Vascular Endothelial Growth Factor Production is Regulated by Gene Polymorphisms

Mojgan Mohammadi[1,3*], Mohammad Reza Bazrafshani[2,4], Philip.J Day[4], William. E.R. Ollier[4]

[1]Departments of Immunology, [2]Medical Genetics, Kerman University of Medical Sciences, Kerman, Iran. [3]Department of Immunology, The Faculty of Life Sciences, Michael Smith Building,[4]Centre for Integrated Genomic Medical Research (CIGMR), Stopford Building, Oxford Road, Manchester, M13 9PT, United Kingdom

ABSTRACT

Background: Vascular endothelial growth factor (VEGF) has a key role in angiogenesis and in transplantation. The level of VEGF is related to the differences in the DNA sequence of its promoter region. **Objectives:** In this study, the association between the combination of VEGF –1154 G and –2578 C alleles and VEGF production by LPS-stimulated PBMCs was investigated. In addition; the relationship between VEGF polymorphisms and the influence of TNF-α and IL-4 on VEGF production was studied. **Methods:** VEGF –1154 G/A and –2578 C/A were detected using ARMS-PCR. To determine the impact of combinations of these two polymorphisms on VEGF production; PBMCs were stimulated by LPS and VEGF production was measured by ELISA. **Results:** The combinations of –1154 GG/-2578 CC and –1154 GG/-2578 CA were significantly associated with higher VEGF production ($p<0.0001$). Production of VEGF was significantly influenced by TNF-α in individuals who had certain VEGF genotype combinations. Although VEGF production was dramatically suppressed by IL-4, it was not dependent on VEGF genotype. **Conclusions:** Since TNF-α has influence on the graft outcome, to avoid allocation of grafts from high TNF-α producer donors to recipients, it might be useful to predict and minimize graft rejection by having prior knowledge of TNF-α and also VEGF genotypes especially -1154 G/A and -2578 C/A VEGF.

Keywords: VEGF, TNF-α, IL-4

INTRODUCTION

VEGF is a powerful stimulator and regulator for endothelial cells and vascular permeability, and it has a key role in normal (wound healing) and abnormal (tumour growth) angiogenesis (1). VEGF is one of the most important angiogenic factors. A fundamental effect is attributed to VEGF in the regulation of pathological angiogenesis of ischemic

*Corresponding author: Dr. Mojgan Mohammadi, Department of Immunology, Kerman University of Medical Sciences, Kerman, Iran. Tel: (+) 98 341 3224617, Fax: (+) 98 341 3224618, e-mail: mo_mohammadi@kmu.ac.ir

retinal and tumour disease (2). VEGF can also be detected in the synovial pannus which develops in rheumatoid arthritis (3) and in keratinocytes during the process of wound healing (4). VEGF levels are elevated in many human diseases which are characterised by inflammation, such as in cerebral ischemia (5), early diabetic retinopathy (6) and inflammatory bowel disease (7). In addition, the rejection of kidney (8), heart (9) and lung (10) transplants, is related to high levels of VEGF. Expression and permeability of endothelial cell adhesion molecules are up-regulated by VEGF. This is due to VEGF's ability to act as a pro-inflammatory cytokine and monocyte chemoattractant. VEGF is stimulated mainly by hypoxia. However it can be up-regulated by other stimulatory factors such as protein kinase C, nitric oxide, lipopolysaccharide (LPS), local glucose concentrations, hormones, cytokines and prostaglandins (11,12). Results of a recent study showed that up-regulation of VEGF mediated via LPS are carried out through the activation of SP1 which is a transcription factor that binds within the VEGF promoter region in human monocytic cells (13). Additionally, AP-1 is another transcription factor involved in VEGF expression (14). Several polymorphisms have been described within the VEGF promoter (15) and 5′UTR regions (16), which regulate VEGF expression at a post-transcriptional level. Polymorphisms at positions -1154 and -2578 of the VEGF gene have been shown to correlate with both increased expression of VEGF and risk of renal allograft rejection (17). Based on these observations our aim was to investigate functional associations between the production of VEGF in LPS-treated PBMCs and combinations of -1154 G/A and -2578 C/A VEGF genotypes. Vascular permeability and the infiltration of pro-inflammatory granulocytes into inflammatory sites are promoted by the proinflamatory cytokine, TNF-α (18). Transplant rejection is correlated with high levels of TNF-α expression (19). Furthermore, it is known that stimulation of endothelial cells and macrophages through engagement of cell surface receptors including CD40 ligation, and exposure to inflammatory cytokines and prostaglandins, causes increased production of VEGF (20). We therefore also attempted to investigate the influence of TNF-α on VEGF production and the possible interaction between TNF-α-induced VEGF production and the combinations of -1154 G/A and -2578 C/A VEGF genotypes. On the other hand, results from studies on IL-4 gene polymorphisms and transplant outcome reveal that transplanted hearts from donors with the IL-4 -590 T allele as high producers of IL-4 are rejected significantly less than those from donors without this allele (21). As IL-4 is also an important regulatory cytokine known to suppress production of VEGF (22) and TNF-α (23) and is associated with long-term allograft survival (24), we also investigated whether VEGF production can be regulated by IL-4.

MATERIALS AND METHODS

Informed consent was obtained from all human adult volunteers who donated their blood at the national blood centre in Manchester.

Subjects. Ten ml of fresh blood samples from healthy volunteers were taken into Vacutainer EDTA tubes (Becton Dickinson, UK). Eight ml of these samples were immediately processed for cell culture and 2 ml were used for DNA extraction and subsequent genotyping analysis. All 384 controls used were UK Caucasoid, aged between 19 and 65 (mean 42 ± 15 years) and the gender split was approximately equal.

Identification of VEGF Genotype. Transitions of G to A at position -1154 and C to A at nucleotide position -2578 were characterised in the promoter of VEGF by ARMS-PCR (15). Briefly, DNA extracted from controls was amplified in a 10 μl reaction mixture containing 1 x Custom PCR master mix (ABgene, Epsom, UK), 0.5 μM specific primer mix containing 10 μM of a generic primer and 10 μM of one of the two allele-specific primers, 1 μM of human growth hormone (hGh, internal control primer mix) and 100-200 ng of DNA. A PCR product size of 429 bp from the hGh gene was amplified by the internal control primers. Amplifications were performed using annealing temperatures of 65°C (10 cycles) and 59°C (20 cycles). Amplified products were resolved in 2% agarose gels.

Measurement of VEGF in the Supernatant of PBMCs. Freshly isolated PBMCs from healthy Caucasian volunteers were resuspended at 2 x 10^6/ml in RPMI 1640 culture medium (Gibco, UK) supplemented with 10% foetal calf serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.05% 2-Mercaptoethanol (Gibco, UK) and 1% penicillin-streptomycin solution (Sigma, UK). One ml cell suspension containing 2 x 10^6 cells was cultured in the presence and absence of LPS (2 μg/ml) and incubated at 37°C in 5% CO_2. Additionally, culture medium was also used as a negative control. Released VEGF was measured in a time-dependent manner using the Duoset ELISA Kit (R&D systems, Oxon, UK) and following the kit protocol. The optical density of each well was immediately determined using a microplate reader (Dynex Biotechnologies, USA) set to 450 nm with a wavelength correction at 570 nm.

Genetic Variation in the VEGF Gene Promoter. ARMS-PCR was used on extracted genomic DNA from whole blood of 384 healthy UK Caucasians volunteers to genotype VEGF polymorphisms at positions -1154 and -2578. Hardy-Weinberg equilibrium was used to predict the distribution of genotypes (expected) from allele frequencies (observed). The allelic and composite genotype frequencies are summarised in Table 1.

Table 1. Frequency of VEGF polymorphisms in healthy Caucasoid volunteers

		Observed		Expected	
		N=384	%	N=384	%
VEGF -1154					
Genotype	G/G	200	52.1[1]	204	53.1
	G/A	160	41.7	152	39.6
	A/A	24	6.3	28	7.3
Allele	G	560	72.9	560	72.9
	A	208	27.1	208	27.1
VEGF -2578					
Genotype	C/C	131	34.1[2]	122	31.8
	C/A	171	44.5	189	49.2
	A/A	82	21.4	73	19.0
Allele	C	433	56.4	433	56.4
	A	335	43.6	335	43.6

[1] χ^2 =1.17, p=NS
[2] χ^2 =2.35, p=NS

Our genotyping results show that they were in Hardy-Weinberg equilibrium. Linkage disequelibrium between SNPs were estimated using the EH program. The likelihood estimates of disequilibrium (D' or normalised measure of Lewontin) between two alleles were calculated as follows: $D' = D / D_{max.}$ The levels of significance for D' were estimated via their related p values based on definition of 0.0≤Weak D'≤ 0.4,

0.4≤Moderate D'≤ 0.6 and 0.6≤Strong D'≤ 1.0. Based on this analysis, moderate linkage disequilibrium between -1154 G and -2578 C was observed. In addition, T test was used for calculation of the P-Value for comparisons between the individuals with different genotypes regarding the VEGF production.

Detection of VEGF Production in Presence of Cytokines. To determine the optimal concentration of cytokines for regulation of VEGF secretion, stimulated PBMCs (2 x 10^6/ml) with 2 μg/ml LPS were incubated with a range of TNF-α pro-inflammatory cytokine concentrations of 0.005, 0.01, 0.02, and 0.05 μg/ml. Similarly, treatment with IL-4 was performed using two different concentrations (0.01 and 0.1 μg/ml, respectively). LPS-stimulated PBMCs (2 x 10^6/ml) isolated from controls possessing a combination of the two VEGF gene polymorphisms containing -1154 G/A and -2578 C/A were incubated with optimal concentrations of cytokines in order to investigate the association of VEGF genotype combination with the regulation of VEGF secretion by pro and anti-inflammatory cytokines.

RESULTS

Time Course Study of Various Stimulators for VEGF Production by PBMCs. PBMC cultures of 10 healthy control subjects were performed using various stimulators including LPS, phytohemagglutinin (PHA), phorbol myristate acetate (PMA) and ionomycin, while non-stimulated PBMCs were considered as reference controls. Production of VEGF was measured in culture supernatants from day one to day four by ELISA. Results of this experiment showed that VEGF production by LPS-stimulated PBMCs was significantly ($p<0.0001$) higher than the other stimulators, at all times. The highest level of VEGF production was observed with 2.0 μg/ml of LPS at day four with the mean production level ± SEM of 1620.0 ± 152.4 pg/ml.

Dose Response of TNF-α for Increasing VEGF Production. The effect of four different concentrations of human recombinant TNF-α (0.005, 0.01, 0.02 and 0.05 μg/ml) on VEGF production was examined in LPS (2.0 μg/ml) stimulated-PBMCs from ten healthy controls. Treated cells with and without LPS were used as controls in these experiments. Although a significant difference was not detected among three different concentrations of TNF-α in stimulating VEGF production in the LPS-stimulated PBMCs, 0.005 μg/ml TNF-α was chosen for evaluating its effect on more individuals in relation to their VEGF genotype. More VEGF was significantly produced in the treated PBMCs with TNF-α alone, in comparison to non-stimulated cells, but it was not as high as the level seen in the supernatant of cells treated with both LPS and TNF-α (data not shown).

Dose Response of IL-4 for Decreasing VEGF Production. The effect of two different concentrations of human recombinant IL-4 (0.01 and 0.1 μg/ml) on decreased production of VEGF by LPS (2.0 μg/ml) stimulated PBMCs from 10 healthy individuals was studied in a time course experiment. Non-stimulated cells were considered as controls for this experiment. VEGF levels in the supernatants of LPS stimulated PBMCs decreased significantly ($p<0.0001$) after treating with both concentrations of IL-4 at day four. IL-4 at a concentration of 0.01 μg/ml was chosen for its decreasing effect on VEGF production in LPS-stimulated PBMCs. VEGF was detected in IL-4 (0.01 μg/ml)

and LPS (2.0 μg/ml) stimulated PBMCs with the mean production level ± SEM of 531.0 ± 18.4 pg/ml at day four. Significantly lower VEGF was produced by PBMCs treated with IL-4 alone, in comparison to LPS-stimulated cells (data not shown).

Regulation of VEGF Production by VEGF Genotype. VEGF production level by LPS-stimulated PBMCs from healthy controls was studied in relation to their genotype. Significant differences for VEGF production were revealed for various genotypes (Figure 1). The VEGF level after LPS treatment was calculated as the mean production level ± SEM for -1154 G and -2578 C VEGF genotypes. Individuals who had the GG -1154/CA -2578 (1231.0 ± 74.7 pg/ml) and GG -1154/CC -2578 (1159.4 ± 57.0) genotype were the highest VEGF producers. In contrast, individuals with the AA -1154/AA -2578 (177.8 ± 70.7 pg/ml), GA -1154/AA -2578 (248.5 ± 29.6 pg/ml) and AA -1154/CA -2578 (233.9 ± 56.1 pg/ml) genotypes displayed the lowest level of VEGF production. Moreover, VEGF was observed at an intermediate level for GG -1154/AA -2578 (605.9 ± 52.2 pg/ml), GA -1154/CC -2578 (607.3 ± 68.0 pg/ml) and GA -1154/CA -2578 (530.5 ± 56.8 pg/ml) genotypes. Statistical analysis of VEGF production was performed. Individuals with GG -1154/AA -2578, GA -1154/CC -2578 and GA -1154/CA -2578 genotypes were shown to be intermediate producers of VEGF ($p<0.0001$) in comparison with GG -1154/CC -2578 and GG -1154/CA -2578 genotypes which were high producers. Additionally, p values were calculated for VEGF production between intermediate and low producers of VEGF. The results obtained for VEGF production were as follows: GG -1154/AA -2578>GA -1154/AA -2578 ($p<0.0001$), GG -1154/AA -2578>AA -1154/CA -2578 ($p=0.0038$), GG -1154/AA -2578>AA -1154/AA -2578 ($p=0.0017$), GA -1154/CC -2578>GA -1154/AA -2578 ($p=0.0001$), GA -1154/CC -2578>AA -1154/CA -2578 ($p=0.0157$), GA -1154/CC -2578>AA -1154/AA -2578 ($p=0.0079$), GA -1154/CA -2578>GA -1154/AA -2578 ($p=0.0003$), GA -1154/CA -2578>AA -1154/CA -2578 ($p=0.0079$) and GA -1154/CA -2578>AA -1154/AA -2578 ($p=0.0094$). No difference was observed for VEGF production between the individuals who had GG -1154/CC -2578 and GG -1154/CA -2578 VEGF genotypes who were both high producers of VEGF. Similarly no significant difference was observed among the low producers of VEGF who had GA -1154/AA -2578, AA -1154/CA -2578 or AA -1154/AA -2578 VEGF genotypes. Furthermore, no differences were seen for VEGF production among the individuals who showed intermediate levels of VEGF production (Figure 1).

Regulatory effect of TNF-α on VEGF Production by VEGF Genotype Combinations. The effect of TNF-α on VEGF production was studied in LPS-stimulated PBMCs of healthy controls in relation to their VEGF genotypes. VEGF level was only increased significantly after treating with TNF-α in LPS-stimulated PBMCs in individuals with certain VEGF genotypes (Figure 2). No significant difference was observed for individuals with AA -1154/CA -2578, GA -1154/CC -2578 and AA -1154/AA -2578 genotypes in relation to the increased level of VEGF production stimulated by TNF-α in comparison with LPS-induced VEGF production. In contrast, significant differences were seen for individuals with genotypes GG -1154/CC -2578 ($p=0.0026$), GG -1154/CA -2578 ($p=0.0281$), GG -1154/AA -2578 ($p=0.03$), GA -1154/CA -2578 ($p=0.0393$) and GA -1154/AA -2578 ($p=0.0034$).

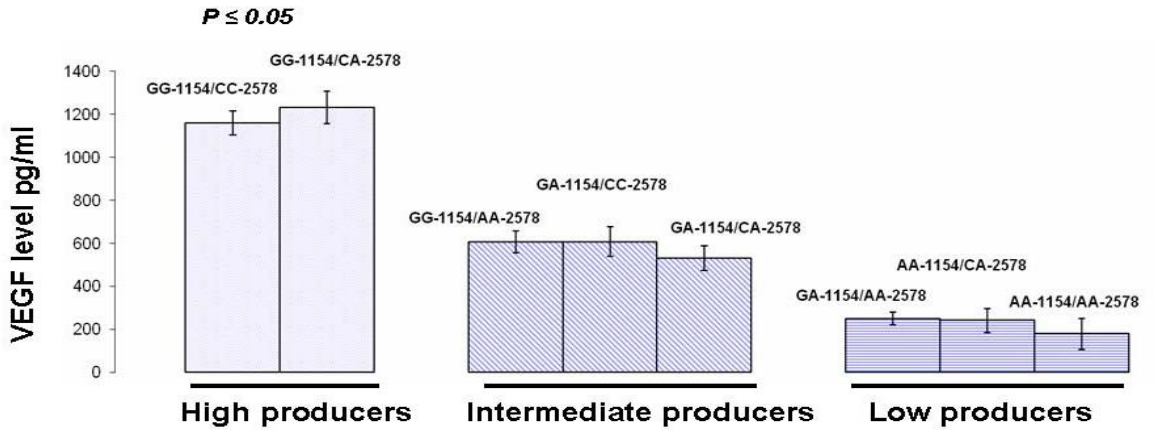

Figure 1. Regulatory effect of VEGF -1154 G/A and -2578 C/A genotype combination on VEGF production in LPS-stimulated PBMCs. Expression of VEGF in completed medium was determined by sandwich ELISA and compared with non-stimulated PBMCs. Each bar is the mean ± SEM of LPS-stimulated PBMCs from 10 determinations except the genotype combination of AA-1154/CA-2578 and AA-1154/AA-2578 which were averages of 3 determinations because of low frequency of these genotypes. High producers of VEGF are shown to express significantly more VEGF level ($p \leq 0.05$) than the intermediate and low producers. For p value calculation, t test was used.

Regulatory effect of IL-4 on VEGF Production by VEGF Genotype Combinations. The influence of IL-4 on VEGF production was investigated in LPS-stimulated PBMCs of healthy controls with VEGF -1154 G/A and -2578 C/A genotypes. VEGF level decreased significantly in LPS stimulated PBMCs following IL-4 treatment in comparison to LPS-stimulated PBMCs. However this was not dependent on combinations of VEGF -1154 G and -2578 C genotypes (Figure 3). VEGF production was significantly decreased ($p < 0.0001$) following Il-4 treatment of LPS-stimulated PBMCs in individuals who had VEGF GG -1154/CC -2578, GG -1154/CA -2578, GG -1154/AA -2578, GA -1154/CC -2578, GA -1154/CA -2578, or GA -1154/AA -2578 genotypes. Additionally, significant effects were observed ($p < 0.0001$) for individuals with the VEGF GA -1154/AA -2578 genotype receiving the same treatment. Furthermore, VEGF production was reduced significantly for individuals with the VEGF AA -1154/CA -2578 ($p=0.0475$) and AA -1154/AA -2578 genotypes ($p=0.0498$).

DISCUSSION

The human VEGF promoter region is highly polymorphic, and linkage disequilibrium is seen between VEGF -1154 G and -2578 C alleles. VEGF production is also related to both transplant rejection and disease (17), (25-29). We found that VEGF production was significantly up-regulated by LPS in comparison to non-stimulated cells and other mitogens. LPS induces many genes that encode inflammatory mediators such as TNF-α, IL-1 and IL-6 via stimulation of monocytes and macrophages. Results of a recent study showed that SP1 in the VEGF promoter is activated after stimulation of monocytic cells by LPS causing up-regulation of VEGF (13).

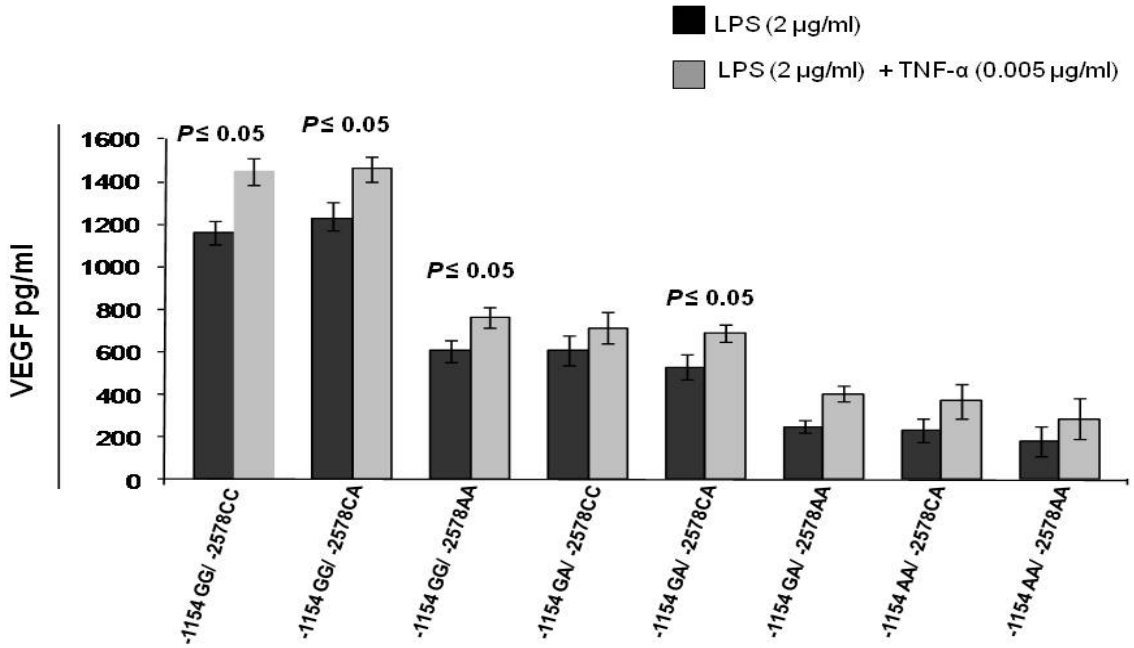

Figure 2 Regulatory effect of TNF-α on VEGF production in LPS-stimulated PBMCs in association with VEGF -1154 G/A and -2578 C/A genotype combinations. Expression of VEGF in completed medium was determined by sandwich ELISA and compared with non-stimulated PBMCs. Black bars are the mean ± SEM of LPS-stimulated PBMCs and grey bars are the mean ± SEM of TNF-α and LPS-stimulated PBMCs from 10 determinations except for genotype combinations of AA-1154/CA-2578 and AA-1154/AA-2578 which were representative of 3 determinations because of low frequency of these genotypes. $P \leq 0.05$ shows significant regulatory effect of TNF-α on VEGF production in LPS-stimulated PBMCs in individuals who carry certain VEGF genotype combinations. For *p* value calculation, t test was used.

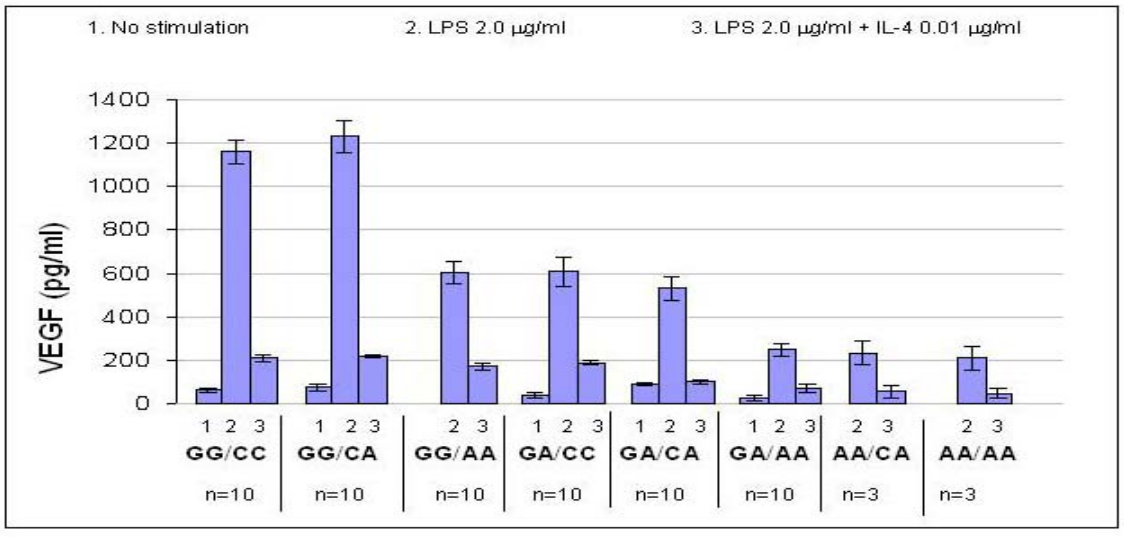

Figure 3. Regulatory effect of IL-4 on VEGF production in LPS-stimulated PBMCs in association with VEGF -1154 G/A and -2578 C/A genotype combinations. Expression of VEGF in completed medium was determined by sandwich ELISA and compared with non-stimulated PBMCs. Each bar is the mean ± SEM of 10 determinations except for the genotype combination of AA-1154/CA-2578 and AA-1154/AA-2578 which were representatives of 3 determinations because of the low frequency of these genotypes.

Additionally, VEGF expression is influenced by activation of AP-1, another transcription factor in VEGF promoter (14) and might be associated with up-regulation of VEGF in LPS-stimulated PBMCs in our experiment. In this work, we defined a functional association between LPS-induced VEGF from PBMCs and a combination of VEGF -1154 G/A and -2578 C/A genotypes. Our results may implicate a predominant influence of individuals with a combination of VEGF GG -1154/CA -2578 and GG -1154/CC -2578 genotypes in producing the highest amounts of VEGF. In contrast, individuals with the AA -1154/AA -2578, GA -1154/AA -2578 and AA -1154/CA -2578 genotype combinations displayed the lowest level of VEGF production. Moreover, VEGF was observed in an intermediate level in individuals with genotype combinations of GG -1154/AA -2578, GA -1154/CC -2578 and GA -1154/CA -2578. Shahbazi and co-workers (17) showed that PBMCs from -1154 GG homozygous individuals can produce significantly more VEGF than cells from -1154 AA homozygotes. Similarly, -2578 CC homozygotes can produce significantly higher amounts of VEGF than -2578 AA. Comparing genotype combinations of high and intermediate VEGF producers with low producers in our results may implicate a predominant influence of -1154 G and -2578 C alleles for producing higher levels of VEGF which is in agreement with the work of Shahbazi et al (17). Employing this knowledge and the significant associations in our results between VEGF production with regard to the combination of two VEGF polymorphic sites at -1154 G and -2578 C, we hypothesised that the VEGF genotypes might be useful indicators for prediction of transplant rejection in heart or other organ transplant recipients. The mechanisms of the influence of these polymorphisms upon VEGF production are unknown. These two polymorphisms may be functional by themselves or may be in linkage with other polymorphisms somewhere else in the VEGF gene. There is a cluster of Sp1 transcription factor sites located 21bp downstream from the polymorphism at position -1154 G/A. An alteration in -1154 G/A may change the secondary structure of DNA and consequently create new binding sites for Sp1 transcription factors.

A significant association between transplant rejection and individuals with high TNF-α producers was previously reported by our laboratory (30). The results of our study showed that the VEGF level increased significantly after treating with TNF-α in LPS-stimulated PBMCs just in individuals who had certain VEGF genotype combinations. Up-regulation of VEGF by TNF-α in our results might be mediated by activating protein 1 (AP1) in the VEGF gene, which is one of the two most important transcription factors responsible for the effects of TNF-α for the initiation of inflammatory cytokine development as well as cell-adhesion molecules, growth factors, metalloproteinases and other proteins (31,32). Statistical analysis showed that VEGF was not elevated significantly by TNF-α treatment in LPS-stimulated PBMCs from individuals who had AA -1154/CA -2578, GA -1154/CC -2578 and AA -1154/AA -2578 genotype combinations. However significant p values were calculated for the same comparison for individuals with other combinations of VEGF -1154 G and -2578 C. Up-regulation of VEGF in individuals with the GG -1154/CC -2578 and GA -1154/AA -2578 genotype combinations seemed to be significantly more influenced by TNF-α than other genotypes. At present no single genotype has the power to predict acute rejection. Although in previously published studies from our laboratory, the association between acute rejection and the high TNF-α producer allele was reported (30,32), there are many high TNF-α producers who do not suffer acute rejection. Therefore, based on our results, we suggest that further studies of VEGF and TNF-α genotypes (high producers of TNF-α) might be

useful in predicting acute rejection. Avoidance of allocation of grafts from donors positive for high production of TNF-α to recipients with certain VEGF genotypes (e.g. GG -1154/CC -2578 and GA -1154/AA –2578) might be a good strategy for minimising the risk of rejection.

In addition to the importance of our finding about VEGF polymorphisms which may influence graft rejection, there are other results about association of certain VEGF polymorphisms and diseases. Comparison and discussion between these findings might be a good approach in better understanding the influence of VEGF genotypes on susceptibility or resistance to a disease.

VEGF is believed to be a major mediator of breast cancer angiogenesis. Three polymorphisms in the VEGF gene including -2578C/A, -1154G/A and +936C/T were examined in a recent study to elucidate the association of the haplotypes with breast cancer risk and the prognostic characteristics of the tumors. The results showed that the VEGF haplotype -2578/-634 CC was associated with more tumor aggressiveness. In addition, these results demonstrated evidence for the correlation of VEGF -2578AA genotype and VEGF haplotype -2578/-634AG with low-grade tumors (33). Comparison of these findings with our results may provide evidence that some genotypes and haplotypes in the VEGF gene may have an effect on VEGF production and the breast tumor growth.

Results of a very recent study have revealed that the frequency of the VEGF –1154 A/A and -2578A/A homozygote was significantly lower in patients with endometriosis in Chinese women compared with control women and both genotypes could significantly decrease the risk of endometriosis development (34). Comparing these results with our results indicate similarities with regard to VEGF -1154 AA and -2578 AA genotypes, and low production of VEGF in individuals who carry these genotypes might be valuable markers for prediction of susceptibility to endometriosis in Caucasian patients too.

As we mentioned in the introduction, IL-4 is an important regulatory cytokine which is known to suppress the production of VEGF (22) and TNF-α (23) and is associated with long-term allograft survival (24). Results of our experiments suggest that suppression of VEGF by IL-4 does not seem to be limited to certain combinations of VEGF polymorphisms at -1154 G and -2578 C. Therefore; IL-4 genotypes (high producers) may not be associated with the combinations of the two VEGF polymorphisms at -1154 G and -2578 C with regard to transplant rejection or control of inflammatory disease. However, VEGF is induced in tissues by hypoxia as a usual consequence of the transplantation procedure. A suppressive effect of IL-4 on hypoxia-induced VEGF production might be dependent on VEGF polymorphisms *in vivo* but not *in vitro* and in normoxic conditions. To answer this question, a valuable *in vivo* study may be useful in examining transplant recipients or patients with inflammatory disease such as inflammatory bowel disease, with high IL-4 producer genotype and high VEGF production with regard to combinations of VEGF –1154 G/A and –2578 C/A polymorphisms to evaluate the anti-inflammatory effects of IL-4 on certain VEGF genotypes.

ACKNOWLEDGMENTS

This research was financially supported by CIGMR (the Centre for Integrated Genomic Medical Research), University of Manchester with the grant number G20. The authors would like to thank staff members of the National Blood Centre in Manchester, who

helped us with the blood collection and Dr Houshang Rafatpanah who assisted with cell culture assay.

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