PECAM-1 (CD31) gene polymorphisms in type 1 diabetes and its microangiopathic complications

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Abstract

**Background:** Platelet-endothelial cell adhesion molecule 1 (PECAM-1) is a widely distributed adhesion molecule and is considered as a candidate molecule in vascular pathologies including diabetic microvascular complications. Endothelial cells have a crucial role in the pathogenesis of diabetic complications. PECAM-1 is expressed on both residential endothelial and circulatory blood cells.

**Methods:** In the present study, at first the PECAM-1 gene promoter region was explored for detection of novel polymorphism using PCR-SSCP technique. Genotyping of polymorphisms was carried out using ARMS-PCR technique on type 1 diabetic patients (N=251) and normal controls (N=86). Subgroup analysis was performed on type 1 diabetic patients with various microvascular complications. In order to investigate the effect of novel polymorphism on PECAM-1 cell surface expression, functional or phenotypic value of novel polymorphism in quantitative level was assessed using flow cytometry of PBMC’s carrying different genotype.

**Results:** We found a novel polymorphism in PECAM-1 gene promoter (-265*C/T) (Genebank acc. No. AJ313330). Flow cytometry analysis showed that this polymorphism had no effect on PECAM-1 cell surface expression. However, flow cytometry analysis for another polymorphism at codon +125*G/C has shown that it was correlated with the level of PECAM-1 cell surface expression in PBMCs. No significant association between these two polymorphisms and type 1 diabetes or diabetes microvascular complications were found.

**Conclusion:** The polymorphisms in PECAM-1 gene do not appear to be genetic risk factors for type 1 diabetes or diabetes microvascular complications. However this needs to be further confirmed.

**Keywords:** Polymorphism, Type 1 diabetes, Microvascular complications, PECAM-1 gene

**Abbreviations**
PECAM-1, Platelet endothelial cell adhesion molecule-1; T1DM, type 1 diabetes mellitus; DN, diabetic nephropathy; DR, diabetic retinopathy; DNU, diabetic neuropathy.

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Introduction
Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31, or EndoCAM in the mouse) is a 130 kDa adhesion molecule. It belongs to the IgG supergene family of receptors (1). As a transmembrane glycoprotein, PECAM-1 is highly expressed at the borders between endothelial cells and at a lower level on the surface of circulating platelets, monocytes, neutrophils, and certain T cell subsets, particularly naive CD8+ T cells. With a few minor exceptions, it is not present on fibroblasts, epithelium, muscle or other non-vascular cells (2). The human PECAM-1 gene is a single copy and TATA-less gene, which is a characteristic of constitutively expressed genes (3). It is located at the end of the long arm of chromosome 17 (17q23) (4), along with other adhesion molecules found on the surface of platelets and endothelial cells. The PECAM-1 gene possesses 16 exons and intermediate introns and spans about 75 kb in length.

The sequence homology of PECAM-1 among different species is highest at about 40 amino acids distal to the transmembrane domain (5), suggesting the functional importance of this segment of the molecule (6).

With regard to the structure of the PECAM-1 molecule, the cytoplasmic tail plays an important role in the mediation of PECAM-1 function, and mutation of the tyrosine residues at position 663 or 686 in the cytoplasmic tail reduces phosphorylation. Mutation at position 686 is also associated with a reduction in PECAM-1-mediated inhibition of cell migration (7).

PECAM-1 leads transendothelial migration of leukocytes in the adhesion cascade. Beside that PECAM-1 is also a downstream signalling molecule for diverse stimuli (8). In heterophilic interaction, PECAM-1 is able to interact with a number of molecules, including integrin αvβ3 and CD38.

The new role of PECAM-1 as a downstream signalling molecule is mediated by phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) domain in the cytoplasmic tail (9). In endothelial cells, PECAM-1 phosphorylation may occur as a response to diverse stimuli, like shear stress, hypoxia, VEGF, TNF-α and cross linking of PECAM-1 itself. While the consequences of PECAM-1 signalling in endothelial cells are not well defined, its signalling in T cells has been shown to influence proliferation, IL-2R expression, TCR signalling and production of several cytokines and chemokines, through the activation of integrins (10).

PECAM-1 regulates the cytoplasmic level of β-catenin, which plays a dual role as a structural (in adherence junction complex) and signalling protein (translocation of bound transcriptional factors to the nucleus), through the modulation of its phosphorylation state (11).

Recently, PECAM-1 expression in endothelial cells has been proposed as a mechanism for the cell associated mechanical forces underlying cell “tensegrity” (structural or tensional integrity). PECAM-1 is diffusely distributed in the plasma membrane of solitary endothelial cells, i.e. following a stimulus to migrate, but it accumulates at intercellular junctions after formation of cell-cell contact. Confluent endothelial cells display a low migrating rate and a high level of PECAM-1 tyrosine phosphorylation, which is in contrast to migrating endothelial cells (12). The low expression of PECAM-1 in sparse or migrating endothelial cells, as a transitional stage toward endothelial cell proliferation, is not linked to the ability to proliferate itself (13). Accordingly, PECAM-1 is a central player in the maintenance of the functional integrity of endothelial monolayers through the formation of cell-cell junctions (14). In PECAM-KO mice, which were expected to show reduced or abolished leukocyte transendothelial migration, unexpectedly augmented emigration of T cells across endothelium was evident in EAE. This is apart from PECAM-1 expression on transmigrating T cells. The endothelial cells of PECAM-KO mice also exhibited prolonged permeability changes (15).

Oxidative stress, which is characterised as one of the chief mechanisms for the development of late diabetic complications and also for other vascular-based pathologies, also operates to some extent through the phosphorylation of PECAM-1. Further to increased adhesion of monocytes and neutrophils to the endothelium as an initial effect, induction of oxidant stress also leads to a five-fold increase in PECAM-1 phosphorylation and a two-fold increase in the transendothelial migration of monocytes (16).
Since shear stress or haemodynamic factors have been identified as other key mechanisms in the evolution of diabetic complications (17, 18), it is interesting to envisage the role of PECAM-1 in this regard as well. As a mechano-sensing and responding molecule, PECAM-1 is the first molecule in mammalian cells that is chemically modified when direct mechanical force is applied to it (5,19). The areas of vessel wall with steady laminar flow of high shear stress are resistant, while temporal shear stress or areas with disturbed flow (more reduced shear stress) are prone to atherogenic injuries. All these data propose that PECAM-1 may play a crucial role in development of diabetic microvascular complications. The aim of present study was first to explore the PECAM-1 gene promoter and 5’UTR region for detection of new polymorphisms and then genotyping PECAM-1 gene polymorphism at codon +125*G/C [Leu (C) → Val (G)] for investigating possible association with type 1 diabetes or its microvascular complications. Functional or phenotypic value of PECAM-1 gene polymorphism in quantitative level has also been assessed by flow cytometry analysis.

Methods

Patients and controls

As an out-patient clinic in the North West region of the UK, about 5000 T1DM registered patients are regularly attending to the Manchester Diabetes Centre. In the present cross-sectional study, unrelated attendees with T1DM were randomly selected during 1999-2002 as the patient group. The ethical approval was obtained from the Manchester Royal Infirmary. All patients fulfilled the relevant criteria for related diagnosis as are detailed later. To be on the safe side, patients who had diabetes less than 3 years were excluded from analysis. Diabetes was diagnosed according to the criteria, which was suggested by an expert committee in 1997 (report of the expert committee on the diagnosis and classification of diabetes Mellitus, 1997). The diabetic patients included in the present study fulfilled at least one of the triple criteria recommended by the expert committee, detailed as follows:

a) symptoms of hyperglycaemia (polyuria, polydipsia, unexplained weight loss) plus random plasma glucose ≥200 mg/dL (11.1 mmol/L). Random is defined as any time of day without regard to time since last meal.
b) fasting plasma glucose (FPG) ≥126 mg/dl (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 hours.
c) 2-hour plasma glucose (PG) ≥200 mg/dl (11.1 mmol/L) during an oral glucose tolerance test (OGTT). The test should be performed as described by the World Health Organization (WHO), using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water. Diabetes was regarded as T1DM if it was diagnosed before age of 30 years and accompanied with acute onset and treatment with insulin began within the first year of diagnosis and continued thereafter. The retina was examined by fundoscopy (after pupillary dilatation) and when more than five dots or blots per eye, hard or soft exudates or new vessels were evident, the diagnosis of retinopathy was applied. Patients who had a history of laser treatment were also diagnosed as retinopathy. The elevation of AER (>300mg-2g/day) at least on two of three occasions and/or 3 positive Albustix over the past 12 months were evident to mark patients as nephropath, while a urinary tract infection (UTI) was ruled out already. Neurothesiometer - a clinical electromagnetic vibrating device- was applied for screening of peripheral diabetic neuropathy, which quantifies the vibration sensitivity through the measurement of vibratory perception threshold (VPT), while the patient’s eye is closed and the probe of neurothesiometer is placed on the hallux of the toe. An average of 3 readings were taken. DNU was diagnosed when VPT was more than 25 volts (vibration threshold above 25 volts indicates a high risk of ulceration). The symptoms of sensory and/or motor neuropathy were looked for, like paresthesia, numbness, tingling, nocturnal rest ache, all in the absence of peripheral vascular disease as non-specific (non-diabetic) underlying cause. The excluding of peripheral vascular disease was approved by palpable pulses and measuring of ankle brachial pressure index.
**PCR-SSCP for mutation detection**

The SSCP method was applied to screen potential polymorphisms in the promoter regions of PECAM-1 genes (Genbank accession no. X96848) (20) in 40 British-Caucasian healthy individuals as controls. In order to fully cover the screened regions and to avoid missing potential polymorphisms within the primer binding sites themselves, primers were designed to produce overlapping DNA fragments. The first 1550 base pairs of PECAM-1 promoter and 5’UTR regions were divided into 7 fragments and each fragment was amplified by PCR-SSCP using specific primers (primers’ sequences are available upon request). The amplified DNA products were separated by electrophoresis in silver stained polyacrylamide gels. To confirm that the dissimilarity of DNA bands in SSCP gel is meaningful (mutation/polymorphism) or not, DNA sequencing was carried out by application of the ABI Prism Big Dye DNA sequencing kit (Applied Biosystems, UK).

**Genotyping of PECAM-1 gene polymorphism using ARMS-PCR**

Genotyping of polymorphisms was carried out on type 1 diabetic patients (N=251) and normal controls (N=86). ARMS-PCR was set up for genotyping of PECAM-1 gene polymorphisms using primers shown in Table 1. Figure 1 shows the PCR product for genotyping of PECAM-1 gene polymorphism at position –265*T/C.

**Flow cytometry analysis for PECAM-1 expression analysis**

By flow cytometry, PECAM-1 cell surface expression in PBMCs was studied in both fresh and activated cell populations. For stimulation, 1x10^6 cells/ml were cultured and stimulated with 4 µg phytohemagglutinin (PHA, Sigma, UK) and 3 µg lipopolysaccharide (Escherichia coli serotype, Sigma, UK) for 48 hours in 12 well flat bottomed tissue culture plates (Helena Bioscience, UK) at 5% CO₂, 37°C and 95% humidity. From each population (activated and non-activated), approximately two million PBMCs were isolated and washed twice with PBS, which contained 2% FCS and 1% sodium azide (FACS buffer). Twenty µl of fluorescein isothiocyanate (FITC) conjugated mouse anti-human PECAM-1 monoclonal antibody (BD Pharmingen, UK) was added to 1x10^6 cells in suspension. In parallel, 1x10^6 cells were separately stained with 20 µl of FITC conjugated mouse IgG1 isotype monoclonal antibody (DAKO, Denmark) as a negative control. Then the cells were incubated at 4°C for 30 minutes in the dark and after that the cells were washed twice with FACS buffer. About 500 µl of cells suspended in FACS buffer were analysed on a FACS machine (Becton Dickinson), using the “FACScan CellQuest” software.

**Statistical analysis**

Strength of association between different groups and alleles or genotypes of PECAM-1 polymorphisms were estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either Chi-square or Fisher exact analysis using the STATA (v8) software. The significance of differences for level of expression was established by one-way analysis of variance followed by Dunnett post hoc multiple comparison tests using SPSS software version 11.5. The significance level was set at p < 0.05.

**Results**

In SSCP-PCR analysis, the promoter region of PECAM-1 gene was screened in 40 healthy controls and while the banding pattern of one fragment of promoter suggested a likely base substitution, the subsequent DNA sequencing revealed a T to C substitution polymorphism in that fragment, which was sited at position –265* and the substitution was T to C (Figure 2). It was directly submitted to Genebank with accession number AJ313330.

To examine whether that polymorphism correlates with the level of PECAM-1 cell surface expression, the level of expressed PECAM-1 was studied in PBMCs by flow cytometry. The collected PBMCs from peripheral blood were tested in two conditions, with no treatment (fresh WBC), and also after stimulation with phytohemagglutinin (PHA) (4µg/ml) and lipopolysaccharide (LPS) (2µg/ml). There was a remarkably decrease in PECAM-1 expression after stimulation. The maximum expression was evident in fresh samples without any treatment (Figure 3). To complete the quantitative analysis of PECAM-1 expression by flow cytometry in addition to
the amount of expression, the fluorescence intensity of PECAM-1 expression, which represents its density on the cell surface membrane, was also studied. The distribution of PECAM-1 expression and intensity among the healthy controls are depicted in Figure 4. To assess if there is any correlation between PECAM-1 molecule expression or fluorescence intensity with the allele/genotype variants at position –265*C/T the control population were categorised according to their genotypes, but there was no association between that polymorphism and PECAM-1 expression or fluorescence intensity levels (Table 2). With regard to another polymorphism of the PECAM-1 gene (previously reported, at codon 125*G/C) it was correlated with the level of PECAM-1 expression, but not with the fluorescence intensity (Table 3). The frequency of PECAM-1 gene polymorphism at codon 125*G/C and at position –265*C/T also was studied in healthy controls and diabetic individuals. The distribution of genotypes in both positions was in Hardy-Weinberg equilibrium in cases and controls and there was no gender or age-dependent difference in distribution of attributed polymorphic genotype/alleles. There was no association between the polymorphisms and diabetes or its chronic complications (Table 4).
A. Single-stranded conformational polymorphism (SSCP) analysis of 5’ flanking region of PECAM-1 gene Silver stained polyacrylamide gel revealed that the amplified fragment 4 in different individuals (different lanes) has created bands (the DNA product) with different migration pattern, which could be explained by homo- (single band) or heterozygosy (double band) of mutated allele. Sequencing later confirmed the presence of a polymorphism in that fragment. The genotype of each individual is depicted according to subsequently developed ARMS-PCR.

B. Sequencing of the DNA fragment, which contains the novel polymorphism at position –265*T/C Sequencing confirmed the observed disparities in the pattern of DNA bands in SSCP was due to substitution of nucleotides. a (TT), b (CC), c (CT) (Genebank accession no. AJ313330).
Figure 3. Analysis of PECAM-1 expression in un-stimulated PBMCs from healthy controls by flow cytometry

The expressed PECAM-1 molecules are stained by mouse anti-human PECAM-1 monoclonal antibody (c, d) while mouse IgG1 isotype monoclonal antibody was used as negative control (a, b). a,c) Gated cells (red) were targeted for analysis of PECAM-1 expression, b,d) Lower left quadrant represents isotype control and lower right represents PECAM-1 expression.
Figure 4. The distribution of PECAM-1 expression and intensity among the healthy controls.

A. The number of PECAM-1 positive cells in non-stimulated PBMCs from 20 healthy controls. The expression of PECAM-1 was assessed on non-stimulated PBMC’Ss cell surface via flow cytometry. The mean number of PECAM-1 positive cells is depicted (52.30 ± 9.46) (Mean± SD), (Min. 39.90, Max 67.93).

B. PECAM-1 fluorescence intensity in non-stimulated PBMCs from 20 healthy controls. The fluorescence intensity of PECAM-1 was assessed on non-stimulated PBMC’S cell surface via flow cytometry. The mean level of PECAM-1 fluorescence intensity is depicted (68.6 ± 6.37) (Mean ± SD), (Min. 57.53, Max. 77.74).
Table 2. Analysis of the PECAM-1 expression by flow cytometry in relation to PECAM-1 gene –265*C/T polymorphism.

A. The expression of PECAM-1 molecule in non-stimulated PBMCs from 20 healthy controls relative to –265*C/T polymorphism in PECAM-1 gene promoter was assessed. The mean value (and standard error) of PECAM-1 expression is depicted with reference to different genotypes. There was no significant association among (P = 0.52) or between any of these genotypes with regard to PECAM-1 expression level (CC vs. TT, P = 0.79; CC vs. CT, p = 0.29; CT vs. TT, p = 0.44).

B. The expression of PECAM-1 molecule in non-stimulated PBMCs from 20 healthy controls relative to -265*T/C polymorphism in PECAM-1 gene promoter was assessed by their fluorescent intensity. The geometrical mean value (and standard error) of fluorescence band intensity of expressed PECAM-1 is depicted with reference to different genotypes. There was no significant association among (p = 0.29) or between any of these genotypes with regard to the fluorescence intensity of expressed PECAM-1 molecule (CC vs. TT, P= 0.15; CC vs. CT, p = 0.94; CT vs. TT, p = 0.28).
Table 3. A. Analysis of the PECAM-1 expression by flow cytometry in relation to PECAM-1 gene polymorphism at codon 125*G/C. The expression of PECAM-1 molecule in non-stimulated PBMCs from 20 healthy controls relative to 125*G/C polymorphism in PECAM-1 gene exon was assessed. The mean value (and standard error) of PECAM-1 expression is depicted with reference to different genotypes. There was a significant difference among these three genotypes in terms of the PECAM-1 expressed level ($p = 0.025$, $\chi^2 = 7.35$). In further analysis, the highest difference was evident between GG and CC ($p = 0.006$). The proportionate difference was present between GC and CC ($p = 0.042$), while the difference between GG and GC was not significant ($p = 0.67$).

B. Analysis of the fluorescence intensity of PECAM-1 expression by flow cytometry in relation to PECAM-1 gene polymorphism at codon 125*G/C. The expression of PECAM-1 molecule in non-stimulated PBMCs from 20 healthy controls relative to 125*G/C polymorphism in PECAM-1 gene exon was assessed by their fluorescent intensity. The geometrical mean value (and standard error) of fluorescence band intensity of expressed PECAM-1 is depicted with reference to different genotypes. There was no significant association among ($p = 0.97$) or between any of these genotypes with regard to the fluorescence intensity of expressed PECAM-1 molecule (GG vs. CC, $p = 0.89$; GG vs. GC, $p = 0.95$; GC vs. CC, $p = 0.78$).
Table 4. Distribution of genotype and allele frequencies of the PECAM-1 –265*C/T, codon 125*G/C polymorphism in healthy controls (C), T1DM subjects (P), diabetic retinopaths (DR), nephropaths (DN), neuropaths (DNU) and complication free (CF) group

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<th>-265*</th>
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<th>P</th>
<th>DR</th>
<th>DN</th>
<th>DNU</th>
<th>CF</th>
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<td>CT</td>
<td>41 (47.7)</td>
<td>132 (52.6)</td>
<td>70 (51.9)</td>
<td>43 (49.4)</td>
<td>43 (53.0)</td>
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<td></td>
<td>TT</td>
<td>21 (24.4)</td>
<td>54 (21.5)</td>
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<td>19 (21.9)</td>
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<td>262 (52.2)</td>
<td>138 (51.1)</td>
<td>93 (53.5)</td>
<td>85 (52.5)</td>
<td>104 (52.5)</td>
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<td>240 (47.8)</td>
<td>132 (48.9)</td>
<td>81 (46.5)</td>
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<td>133 (49.3)</td>
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<td>88 (51.8)</td>
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*No significant difference was evident.

Discussion

In this study the 5’ flanking region of PECAM-1 gene (containing promoter and 5’ UTR regions) was analysed by SSCP over a span of 1460 nucleotides upstream of the transcriptional start site. The polymorphism at codon +125*G/C, a missense mutation Leu (C) → Val (G) (21) which is located in Ig-domain 1 and resides in the homophilic interaction zone, also was considered with the novel polymorphism (-265*C/T) to find more information about the impact of PECAM-1 gene variation on its own expressed gene product. A given gene variation actually may create a phenotypic variation or even a new manifestation. In order to answer this question a functional study was performed. In this study only PECAM-1 gene polymorphisms at codon +125*G/C showed significant correlation with the level of PECAM-1 molecule expression. However our study using flow cytometry analysis could not rule out the absolute functionality of the –265*C/T polymorphism, and moreover even in the case of non-functionality, a polymorphic allele in a candidate gene can be used as a marker to explore and dissect the genetic constitution of a phenotype due to its feasible linkage disequilibrium with the functional and relevant allele(s) in close vicinity.

Stimulation of the lymphocytes in the culture revealed that PECAM-1 as a constitutively expressed molecule is expressed at the highest level in the non-activated state, which is compatible with the data of Stewart et al. (22). The idea behind applying stimulation was to determine if there is any correlation between the level of PECAM-1 down-regulation and its allelic construction, given that the higher suppression in PECAM-1 expression level might facilitate involvement of a pro-inflammatory/pathological phenomenon. There was a considerable down-regulation in PECAM-1 expression, on average 13%, 22% and 38% reduction was evident at 18 hrs, 24 hrs and 48 hrs after stimulation, respectively; but there was no correlation between the rate of post-stimulation reduction and PECAM-1 gene polymorphisms.

Genotyping was performed on type 1 diabetes patients and normal controls using two polymorphisms within PECAM-1 gene. Both polymorphisms had a high frequency of both wild/mutant alleles, which was an advantageous point to utilize them in studying common traits like diabetes and its complications. The high frequency of alleles may also imply that they have limited functional importance because differences in PECAM-1 biology are tolerated.
There was no significant association between any of those polymorphisms and T1DM. In next step, to define whether these polymorphisms have any role in the development or otherwise of diabetic complications, the diabetic subjects in different subgroups were studied according to these polymorphisms. There was no significant association between each of the late complications (DR, DN, DNU) and PECAM-1 gene polymorphisms. The complication free population group also did not show any association with these polymorphisms.

Our results showing a negative association at both loci with similar distribution in patients and controls groups/subgroups discount the role of examined PECAM-1 gene polymorphisms in development of T1DM and its chronic complications. However, our data can not mitigate the overall role of the PECAM-1 molecule polymorphisms. These data need to be repeated in different population and larger number of samples to be confirmed.

Acknowledgment
This work was supported in part by a fellowship grant from Iranian ministry of health and medical education

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