Monocyte Telomere Shortening and Oxidative DNA Damage in Type 2 Diabetes

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OBJECTIVE — Telomeres are DNA sequences necessary for DNA replication, which shorten at cell division at a rate related to levels of oxidative stress. Once shortened to a critical length, cells are triggered into replicative senescence. Type 2 diabetes is associated with oxidative DNA damage, and we hypothesized that telomere shortening would characterize type 2 diabetes.

RESEARCH DESIGN AND METHODS — We studied 21 male type 2 diabetic subjects (mean age 61.2 years, mean HbA$_{1c}$ 7.9%) selected to limit confounding effects on telomere length and 29 matched control subjects. Telomere length was measured in peripheral venous monocyte and T-cells (naïve and memory) by fluorescent in situ hybridization and oxidative DNA damage by flow cytometry of oxidized DNA bases. Peripheral insulin resistance (homeostasis model assessment) and high-sensitivity C-reactive protein (hsCRP) were measured.

RESULTS — Mean monocyte telomere length in the diabetic group was highly significantly lower than in control subjects (4.0 [1.1] vs. 5.5 [1.1]; $P < 0.0001$), without significant differences in lymphocyte telomere length. There was a trend toward increased oxidative DNA damage in all diabetes cell types examined and a significant inverse relationship between oxidative DNA damage and telomere length ($r = -0.55$; $P = 0.018$) in the diabetic group. Telomere length was unrelated to plasma CRP concentration or insulin resistance.

CONCLUSIONS — Monocyte telomere shortening in type 2 diabetes could be due to increased oxidative DNA damage to monocyte precursors during cell division. This data suggests that monocytes adhering to vascular endothelium and entering the vessel wall in type 2 diabetes are from a population with shorter telomeres and at increased risk of replicative senescence within vascular plaque.

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Telomeres are tandem repeats of the DNA sequence TTAGGG extending over 6–15 kb at the end of eukaryotic chromosomes and are necessary for both successful DNA replication and chromosomal integrity (1–3). Telomeres in somatic human cells shorten by 30–200 bp each cell division, and once shortened to a critical length, cells are triggered into replicative senescence, an irreversible cell cycle block in G0/G1 (1–5) where cells function differently (1,2,5,6) and are more likely to undergo apoptosis if exposed to increased oxidative insult (7). Rates of telomere shortening, and therefore telomere length, are highly dependent on oxidatively induced strand breaks in telomeric DNA and on cellular oxidant balance (1–3,5,6,8,9), and we and others have shown that lymphocyte DNA from subjects with type 2 diabetes is characterized by increased susceptibility to oxidative damage (10–12). Telomeric DNA is particularly prone to oxidative damage at the GGG sequence (13,14), and it is probable that oxidatively induced single- and double-strand DNA breaks in people with type 2 diabetes (10–12) would translate into accelerated telomere shortening and a progression to replicative senescence (1–3). Many of the dysfunctions described in vascular endothelial cells in type 2 diabetes, as well as in ageing and atherosclerosis, are similar to the phenotype of in vitro senescent vascular endothelial cells (6,15). Telomere attrition has attracted attention as a possible associate of cardiovascular disease, vascular senescence, and vascular ageing (16–19) and appears to be an age-independent predictor of coronary mortality in adults (20). It has also been suggested that in utero programming of telomere length contributes to later risk of cardiovascular disease and type 2 diabetes (21,22). There are no adequate data on telomere length and DNA damage in human type 2 diabetes, and we hypothesized that type 2 diabetic patients would demonstrate shorter telomeres in peripheral monocytes and lymphocyte subsets compared with control subjects and that this would be directly related to markers of oxidative DNA damage.

RESEARCH DESIGN AND METHODS — All subjects gave written informed consent to participate in this study, which was approved by the local ethical committee. To lessen the substantial confounding effects of age, sex, smoking, ethnicity, and inflammatory processes such as atherosclerosis on telomere length (1–6,23,24), all subjects were Caucasian males; never smokers; within a narrow age range of 50–65 years; with no clinical history of angina, myocardial infarction, treated hypertension, or ischemic stroke; and with a normal 12-lead electrocardiogram. Subjects with type 2 diabetes ($n = 21$) were recruited if they had never received gliclazide, antihypertensives, or ACE inhibitors, which have antioxidant or anti-inflammatory properties (25,26). The type 2 diabetic patients had no macroproteinuria or an elevated urinary albumin-to-creatinine ratio, and no sight-threatening diabetic eye disease on digital retinal imaging, and all had been diagnosed as having diabetes after the age of 40 years, with no history of ketosis, and with no insulin therapy requirement for 3

years after diagnosis. The type 2 diabetic patients were treated with diet alone ($n = 5$), metformin alone ($n = 4$), sulfonylureas alone ($n = 4$), metformin and sulfonylureas in combination ($n = 3$), or subcutaneous insulin alone or in combination with metformin ($n = 5$). Fourteen of the 21 type 2 diabetic patients were taking an hydroxymethylglutaryl CoA reductase inhibitor ("statin"). Control subjects without diabetes were recruited from the general population and met the same inclusion criteria, and none were taking any medication. All control subjects had a fasting plasma glucose <6.1 mmol/l and all type 2 diabetic patients had paired fasting plasma glucose concentrations >7.0 mmol/l and/or a 2-h plasma glucose concentration of >11.1 mmol/l after a standard 75-g oral glucose tolerance test (27).

RESEARCH DESIGN AND METHODS

Preparation of mononuclear cells
Donor blood was collected into Vacutainer CPT tubes (Becton Dickinson, Oxford, U.K.) and the peripheral blood mononuclear cells (PBMCs) isolated by centrifugation at 1500g for 20 min. PBMC were washed once, cryopreserved in liquid nitrogen, and stored until analysis.

Reagents and monoclonal antibodies
PBS, BSA, sodium azide, bis(sulfosuccinimidyl)suberate (3), Tris-HCl, sodium chloride, and 7-aminoactinomycin were all purchased from Sigma (Poole, U.K.). Anti-human CD3-AF647 antibody was purchased from Invitrogen (Paisley, U.K.). Anti-human CD45RA-PE and purified anti-human CD-14 were purchased from Serotec (Oxford, U.K.). Anti-human CD14-AF647 was prepared by conjugating purified CD14 monoclonal antibody to Alexa Fluor 647, using an Alexa Fluor 647 monoclonal antibody labeling kit (Invitrogen).

Surface staining of cell surface antigens
Cryopreserved PBMCs were thawed and washed in PBS + 0.1% BSA + 0.02% azide. A total of 1×10^6 cells were placed in 12×75-mm tubes and stained with either anti–CD3-AF647 or anti–CD14-AF647 at 4°C for 25 min. The cells were washed in PBS and antibodies crosslinked to the cell surface by the addition of 1 mmol/l bis(sulfosuccinimidyl)suberate (3) and incubated for 30 min at 4°C.

Excess reagent was quenched by adding 1 ml of 100 mmol/l Tris-HCl, pH 7.0, and 150 mmol/l NaCl for 20 min at room temperature.

Flow-fluorescent in situ hybridization
Telomere length in individual cells was measured using a Dako Telomere peptide nucleic acid kit/fluorescein isothiocyanate (FITC) (Dako Cytomation, Ely, U.K.) modified to identify specific leukocyte subsets as per the methods of Baerlocher and Lansdorp (28) and Batliwalla et al. (29). In brief, the surface-stained cells were centrifuged and resuspended in either hybridization buffer 2 (containing probe) or in hybridization buffer 1 (no probe) as negative control cells. The cells were heated at 82°C for 10 min in a water bath, followed by hybridization at room temperature overnight in the dark. Cells were then incubated twice for 10 min at 40°C with wash buffer. Cells were centrifuged and resuspended in 100 ul PBS azide. The cells stained with anti–CD3-AF647 were then incubated with anti–CD45RA-PE for 25 min at 4°C, washed once, and resuspended in 100 ul of PBS azide. A subsaturating amount of 7-aminoactinomycin (100 ul at 1.0 ug/ml) was added to all cells, to identify and remove aggregates from subsequent analysis, and incubated for at least 30 min before acquisition of at least 20,000 events (cells) on an Altra flow cytometer (Beckman Coulter). Monocytes were identified as CD14+, naïve T-cells as CD3+/CD45RA+, and memory T-cells as CD3+/CD45RA−. The mean telomere fluorescence was calculated as the difference between the mean fluorescence of cells hybridized in the presence of the FITC-PNA probe and the background control cells. Telomere fluorescence data were then converted into molecular equivalent of soluble fluorochrome units (MESF) using Quantum premixed low-level MESF beads, which were run before and after each experiment.

Oxidative DNA damage
A Biotrin OxyDNA test kit (Biotrin, Dublin, Ireland) was used to evaluate oxidative DNA damage, following the manufactures recommendations, with some modifications. The assay is an in vitro fluorescent protein-binding method used to detect oxidative DNA in fixed permeabilized cells. The probe is specific for 8-oxoguanine (as part of the oxidized nucleotide 8-oxoguanosine), which is

formed during free radical damage to DNA and is a sensitive and specific indicator of oxidative DNA damage (30,31). Briefly, 1×10^6 cells were incubated immediately (to prevent ex vivo oxidative damage) with 1% paraformaldeyde for 15 min on ice, washed once with PBS, and resuspended in 70% ethanol and kept at −20°C until staining. Cells were washed in PBS, then the T-cell subsets were identified with anti–CD3-AF643 and anti–CD45RA-PE labeling for 25 min at 4°C then washed. Comparable studies on the monocyte populations were not possible as CD14 expression could not be detected following the fixation process. The cells were then incubated for 1 h at 37°C with 50 ul of Biotrin blocking buffer, washed twice, then incubated for 1 h at room temperature in the dark with 100 ul of FITC-labeled 8-oxoguanine probe. The cells were washed twice and analyzed by flow cytometry and the mean fluorescent intensity of the different cell populations were recorded.

Blood pressure was measured after 5-min seated rest with a Hawksley random zero sphygmomanometer with three readings at 1-min intervals, and blood pressure was taken as mean of the last two readings.

Insulin, glucose, homeostasis model assessment of insulin resistance, and β-cell function
Plasma insulin was measured using a human insulin–specific (no cross-reactivity with proinsulin) enzyme-linked immunosorbent assay (Dako Cytomation) and glucose by the glucose oxidase method. Homeostasis model assessment (HOMA) of insulin resistance was determined as described previously using these measurements (32).

HbA$_{1c}$ and lipid profiles
Plasma lipid profiles were measured on an automated biochemistry analyzer with estimation of LDL cholesterol. HbA$_{1c}$ (A1C) was assessed using a commercially available kit (Roche Diagnostic, Welwyn, U.K.) on an automated biochemistry analyzer (COBAS MIRA; Roche Diagnostic systems) with the normal range quoted as 4.5–5.7%.

High-sensitivity C-reactive protein
This inflammatory marker was measured using a commercially available high-sensitivity C-reactive protein (hsCRP) enzyme-linked immunosorbent assay (Kalon Biological, Aldershot, U.K.).

Table 1—Clinical features of type 2 diabetes and control groups

	Control subjects	Type 2 diabetes
n	28	21
Age (years)	61.2 ± 5.3	62.0 ± 5.6
Diabetes duration (years)	—	3.0 (6.5)
Waist-to-hip ratio	0.92 ± 0.04	0.93 ± 0.07
BMI (kg/m^2)	17.3 ± 3.3	29.5 ± 3.6*
Fasting plasma glucose (mmol/l)	4.9 ± 0.5	9.1 ± 2.5†
Fasting plasma insulin (mU/l)	58.4 ± 30	51.8 ± 21
2-h plasma glucose (mmol/l)	5.8 ± 1.4	17.7 ± 4†
2-h plasma insulin (mU/l)	305 ± 230	170 ± 127*
HOMA-insulin resistance	13.0 ± 7	21.1 ± 10*
A1C (%)	5.4 ± 0.3	7.9 ± 1.5†
Systolic blood pressure (mmHg)	140 ± 19	141 ± 14
Diastolic blood pressure (mmHg	87 ± 11	82 ± 6
Plasma total cholesterol (mmol/l)	5.4 ± 0.8	4.7 ± 0.7*
Plasma HDL cholesterol (mmol/l)	1.3 ± 0.4	1.1 ± 0.3*
Plasma tryiglycerides (mmol/l)	1.3 ± 0.7	1.8 ± 1.6
Plasma LDL cholesterol (mmol/l)	3.5 ± 0.7	2.2 ± 0.6†
hsCRP (μg/l)	905 (969)	801 (1,045)

Data are means ± SD or median (interquartile range); all study subjects were male and receiving no medication. *$P < 0.05$; †$P < 0.0001$.

Data analysis and power calculations

Data are expressed as mean ± SD or as median (interquartile range) as appropriate. Differences between groups were analyzed by unpaired *t* or Mann-Whitney *U* tests as appropriate and significance taken as $P < 0.05$. Relationships between variables were analyzed on simple linear regression and stepped multiple regression, using MESF in cell types as the primary dependent variable. No adequate previous data with this methodology in type 2 diabetes was available to allow sample size calculations, but a total sample size of 40 between two groups would give 80% power at the 5% level to detect a 1-SD difference between group means, which appears to be a biologically relevant difference (16–19).

RESULTS

Clinical features

The diabetic subjects were almost identical in age and blood pressure to control subjects but were significantly more obese ($P < 0.05$), with a higher index of HOMA insulin insensitivity ($P < 0.05$). The diabetic group members were in reasonable glycemic control (mean A1C 7.9%) after a median diabetes duration of 3.0 (6.5) years. There was no significant difference between groups in median hsCRP (Table 1).

Telomere length by flow-fluorescent in situ hybridization and oxidative DNA damage

In the diabetic group, monocyte (CD14-labeled) MESF was very significantly lower than in control subjects (4.0 [1.1] vs. 5.5 [1.1]; $P < 0.0001$). PBMC mean telomere length was also lower in the type 2 diabetic group compared with control subjects at borderline significance ($P = 0.062$). There was no difference in mean telomere length in naïve or memory T-cell populations between groups, but, as reported by others (33,34), telomere lengths were significantly shorter in memory T-cells compared with naïve T-cells from the same individuals, within

both groups ($P < 0.05$ in both groups). Due to the spectral overlap of FITC (telomere probe) and phycoerythrin (CD45RA) and the necessary compensation required to correct for this, the difference in telomere length observed between the T-cells and monocytes may be an experimental artifact. However, this does not affect all other comparisons, where cells were labeled using both FITC and phycoerythrin stains. Oxidative DNA damage (8-oxoguanine levels) in PBMC and naïve and memory T-cell populations was higher in the type 2 diabetic group at borderline significance (Table 2).

Determinants of telomere length in type 2 diabetes

In the diabetic group, PBMC telomere length was significantly inversely related to PBMC oxidative DNA damage ($r = -0.55$; $P = 0.018$), a relationship that was also evident in memory and naïve T-cell populations (Fig. 1). Monocyte DNA damage could not be measured in any subject because of the loss of CD14 expression on monocytes following the fixation process. There was no relationship between PBMC telomere length and HOMA of insulin resistance ($r = 0.08$; $P = 0.2$), hsCRP ($r = 0.29$; $P = 0.2$), A1C ($r = 0.25$; $P = 0.26$), fasting or 2-h plasma glucose and insulin concentrations during a glucose tolerance test, waist-to-hip ratio, diabetes duration, or age (all $P > 0.1$). These relationships were also all nonsignificant for the monocyte and lymphocyte subgroups (all $P > 0.1$).

Determinants of telomere length in control subjects

In the control subjects, there was no relationship between PBMC telomere length

Table 2—Telomere length and oxidative DNA damage in subjects with or without type 2 diabetes

	Control subjects	Type 2 diabetes	P
n	28	21	
Telomere length			
PBMC	8.9 ± 3.1	7.3 ± 2.5	0.062
Naïve T-cells	8.7 ± 3.0	8.1 ± 2.7	0.53
Memory T-cells	7.4 ± 2.9	7.0 ± 2.4	0.60
Monocytes	5.5 ± 1.1	4.0 ± 1.1	0.0001
Oxidative DNA damage			
PBMC	442 ± 86	483 ± 96	0.12
Naïve T-cells	390 ± 106	447 ± 117	0.07
Memory T-cells	473 ± 70	508 ± 93	0.14

Data are means ± SD. Data as MESF for telomere length and mean fluorescent intensity for 8-oxoguanine as a marker of oxidative DNA damage. Monocyte-specific DNA damage was not measurable.
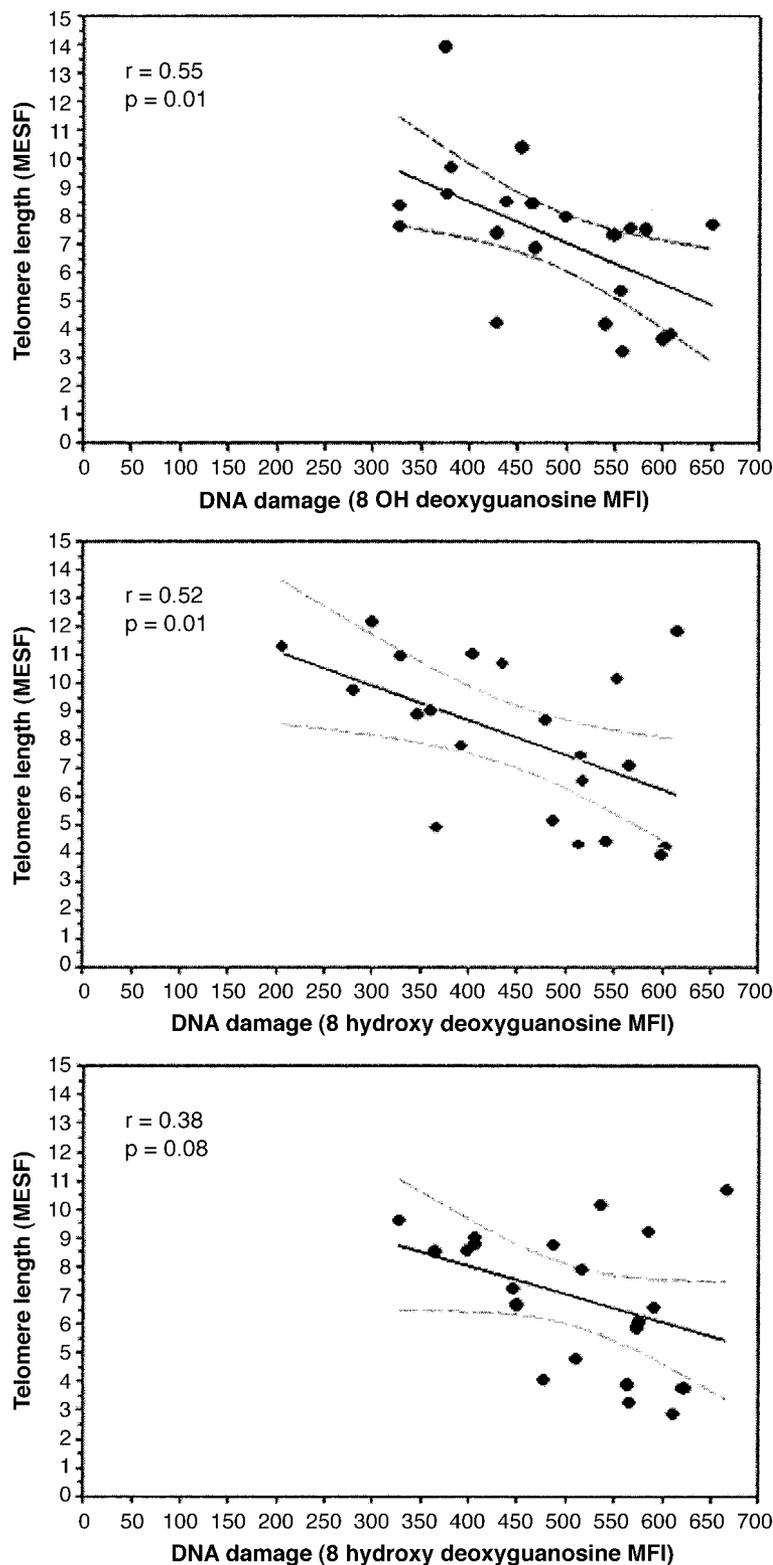
Figure 1—*Relationship between oxidative DNA damage and telomere length in type 2 diabetes PBMCs and lymphocytes. Data shown for 21 type 2 diabetic patients for all PBMCs* (upper panel), *naïve T-cells* (middle panel), *and memory T-cells* (lower panel). *MESF by flow-fluorescent in situ hybridization and DNA damage by flow cytometry of 8-oxoguanine as described in text. Regression line and 95% CI for regression shown.*

and PBMC oxidative DNA damage ($r = 0.17$; $P = 0.4$), as there was in the type 2 diabetic group, and no other measured variable was significantly independently related to PBMC telomere length except for hsCRP ($r = -0.44$; $P = 0.02$). This relationship was not apparent in the monocyte or lymphocyte subgroups.

CONCLUSIONS — The main findings in this study are that peripheral venous monocyte telomere length is very significantly shorter in type 2 diabetic compared with control subjects, and that variability in telomere length was independent of glycemic control, peripheral insulin resistance, and inflammatory markers but inversely related to levels of oxidative DNA .

One strength of this study is that the diabetic group was selected to limit confounding by variables that influence telomere length such as age, sex, ethnicity, drug therapy, smoking, and inflammatory processes such as established vascular disease or hypertension, which make clinical studies on telomere length less easy to interpret (1–3,15–19,23,24). Monocyte–endothelial cell adhesion is enhanced in type 2 diabetes (35), and in other studies we have shown that monocytes from type 2 diabetic subjects demonstrate increased adhesion molecule expression during glycemic excursions and increased expression of the LDL scavenger receptor, CD36 (36,37). The present data indicate that these abnormal peripheral venous monocytes (35–37) also appear to have significantly shortened telomeres. This suggests that the peripheral venous monocyte population adhering to the vascular endothelium in type 2 diabetes (35), entering the vascular wall and undergoing macrophage and foam cell transformation (38), are a population with shorter telomeres. Telomere shortening indicates a cell population at increased risk of replicative senescence and apoptosis at cell division (1–3,6,7), and active monocyte and macrophage proliferation occurs within developing vascular plaque (39–41), although rates of proliferation vary between models (38–41). In some studies, macrophages have been the dominant proliferating cell type (39,42), and proliferative activity and macrophage density are directly related to areas of plaque degeneration and instability (39,42,43). The data in the present study would support the original hypothesis that increased oxidative DNA damage in type 2 diabetes is associated

with telomere attrition and shortening, at least in monocytes. It would also imply that transformed macrophages in type 2 diabetes would be at increased risk of further telomere attrition, senescence, and apoptosis, leading to enhanced atherogenesis and plaque instability in type 2 diabetes (1,6,7). This suggestion would be supported by the observation of Cawthon et al. (20) that telomere length in the peripheral blood of adult males and females without diabetes is strongly associated (odds ratio 4.87 [95% CI 1.59–14.9]) with coronary death. Vascular endothelial cells, smooth cells, and monocyte-macrophage cells within vascular plaque have all been described as demonstrating senescent and apoptotic phenotypes (6,44,45).

A weakness of the present study is that the mechanism for monocyte telomere shortening in type 2 diabetes is unclear. Increased susceptibility to oxidative DNA damage in type 2 diabetes is well recognized (10), and monocytic/endothelial cell precursors from diabetic and nondiabetic animal models are susceptible to oxidative damage in vitro (46). Therefore, it is probable that monocyte telomere shortening reflects increased oxidative damage to monocytic marrow precursors, a suggestion supported by the inverse relationships between telomere length and oxidative DNA damage in PBMC. The increased susceptibility of the GGG sequence in telomeres to oxidative damage compared with the rest of the chromosomal DNA (14) could account for the trend toward higher levels of oxidative DNA damage in the diabetic group but very much more significantly shorter monocyte telomere lengths than control subjects. In other studies we have shown that type 2 diabetes is characterized by increased susceptibility to oxidative DNA damage (10), and the present study extends these observations and suggests that telomere attrition is an outcome of this increased DNA oxidative susceptibility. Jeanclos et al. (47) described significantly shorter telomere lengths in peripheral leukocytes of patients with type 1 diabetes and perhaps a shift in distribution toward shorter telomere length in type 2 diabetes (Fig. 2 in 47). This observation in type 1 diabetes would support the argument that increased oxidative stress (and increased DNA damage) is the dominant contributor to telomere attrition in diabetes. Recently, total venous leukocyte (rather than monocyte) telomere shortening has been described in a heterogenous

group of South Asian patients with type 2 diabetes using a Southern blot analysis for telomere length (48). Telomere length was inversely related to measures of lipid peroxidation in this study, but DNA damage was not assessed (48) and the relative contributions of sex, smoking, and antihypertensives or other medication to telomere length in type 2 diabetes not examined. However, this data (48) would support a significant role for oxidative damage in telomere attrition in type 2 diabetes, and the relationship between oxidative DNA damage and telomere shortening seen in the present study has not been described previously. Most type 2 diabetic patients in this study were taking a statin, but this would not account for our observations, as the available data suggest a neutral or protective effect of statins on telomere length or damage in vitro (49). There was no significant difference in telomere length between diabetic subjects treated or not treated with a statin, and it would have been unethical to withdraw statin therapy for research purposes in this study.

There was no relationship between hsCRP and telomere length in the diabetic group, but there was a significant inverse relationship between hsCRP and telomere length in control subjects. It is possible that this disparity between groups could reflect the anti-inflammatory actions of statins (50), used in the diabetic group, blunting such a relationship in diabetic patients. Similarly, the control subjects did not demonstrate the significant inverse relationship between telomere length and oxidative DNA damage seen in the diabetic group. This might reflect increased levels of oxidative stress in the type 2 patients, driving a trend toward both the increased DNA damage seen in this study and in our other studies (10) and telomere shortening and producing a relationship absent in control subjects with lower levels of oxidative stress. It should be stressed that telomeres are particularly prone to damage at the GGG sequence (14) compared with the rest of chromosomal DNA and that relatively modest degrees of oxidative DNA damage could be expressed as substantial telomere attrition, as in the present study.

We cannot exclude the possibility that the type 2 diabetic monocyte population has undergone more developmental cell divisions, remains in the circulation for longer, or that increased inflammation in type 2 diabetes has led to increased monocyte turnover (16–18),

although hsCRP concentrations did not differ between groups as a marker of inflammation, and the observation was not apparent in T-cells. Similarly, we cannot exclude the possibility that the type 2 diabetic population is preprogrammed toward both shorter telomere length and type 2 diabetes, although this is highly unlikely and has not been examined in this study (51,52).

The monocyte abnormalities in the present study cannot be extended to other cell types. However, endothelial cell precursors in the marrow are susceptible to oxidative damage (46), vascular endothelium is constantly repopulated by endothelial progenitor cells in adult life (51), and senescent endothelial cells appear to be a feature of type 2 diabetes and atherosclerosis (6,51,52).

In summary, we have shown that peripheral blood monocytes from type 2 diabetic patients are characterized by significant telomere shortening. This implies that the peripheral venous monocyte population adhering to the vascular endothelium in type 2 diabetes, entering the vascular wall, and undergoing macrophage transformation are a population at increased risk of replicative senescence and apoptosis within plaques. This suggestion is valid regardless of the mechanisms underlying this observation, which is likely to be due to oxidative DNA damage to monocyte precursors during replication.

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