increased oxidative stress in a human monocytic cell line cultured with elevated levels of AA. In addition, cell culture studies have shown that AA can induce apoptosis and inhibit cell proliferation in U937 cells. It is known that hydroperoxides, such as tertiary-butylhydroperoxide (TBH) and hydrogen peroxide (H_2O_2), which cause cellular lipid peroxidation, inhibit cellular growth in cultured adult cardiac myocytes (Vlessis *et al.*, 1991), LLC-PK cells (Chen and Stevens, 1991), and human proximal tubular cells (Jain *et al.*, 1996). Treatment with standard MDA, a product of lipid peroxidation, is also known to inhibit growth in human proximal tubular cells (Jain *et al.*, 1996). A recent study has demonstrated induction of apoptosis by H_2O_2 in a fibroblast cell line (Gardner *et al.*, 1997). Thus, increased oxidative stress can play a role in the induction of apoptosis in these cells and thereby reduce the overall cell survival and proliferation in the culture. Our data support this concept, as shown in the cultured U937 cells treated with AA.

The *in vitro* studies described here using cultured U937 cells exposed to elevated AA levels and the *in vivo* data on the lower monocyte count in type-I diabetic patients suggests that ketosis can induce apoptosis and reduce the viability of monocytes in type-I diabetes. Cell–cell communication between endothelial cells, monocytes, resting and activated macrophages, and T lymphocytes are very complex. Although the reduced monocyte count may be due to apoptotic cell death over a period of time, it does not fully explain the inflammatory process that could be initiated by macrophages reported in the literature. It is reasonable to hypothesize that AA may cause PS externalization and monocyte activation resulting in the secretion of proinflammatory cytokines. This, in turn, might chemoattract monocytes and macrophages to the site of lesion, starting an immunological cascade of events leading to inflammation and microvascular lesions. With the involvement of endothelial cells and activated immunocompetant cells, atherosclerotic lesions and plaque formation are set in motion and are often difficult to reverse. On the other hand, increased triglyceride level in plasma can promote monocyte adhesion to human endothelial cells (De Gruijter *et al.*, 1991). These probable mechanisms may partly explain cellular dysfunction and the lower level of blood monocytes seen in type-I diabetic patients. We did not determine

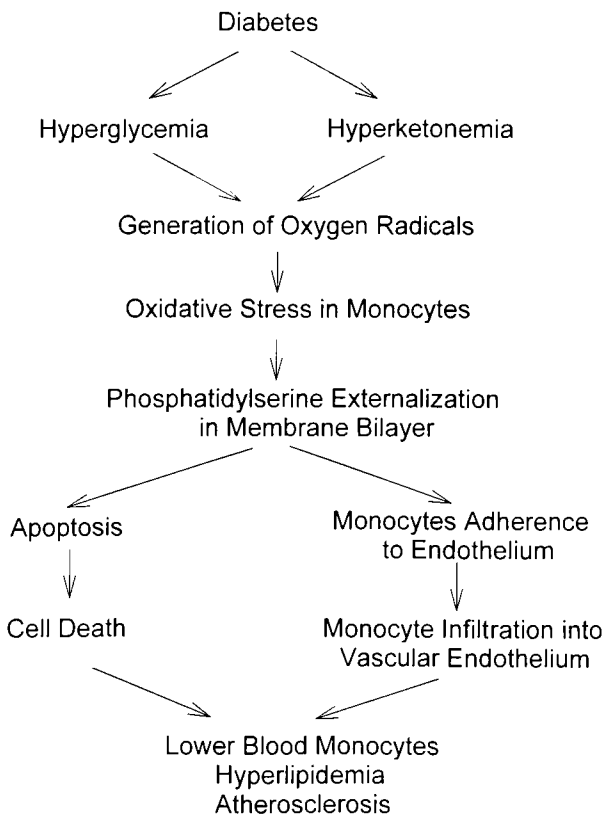


FIG. 5. Proposed scheme for the effects of diabetes and ketosis on blood monocytes and atherosclerosis.

MDA levels in monocytes isolated from blood of diabetic patients. However, there was a significant inverse relationship between plasma MDA level and monocyte count in the blood of diabetic patients. The relationship between the levels of AA and monocyte counts was not statistically significant. This suggests that in *in vivo* conditions, other factors such as hyperglycemia may also contribute in lowering the monocyte count in the peripheral blood of diabetic patients (Jain, 1989).

Figure 5 gives a proposed scheme by which hyperketonemia can lower circulating monocyte level, which in turn may influence events leading to the atherosclerosis in diabetes. It appears that ketosis can induce apoptosis and reduce monocyte viability by increasing membrane lipid peroxidation. Phosphatidylserine externalization (associated with apoptosis) is probably a cause for enhancement of monocyte adhesion to the endothelium, which subsequently can initiate the atherosclerotic process.

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ABBREVIATIONS

AA, acetoacetate; ATCC, American Type Culture Collection; BHB, β-hydroxybutyrate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GSH, glutathione; H_2O_2, hydrogen peroxide; HbA_1, glycated hemoglobin; HPLC, high-pressure liquid chromatography; IRB, Institutional Review Board; MFI, mean fluorescence intensity; MPA, malondialdehyde; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PPARS, peroxisome proliferator-activated receptors; PS, phosphatidylserine; TBH, tertiary-butylhydroperoxide; TdT, terminal deoxynucleidyl transferase; TUNEL, TdT-mediated dUTP biotin nick end-labeling.

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