Damage to the vascular endothelium of diabetic patients can be assessed by analysing blood samples for the number of circulating endothelial cells with mitochondrial DNA deletions

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We report that diabetic patients with both (a) persistent hyperglycaemia, indicated by HbA1C > 9% of total haemoglobin (Hb) and (b) clinically evident micro- or macrovascular disease have a significant increase in the number of detached vascular endothelial cells circulating in their blood relative to age-matched diabetic controls without clinically evident vascular disease. These increased levels of CECs have previously been identified in patients suffering from Mediterranean spotted fever, a disease produced by Rickettsia conorii, a bacterial parasite of endothelial cells [1], (i) in smokers [2], (ii) in diabetes [3] and (iv) in non-diabetics with peripheral vascular disease (PVD) [4].

With ethical permission, 15ml of citrate-treated blood was obtained from diabetic patients using vacutainers and diluted three-fold with phosphate buffered saline (PBS, pH 7.4). The diluted blood was carefully layered onto a cushion of Histopaque-1077 (Sigma) and centrifuged at 800g for 30 min at room temperature. The mononuclear cell band, free of erythrocytes and containing mostly lymphocytes, monocytes and circulating endothelial cells, was drawn off and diluted ten-fold with PBS. After thorough mixing, the cells were harvested by centrifugation at 400 g for 20 min at 4°C. The supernatant was carefully discarded and the cells resuspended in 1 ml of PBS. Three densities of Percoll (1 060 g/ml, 1 070 g/ml and 1 082 g/ml) were then made up as series of stepped gradients [5]. 0.5 ml of the mononuclear cell fraction was layered on top and centrifuged at 800 g for 30 min at 4°C. 0.5 ml of the same mononuclear fraction was retained for PCR. Cells with banding in fractions (a) ≤ 0.060 g/ml (b) > 1.060 to ≤ 1.070 g/ml (c) > 1.070 to < 1.082 g/ml and (d) ≥ 1.082 g/ml were isolated, washed with PBS and analysed by PCR. Typical results obtained from a diabetic patient with peripheral vascular disease are shown in Fig 1. PCR analysis of fraction (a) using primers DEL1 (np 8345-8364) and DEL2 (FL1) versus forward scatter are shown in Fig 1. The population of cells exists in the mononuclear cell fraction which has a relatively high fluorescence intensity and a moderately large forward scatter (Fig 2A, encircled area). This profile appears to be very similar to that obtained when 1 x 10^6 HUVE cells were added to the mononuclear cell fraction from a non-diabetic age-matched control immediately prior to immunostaining (Fig 2B). This population of cells was absent from the same non-diabetic control in the absence of added HUVE cells (Fig 2C) and is also absent from a diabetic patient with no clinically evident vascular disease (Fig 2D).

Fig 1) or weak (+) (< 20% intensity of the strong band in Fig 1). The results of a preliminary survey are presented in Table 1. All patients studied had a HbA1C > 9%. Patients with PVD or nephropathy (proteinuria without renal failure) appeared to have highest levels of both CECs and the 4977 bp mt DNA deletion. Both non-diabetic and diabetic controls without clinically evident vascular disease had no detectable CECs. It remains to be established: (a) if CECs are the only type of cells present in the blood which harbour the 4977 bp mt DNA deletion (b) if the 4977 bp deletion generated by hyperglycaemia is a key event in the aetiology of diabetic vascular complications (c) if these observations are relevant: (i) The 4977bp deletion removes several mitochondrial genes including one which codes for subunit 6 of the mitochondrial ATP-synthase [9]. Thus, as the level of 4977bp deletion increases in a cell, the level of ATP synthesis by oxidative phosphorylation should fall. (ii) When the 4977bp deletion reaches high levels in ex vivo cultured endothelial cells (there are several hundred copies of mtDNA per cell), they detach from their substratum [6]. This observation could explain the origin of CECs in vivo in patients with persistent hyperglycaemia. In conclusion, we have demonstrated for the first time the presence of a major deletion in the mt DNA (~ one third of the total mt genome) in CECs obtained from diabetics with vascular complications.

Figure 1 PCR analysis of blood fractions obtained from a diabetic patient with PVD using primers DEL1 and DEL2, specific for the 4977 bp mt DNA deletion. Lane 1: 1 kb DNA ladder (size markers). PCR was performed on DNA from 5 x 10^5 cells as follows: Lane 2: mononuclear cells isolated from the Histopaque-1077 fraction. Lane 3: cells isolated from the ≥ 1.060 g/ml Percoll fraction. Lane 4: cells present in the >1.060 to <1.070 g/ml Percoll fraction. Lane 5: cells present in the Percoll fraction >1.070 to <1.087 g/ml. Lane 6: cells present in the ≥ 1.082 g/ml Percoll fraction. Lane 7: mononuclear cells from a non-diabetic control following fractionation on Histopaque-1077. Lane 8: Cells from the same non-diabetic control further purified and present in the ≥ 1.060 g/ml Percoll fraction.

Figure 2. Plot of fluorescence intensity (FL1) versus forward scatter following FACS analysis of immunostained mononuclear cells isolated by fractionation on Histopaque-1077 from: (A) diabetic patient with PVD (B) non-diabetic age-matched control following addition of 1 x 10^6 HUVE cells. (C) the same non-diabetic age-matched control without added HUVE cells. (D) diabetic patient with no clinically significant vascular disease. Cells were stained with an anti-human von Willebrand factor rabbit IgG (dilution 1/200 in PBS) followed by an FITC-labelled goat anti-rabbit IgG (dilution 1/50 in PBS).

Table 1 Numbers of diabetic patients with the 4977 bp mt DNA deletion. DNA from 5 x 10^5 cells was analysed for the level 4977 bp mt DNA deletion in partially purified CECs in ≤ 1.060 g/ml Percoll fraction. Levels of deletion were classified as strong (+ + +), intermediate (+++) or weak (+) as defined in the main text.

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