ORIGINAL ARTICLE

Increased expression of activation markers on monocytes and neutrophils in type 2 diabetes

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ABSTRACT

Background: Activation of leukocytes is obligatory for adherence to the endothelium and atherogenesis. Since leukocyte activation by triglycerides (TG) and glucose has been described *in vitro*, we hypothesised higher leukocyte activation in patients with type 2 diabetes.
Methods: Using flow cytometry, we studied the expression of the leukocyte activation markers CD11A, CD11B, CD62L and CD66B in 15 patients with type 2 diabetes without clinical evidence of atherosclerosis (55±7 years) and in 15 healthy controls (53±2 years). All patients were on oral antidiabetic treatment (glyHb 6.3±0.9%) and not taking statins or anti-inflammatory drugs.
Results: In comparison with controls, the patients had a higher waist circumference (1.08±0.09 *vs* 0.94±0.11 m, p<0.005) and higher fasting glucose (8.4±2.3 *vs* 5.3±0.7 mM, p<0.005), whereas fasting plasma lipids were not statistically different. The leukocyte count was higher in the patients (6.55±1.55 *vs* 5.07±1.10 x 10⁹cells/l, p<0.005) due to higher neutrophils and lymphocytes (+34% and +24%, p<0.05 for each). CD11B on monocytes and CD11B and CD66B on neutrophils were higher in the patients (+30%, +52% and +43%, p<0.05 for each). Fasting glucose, waist circumference, body mass index and systolic blood pressure were positively associated with the leukocyte and neutrophil count. The expressions of CD11B and CD66B on monocytes and neutrophils were strongly positively interrelated, but unrelated to TG and glucose.
Conclusion: In patients with type 2 diabetes, the expression of activation markers on monocytes and neutrophils is enhanced and not correlated to fasting glucose or TG. These results suggest a proinflammatory situation in type 2 diabetes and most likely represent increased adhesive capacity of neutrophils and monocytes to the endothelium.

INTRODUCTION

It is generally accepted that atherosclerosis is a low-grade, chronic inflammatory disease.[1,2] In response to endothelial injury, atherogenesis is initiated by resident and recruited leukocytes, eventually resulting in release of various inflammatory mediators.[2] A relationship between leukocyte count and the incidence of coronary heart disease (CHD) and mortality has been described.[3] In addition, increased leukocyte counts are positively associated with several traditional CHD risk factors such as smoking, hyperlipidaemia and insulin resistance.[4,5]

A prerequisite for adherence of leukocytes to the endothelium is that both cell types are activated.[6] Studies with animal models have established the importance of the interaction between leukocytes and endothelial cells with regard to atherogenesis, since blocking of specific selectins reduced plaque formation[7,8] and prevented endothelial dysfunction.[9] A plausible explanation for a proinflammatory state in various metabolic disorders could be that triglycerides (TG) and glucose are able to induce activation of leukocytes and endothelial cells. Activation of endothelial cells has been described *in vitro*.[10-12] In contrast, the effects of glucose and TG on the activation of leukocytes have not been studied in detail. Activation of monocytes in particular, but also neutrophils, has been described *in vitro*. In these studies cell activation was established by gene expression,[13,14] cytokine production, nuclear factor kappa B (NF-κB) activation[14] and intracellular signalling.[15] The effects of TG were studied using artificial TG emulsions.[15] A more appropriate way of measuring would be flow cytometric quantification of the expression of leukocyte activation markers that are involved in the interaction with the endothelium.[16]

Increased expression of these markers in ischaemic heart disease has been described earlier,[17,18] but there are only limited data available on patients at risk for CHD without clinical atherosclerosis. Since *in vitro* data are suggestive of activation of monocytes and also neutrophils, in particular in conditions associated to the metabolic syndrome, we carried out the present observational study to determine the expression of leukocyte activation markers in a group of patients with type 2 diabetes and in healthy controls.

METHODS

Subjects and study design

Diabetic patients and healthy controls aged 45 to 65 years were recruited by advertisement. Exclusion criteria for the patients were smoking, alcohol intake >2 units/day, glycated haemoglobin (glyHb) >8.5 %, presence of renal disease or a liver disorder, clinically established CHD and use of insulin. Due to a possible anti-inflammatory effect of statins with regard to leukocyte-endothelium adherence,[19-22] patients on statins were also excluded. Exclusion criteria for the controls were fasting hyperlipidaemia (plasma cholesterol >6.5 mM, plasma TG >2.0 mM), fasting plasma glucose >6.5 mM, body mass index (BMI) >30 kg/m^2, smoking, alcohol intake >2 units/day, presence of clinically established CHD or renal and liver diseases, a family history of premature CHD or type 2 diabetes mellitus and use of any drugs known to affect lipid metabolism. None of the patients and controls were on special diets, antioxidants, antihypertensive or anti-inflammatory drugs. For both groups recent infection suggested by a positive medical history was an exclusion criterion. All subjects gave written informed consent. The study was approved by the Independent Ethics Committee of Institutional Review Board of the University Medical Centre, Utrecht. Subjects visited the hospital after an overnight fast for at least ten hours and they were asked not to drink alcohol on the day before the test. Blood pressure and waist circumference were measured and the BMI was calculated. Venous blood samples were obtained in sodium EDTA (2 mg/ml).

Analytical methods

Blood cell counts and differentials were determined automatically using a Celldyn-3500 (Abbott, Abbott Park, IL, USA). One blood sample was stored at 4 °C to determine expression of leukocyte activation markers. All other blood samples were chilled and centrifuged immediately for 15 minutes at 800 g at 4 °C, after which plasma was stored at -80 °C. Total cholesterol, HDL cholesterol obtained after precipitation with heparin/MnCl2 and TG were measured in duplicate by colorimetric assay with the CHOD-PAP and GPO-PAP kits, respectively (Roche

diagnostics, Germany). LDL cholesterol was calculated using the Friedewald formula. Glucose was measured by glucose oxidase dry chemistry (YSI, USA) and GlyHb was measured photometrically (Hitachi 911, Roche, Germany) in the diabetic patients only.

Leukocyte activation markers

Using fluorescent labelled monoclonal antibodies (MoAbs), the cell surface expression of two pairs of leukocyte activation markers was detected by direct immunofluorescence in duplo and evaluated by flow cytometry: a combination of fluorescein isothiocynate (FITC) conjugated CD66B (CLB, the Netherlands) and phycoerythrin (PE) conjugated CD11B (DAKO, Denmark) and a combination of FITC conjugated CD62L (Cymbus Biotechnology, UK) and PE conjugated CD11A (Diaclone, France). CD11A (also termed LFA-1), CD11B (also termed MAC-1 or CR3) and CD62L (also termed leukocyte selectin) are the most important markers for the early adhesion of leukocytes to the endothelium.[23,24] CD66B (also termed CEACAM8) is a degranulation marker of neutrophils and is not expressed on lymphocytes and monocytes.[25] Using isotype matched MoAbs, nonspecific binding of each label was ruled out in five subjects (data not shown). To avoid *in vitro* activation, the leukocytes were incubated with MoAbs in whole blood at a saturating concentration of 1:10 for 30 minutes in the dark on ice. Erythrocytes were lysed by adding ice-cold isotonic erythrocyte lysing solution (NH$_4$Cl 0.19 M; KHCO$_3$ 0.01 M; Na$_2$EDTA•2H$_2$O 0.12 M, pH 7.2) for approximately 15 minutes and centrifuged at 500 x g for five minutes at 4 °C. The remaining leukocyte suspension was washed twice in ice-cold PBS supplemented with bovine serum albumin (BSA 0.2%). Within one hour a total of 5000 cells/sample was analysed by flow cytometry using a fluorescence activated cell counter (FACS, Becton-Dickinson) and CellQuest software. Neutrophils, lymphocytes and monocytes were identified by their characteristic forward and side scattering properties. Fluorescence intensity of each cell was expressed as the average mean fluorescence intensity (MFI) of the duplo, given in arbitrary units (AU).

Statistics

Data are given as mean ± SD. Differences were tested using a Chi-square or Student's t-test. For TG and leukocyte counts, calculations were performed after logarithmic transformation. Bivariate correlations were calculated using Spearman's correlation coefficients. All significantly correlated variables were used as independent variables in stepwise multiple regression analysis with the average MFI of the leukocyte activation markers as dependent variables. For statistical analysis SPSS version 10.0 was used. P values <0.05 (two-tailed) were considered statistically significant.

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RESULTS

Subject characteristics

All patients were on unchanged oral antidiabetic treatment with either a sulphonylurea derivative (n=6), metformin (n=5) or a combination of both (n=4) for more than three months. In comparison with controls, the patients with type 2 diabetes showed a higher frequency of characteristics of the metabolic syndrome, e.g. central obesity, a higher blood pressure and higher fasting glucose (*table 1*). None of the lipid parameters from *table 1* showed a significant difference between patients and controls. Furthermore, when compared with controls, the patients showed a higher leukocyte count (6.55±1.55 *vs* 5.07±1.10 x 10⁹ cells/l, p<0.005) due to higher numbers of neutrophils and lymphocytes (+34% and +24% respectively, p<0.05 for each, *figure 1*).

Figure 1

*Leukocyte count and differentiation into monocytes, lymphocytes and neutrophils in healthy controls (n=15, open bars) and in patients with type 2 diabetes (n=15, closed bars) Student's t-test: * p<0.05, ** p<0.005 patients vs controls.*

Table 1

Baseline characteristics (mean ± SD) of the study group consisting of healthy controls and patients with type 2 diabetes

	CONTROLS (N=15)	DM TYPE 2 (N=15)
Gender (M/F)	13/2	10/5
Age (years)	53 ± 2	55 ± 5
BMI (kg/m²)	25.7 ± 2.7	31.6 ± 4.8 **
Waist (m)	0.94 ± 0.11	1.08 ± 0.09 **
BP systolic (mmHg)	128 ± 12	148 ± 11 **
BP diastolic (mmHg)	86 ± 5	89 ± 8
Glucose (mM)	5.3 ± 0.7	8.4 ± 2.3 **
GlyHb (%)	ND	6.3 ± 0.9
Triglycerides (mM)	1.69 ± 0.67	2.50 ± 1.45
Cholesterol (mM)	5.8 ± 0.9	6.3 ± 1.5
LDL-c (mM)	3.8 ± 0.7	3.9 ± 1.5
HDL-c (mM)	1.21 ± 0.30	1.27 ± 0.57
Cholesterol/HDL-c	5.0 ± 1.2	5.6 ± 2.1

*ND not determined, Student's t-test: * p<0.05, ** p<0.005 patients vs controls.*

Table 2

Expression of leukocyte activation markers in healthy controls and in patients with type 2 diabetes

	CONTROLS (N=15)	DM TYPE 2 (N=15)
CD11A		
Monocytes	80 ± 18	80 ± 20
Lymphocytes	57 ± 12	60 ± 13
Neutrophils	34.1 ± 7.7	35.2 ± 8.9
CD11B		
Monocytes	194 ± 46	253 ± 89 *
Lymphocytes	31.3 ± 7.2	30.1 ± 10.5
Neutrophils	202 ± 66	307 ± 175 *
CD62L		
Monocytes	96 ± 22	94 ± 22
Lymphocytes	51 ± 8	53 ± 11
Neutrophils	81 ± 25	85 ± 18
CD66B		
Neutrophils	7.5 ± 2.5	10.7 ± 5.0 *

*Data are given as mean fluorescence intensity per cell (MFI, in arbitrary units) ± SD. Student's t-test: * p<0.05 patients vs controls. Note that CD66B is not expressed on monocytes and lymphocytes.*

Leukocyte activation

In comparison with controls, the diabetic patients showed a higher expression of CD11B on monocytes (253±89 *vs* 194±45 AU, p<0.05) and of CD11B and CD66B on neutrophils (307±175 *vs* 202±66 and 10.7±5.0 *vs* 7.5±2.5 AU, respectively, p<0.05 for both comparisons, *table 2* and *figure 2*). The expression of CD11A or CD62L in any of the cell types was not statistically different between patients and controls (*table 2*). Subanalysis of the diabetic patients according to the type of oral antidiabetic treatment did 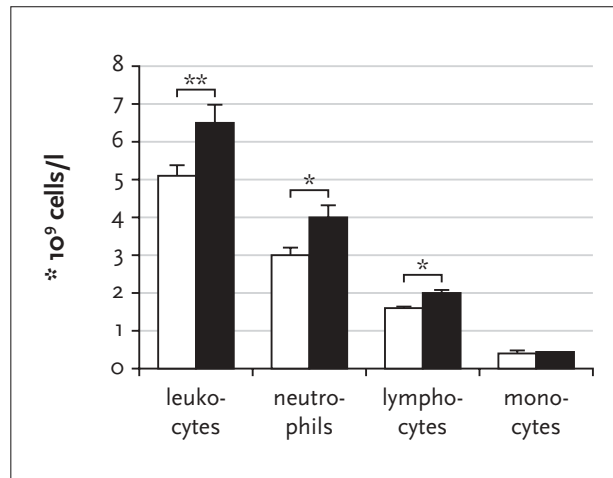not show differences in parameters from *table 1* or *figures 1* and *2*. When patients and controls were analysed together, BMI, waist circumference, fasting glucose and systolic blood pressure were positively related to the leukocyte count (R=0.54, R=0.48, R=0.59 and R=0.43, respectively, p<0.05 for each) and neutrophil count (R=0.53, R=0.50, R=0.53 and R=0.46, respectively,

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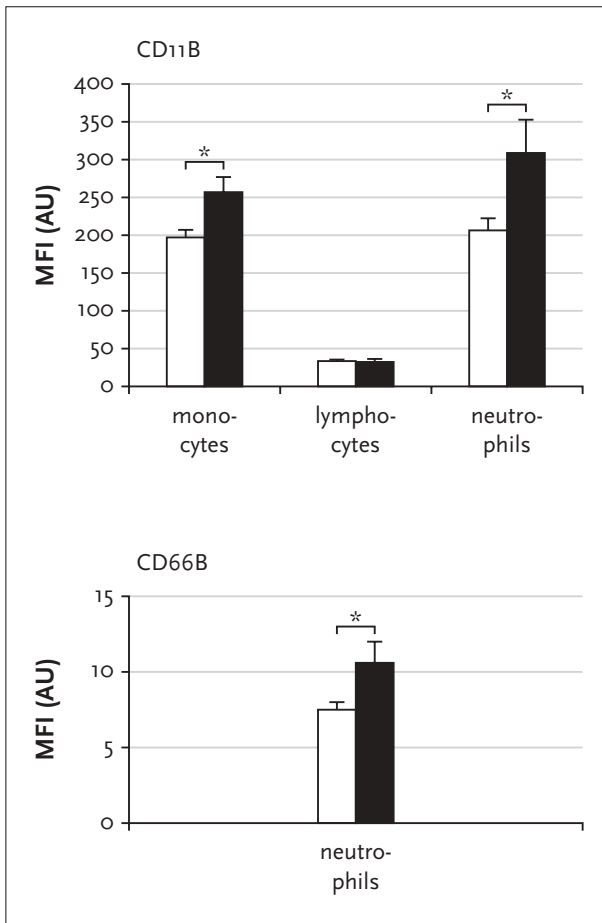OCTOBER 2004, VOL. 62, NO. 9

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Figure 2

*Expression of leukocyte activation markers CD11B on
neutrophils, lymphocytes and monocytes (upper panel)
and of CD66B on neutrophils (lower panel) in healthy
controls (n=15, open bars) and in patients with type 2
diabetes (n=15, closed bars)*
*Data are given as mean fluorescence intensity per cell
(MFI, in arbitrary units). Student's t-test: * p<0.05
patients vs controls. Note: the different Y-axis scale in
each panel.*

p<0.05 for each). From all parameters in *table 1*, age was
significantly related to the expression of CD11B on mono-
cytes and of CD11B and CD66B on neutrophils (R=0.68,
R=0.65 and R=0.47, p<0.01 for all). Furthermore, CD66B
expression on neutrophils was significantly related to
systolic and diastolic blood pressure (R=0.48 and R=0.42,
p<0.05 for each). The expression of these markers was
strongly interrelated (monocyte and neutrophil CD11B:
R=0.83, neutrophil CD66B and CD11B: R=0.57 and
neutrophil CD66B and monocyte CD11B: R=0.57,
p<0.001 for all comparisons). Stepwise linear regression
showed that the models to explain the variation in the
expression of these markers did not improve by adding
age and or blood pressure.

DISCUSSION

When compared with controls, in type 2 diabetic patients,
leukocyte cell counts were increased and neutrophils and
monocytes showed a higher expression of activation
markers. An increased leukocyte count in patients with
type 2 diabetes was observed earlier in the ARIC study.[5]
In that study it was hypothesised that the leukocyte count
reflects the pathogenesis of type 2 diabetes. In line with
this, three traditional CHD risk factors in diabetic patients,
glucose, obesity and blood pressure, were positively related
to the leukocyte and neutrophil count in the present
study and in earlier reports.[4,5]
Leukocyte adherence starts with rolling along the vessel
wall, largely mediated via leukocyte selectin (L-selectin or
CD62L).[6] Upon *in vitro* stimulation, L-selectin is rapidly
shed from the leukocytes, in order to improve a more
tight adherence via β2 integrins (in particular CD11B, but
also CD11A).[23,26] Since we hypothesised a higher degree of
activation in the diabetic patients, we expected to find a
lower expression of CD62L per cell in these subjects.
However, in the present study, which is to the best of our
knowledge the first to compare cellular CD62L in diabetic
patients with healthy controls, we did not observe differential
expression of CD62L. On the other hand, the most
important marker involved in tight leukocyte adherence,
CD11B, showed a higher expression on monocytes and
neutrophils of the diabetic patients. This may suggest that
these cells have increased adhesive properties thereby
potentially enhancing the risk of vascular complications.
Previous reports of CD11B expression in patients with
type 2 diabetes are contradictory. Increased neutrophil
CD11B expression in type 2 diabetes has been reported,[27]
while another study did not show different expression of
CD11A and CD11B on monocytes and neutrophils in diabetic
patients *vs* controls.[28] Furthermore, in comparison with
controls, enhanced neutrophil CD11B expression after
ex vivo stimulation has been described in patients with
type 2 diabetes.[29] This finding may suggest increased
responsiveness of these cells.
CD66B is a marker of release of substances such as
collagenase and gelatinase from specific granules.[30,31]
Upon *ex vivo* stimulation, expression of CD66B is
rapidly upregulated.[25] CD66B has not been associated
with atherosclerosis before, but under proinflammatory
conditions activated neutrophils secreting these enzymes
in the vicinity of the endothelium, could negatively affect
endothelium function and plaque stability. We found a
positive association between neutrophil CD66B expression
and diastolic and systolic blood pressure. In diabetic patients,
besides increased *ex vivo* neutrophil responsiveness, also
impaired *ex vivo* degranulation has been shown and these
effects where most pronounced in the patients with
hypertension and microalbuminuria.[29] We have not

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stimulated leukocytes *ex vivo*, but higher *in vivo* CD66B expression does not exclude degranulation dysfunction in response to acute activating stimuli. In the present study, the higher degree of CD66B expression in the patients is in line with increased expression of CD11B and the strong correlations between the expressions of these markers are suggestive of a common underlying mechanism. Supportive for this is that in resting neutrophils 75% of CD11B is colocalised with CD66B in secretory granules.[32]

It was remarkable that the expressions of CD11B and CD66B were best predicted by each other whereas age and blood pressure, which were also correlated by univariate analysis, did not improve the models. This may also be a result of a common mechanism of expression of these markers resulting in amplification of the effect of age and blood pressure. BMI or gender were not correlated in the whole group correlation analysis, so we do not expect gender and BMI to explain the differences in leukocyte activation between the patients and controls.

On lymphocytes the expression of the studied markers was low and not different between the patients and controls. The selected markers are most certainly not the most appropriate to reflect lymphocyte activation. Indeed, upon *in vitro* stimulation the change in expression of these markers was markedly lower on lymphocytes than on monocytes and neutrophils (data on file).

While the leukocyte count was positively related to fasting glucose, a similar relationship with TG, another important characteristic of the metabolic syndrome, was not observed. In addition, both TG and glucose were unrelated to the expression of activation markers on leukocytes, which is in agreement with other studies.[27,33] From many *in vitro* studies it is known that both TG and glucose can activate leukocytes.[13-15,34-36] and that under hyper-TG or hyperglycaemic conditions adherence of leukocytes to the endothelium is increased due to activation of endothelial cells.[12,20,37] Monocytes from diabetic patients showed increased superoxide production upon *ex vivo* stimulation, suggestive of increased responsiveness of these cells. However, this activation was related to plasma TG only and not to elevated glucose or glyHb.[36] The *in vivo* situation seems even more complex, since in diabetic patients and controls after a glucose load, monocyte but not neutrophil CD11B expression increased, unrelated to the glucose rise.[28] Furthermore, we have recently reported acute increments of neutrophil counts during the postprandial phase after ingestion of a glucose and fat load, suggesting a proinflammatory effect of glucose and TG-rich lipoproteins on leukocytes.[38,39] In another study in healthy subjects we have observed increased expression of monocyte and neutrophil CD11B, CD62L and CD66B after an oral fat load, while only the CD11B increments were related to the TG increment (data on file). A possible explanation for the weak *in vivo* relationship could be that advanced glycaemic end products (AGE) or the type of fatty acids or lipoproteins are more important than glucose or TG.[16,40] Regarding AGEs, in the present study we could not find a relationship between glyHb and the activation of leukocytes. This may be due to the relative tight glucose control of the diabetic patients we studied. In addition, leukocytes could become activated indirectly via triggering of endothelial cells by TG or glucose,[12,20,37] by unstable leukocyte-enriched atherosclerotic plaques,[41] or by endothelial injury in general, since coronary angioplasty has been shown to induce leukocyte activation.[42] We also have to underline that, as has been shown by others, the leukocytes we obtained by peripheral sampling are most certainly less activated than the cells contributing to local inflammatory processes, since activated leukocytes will probably adhere to the endothelium *in vivo*.[41]

In conclusion, the expression of activation markers on monocytes and neutrophils is enhanced in patients with type 2 diabetes, independent of fasting glucose and TG. These results suggest a proinflammatory situation in type 2 diabetes and most likely represent increased adhesive capacity of neutrophils and monocytes to the endothelium. The clinical relevance of leukocyte activation and the exact mechanisms whereby type 2 diabetes leads to this activation need to be studied.

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