

The Influence of Immunosuppressive Drugs on Vascular Endothelial Growth Factor Production in Relation to VEGF - 1154 G and -2578 C Genotypes

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ABSTRACT

Background: The pathogenesis of many diseases is correlated to irregularity in vascular endothelial growth factor (VEGF) expression. Results from several association studies show that variation in the level of VEGF expression is related to polymorphic sequences within the VEGF gene. Additionally, there are many studies showing that some gene polymorphisms significantly influence the pharmacokinetics of immunosuppressive drugs. **Objective:** The aim of this study was to determine the influence of immunosuppressive drugs on VEGF production in individuals with different VEGF genotypes. **Methods:** ARMS-PCR was used to genotype VEGF polymorphisms at positions -1154 and -2578 within the promoter of VEGF gene. A VEGF-specific ELISA was used to determine the influence of immunosuppressive drugs on VEGF production in PBMCs of individuals with different VEGF genotypes.

Results: Suppressive effect of mycophenolic acid was observed just in individuals with GG -1154/CC -2578, GG -1154/CA -2578 and GA -1154/CC -2578 haplotypes. Additionally, VEGF was significantly suppressed in all individuals after treatment with rapamycin except those who had AA -1154/CA -2578 and AA -1154/AA -2578 VEGF genotype combinations. **Conclusion:** Results of a recent study revealed that MMF treatment might be effective in preventing chronic renal rejection only in recipients with IL-10 high producer genotype. Additionally result of another study showed that CYP3A5 genotype markedly influences the pharmacokinetics of rapamycin in kidney transplant recipients. Therefore with regard to our results, different suppressive effect of mycophenolic acid and rapamycin on VEGF production might also be dependent on VEGF genotype.

Key words: gene polymorphisms, immunosuppressive drugs, VEGF

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is a fundamental mediator of physiologic and pathophysiologic angiogenesis. The pathogenesis of many diseases is correlated to the irregularities in VEGF expression (1, 2). Additionally, results from several association studies have shown that variation in the level of VEGF expression is related to the polymorphic sequences within the promoter or 5'-untranslated region (5'-UTR) of VEGF. In one of these studies, a significant association was reported between VEGF expression from LPS stimulated PBMCs and +405 G/C genotype within VEGF gene (3). Results of another study showed that renal graft recipients who were producers of higher levels of VEGF demonstrated significant correlation with acute rejection. These findings identified that a 6.8 fold higher risk of kidney transplant rejection existed among graft recipients with -1154 G/G, compared to individuals with -1154 A/A genotype. Similar results were reported for individuals with -2578 C/C and C/A compared with the A/A genotype (4). In a recent study we have found that the combinations of the above polymorphisms namely -1154 GG/-2578 CC and -1154 GG/-2578 CA, were significantly associated with higher VEGF production ($P < 0.0001$) and VEGF was significantly influenced by TNF- α in individuals possessing certain VEGF genotype combinations (5).

The differences in cell surface protein expression of transplanted organs versus those of the recipients necessitate the lifelong use of immunosuppressive medications. Thus, organ transplantation should be viewed as a treatment rather than a cure. If immunosuppressive medications are discontinued for any significant length of time, rejection of the transplanted organ occurs in most of the patients. Transplantation was revolutionized by the short polypeptide cyclosporine A, which blocks the activation of lymphocytes and other immune system cells, decreasing morbidity and enabling routine organ transplantation. Cyclosporine has been approved for use in organ transplantation to prevent graft rejection in the kidney, liver, heart, lung and combined heart-lung transplants. It is used to prevent rejection following bone marrow transplantation and in the prophylaxis of host-versus-graft disease (6). Mycophenolic acid (MPA) is another immunosuppressive drug that was initially derived from cultures of penicillium fungal spp. by Gosio in 1896 (7). Inosine monophosphate dehydrogenase (IMPDH), which is a key enzyme in the *de novo* pathway of purine synthesis in B and T lymphocytes, is inhibited selectively and reversibly by MPA. Guanine nucleotides are depleted following inhibition of IMPDH (8).

Rapamycin (sirolimus), a microbial product isolated from the actinomycete *Streptomyces hygroscopicus*, was discovered initially as an antifungal agent in the mid-1970s. Rapamycin received approval from FDA for marketing as an immunosuppressive drug for preventing renal graft rejection in 1999 (9) and also for preventing the rejection of several other types of grafts such as heart (10) and liver (11).

Additionally, allograft rejection is prevented by systemic glucocorticoid administration in combination with other immunosuppressive drugs. Results of several *in vitro* studies on suppressive effects of methylprednisolone, prednisolone and cyclosporine demonstrated that suppression of mitogen-stimulated lymphocyte proliferation was reported to be dramatically greater by methylprednisolone than prednisolone (12, 13).

Based on these observations, our aim was to investigate the influence of four immunosuppressive drugs, including cyclosporine A, rapamycin, mycophenolic acid and methylprednisolone on VEGF production in individuals with combinations of -1154 G/A and -2578 C/A VEGF genotypes.

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMCs) isolation/stimulation/suppression and culture for determination of VEGF production and ELISA. Informed consent was obtained from all volunteers who donated their blood at the National Blood Transfusion Centre in Manchester.

Our preliminary work on stimulating PBMCs to produce VEGF using LPS and choosing the subjects and identifying of VEGF genotypes, are available in a recent publication (Mohammadi, et al, 2009). Briefly, 384 healthy UK Caucasoids aged 19 -65 (mean 42 ± 15 years) with approximately equal gender distribution participated as controls in this study. ARMS-PCR was used on genomic DNA from their whole blood to genotype VEGF polymorphisms at positions -1154 and -2578. A VEGF-specific ELISA was used to determine VEGF protein level in supernatants of PBMCs from healthy volunteers using the DuoSet ELISA Kit (R&D systems, Oxon, UK) in accordance to the manufacturer's instructions.

To investigate optimal dose for the suppression of VEGF production, LPS ($2.0 \mu\text{g/ml}$) stimulated peripheral blood mononuclear cells (2×10^6 /ml) from ten healthy controls were cultured per well in the presence and absence of different concentrations of cyclosporine A, mycophenolic acid, rapamycin and methylprednisolone (2.0, 1.0 and $0.5 \mu\text{g/ml}$), while non-stimulated PBMCs were included as controls. The VEGF protein level was measured from day one to day four using an ELISA Kit (R&D systems, Oxon, UK). The optical density of each well was immediately determined using a micro plate reader (Dynex Biotechnologies, USA) set to 450 nm with a wavelength correction at 570 nm.

RESULTS

1. Regulation of VEGF production by VEGF promoter region gene polymorphisms and the influence of immunosuppressive drugs.

The effect of immunosuppressive drugs such as cyclosporine A, mycophenolic acid, rapamycin and methylprednisolone on VEGF production was studied in individuals with different VEGF promoter genotypes by a number of *in vitro* trials. A series of time-dose response experiments established the optimum concentration for the suppression of VEGF production by immunosuppressive drugs. A VEGF-specific ELISA was used to determine VEGF protein level in supernatants of PBMCs from healthy volunteers.

2. Dose response experiments for cyclosporine A, mycophenolic acid, rapamycin and methylprednisolone for the suppression of VEGF production by PBMCs. To investigate the optimal dose for suppression of VEGF production, LPS ($2.0 \mu\text{g/ml}$) stimulated peripheral blood mononuclear cells (2×10^6 /ml) from 10 healthy controls

were cultured per well in the presence and absence of different concentrations of cyclosporine A, mycophenolic acid, rapamycin and methylprednisolone (2.0, 1.0 and 0.5 µg/ml), and non-stimulated PBMCs were included as controls. The VEGF protein level was measured from day one to day four by ELISA (Table 1).

Table 1: VEGF production (pg/ml) using various concentrations of cyclosporine A or mycophenolic acid

Treatment	Hours		
	48	72	96
Non-stimulated	46.1 ± 11.4	77.0 ± 11.3	38.0 ± 5.7
LPS (2.0 µg/ml)	331.2 ± 55.5	427.5 ± 30.0	1602.7 ± 62.1
LPS (2.0 µg/ml) + cyclosporine A (2.0 µg/ml)	158.3 ± 37.5	332.4 ± 39.2	1591.4 ± 61.8
LPS (2.0 µg/ml) + cyclosporine A (1.0 µg/ml)	224.6 ± 47.2	351.5 ± 41.3	1573.9 ± 58.0
LPS (2.0 µg/ml) + cyclosporine A (0.5 µg/ml)	260.1 ± 52.2	406.0 ± 30.3	1565.3 ± 54.9
LPS (2.0 µg/ml) + mycophenolic acid (2.0 µg/ml)	205.8 ± 43.7	402.7 ± 31.3	1578.7 ± 62.6
LPS (2.0 µg/ml) + mycophenolic acid (1.0 µg/ml)	216.5 ± 43.4	419.6 ± 30.8	1576.2 ± 63.5
LPS (2.0 µg/ml) + mycophenolic acid (0.5 µg/ml)	228.6 ± 41.0	414.5 ± 32.5	1594.4 ± 61.7

Results of this study showed that VEGF was suppressed significantly ($p=0.0188$) 48 hours after treatment with 2.0 µg/ml cyclosporine A in comparison with the LPS-stimulated PBMCs of 10 control samples. No significant difference was observed for the suppressive effect of various concentrations of mycophenolic acid at different intervals. However, 2.0 µg/ml of mycophenolic acid was used for studying its suppressive effect on VEGF production in PBMCs among healthy individuals in relation to their VEGF genotypes. The greatest suppressive effect of rapamycin and methylprednisolone on VEGF production was found to be 2.0 µg/ml at day four. The lowest level of VEGF production was detected in the supernatant of PBMCs treated with LPS (2.0 µg/ml) in combination with rapamycin (2.0 µg/ml) at day four with the mean production level ± SEM of 451.0 ± 65.0 pg/ml. In addition, combination of LPS (2.0 µg/ml) and methylprednisolone (2.0 µg/ml) led to the lowest VEGF production at day four with the mean production level of 409.4 ± 77.3 pg/ml (Table 2).

Table 2: VEGF production (pg/ml) using various concentrations of rapamycin and methylprednisolone

Treatment	Hours		
	48	72	96
Non-stimulated	41.3 ± 9.5	84.7 ± 12.7	36.3 ± 4.2
LPS (2.0 µg/ml)	287.5 ± 42.0	605.3 ± 79.8	1026.4 ± 156.7
LPS (2.0 µg/ml) + rapamycin (2.0 µg/ml)	253.6 ± 40.7	457.0 ± 62.8	451.0 ± 65.0
LPS (2.0 µg/ml) + rapamycin (1.0 µg/ml)	279.1 ± 45.9	515.5 ± 75.9	529.4 ± 101.8
LPS (2.0 µg/ml) + rapamycin (0.5 µg/ml)	269.9 ± 43.5	519.2 ± 77.0	739.5 ± 157.9
LPS (2.0 µg/ml) + methylprednisolone (2.0 µg/ml)	257.4 ± 40.9	481.6 ± 73.2	409.4 ± 77.3
LPS (2.0 µg/ml) + methylprednisolone (1.0 µg/ml)	261.6 ± 41.7	544.8 ± 75.8	850.2 ± 158.5
LPS (2.0 µg/ml) + methylprednisolone (0.5 µg/ml)	267.1 ± 40.1	535.3 ± 77.3	893.2 ± 155.4

3. The relationship between VEGF genotypes and the suppressive effects of cyclosporine A and mycophenolic acid on VEGF production by LPS-stimulated PBMCs. The suppressive effect of cyclosporine A and mycophenolic acid on VEGF production was studied in LPS-stimulated PBMCs from 68 healthy controls in relation to their genotypes. LPS-stimulated and non-stimulated PBMCs were considered as controls (Figure 1)

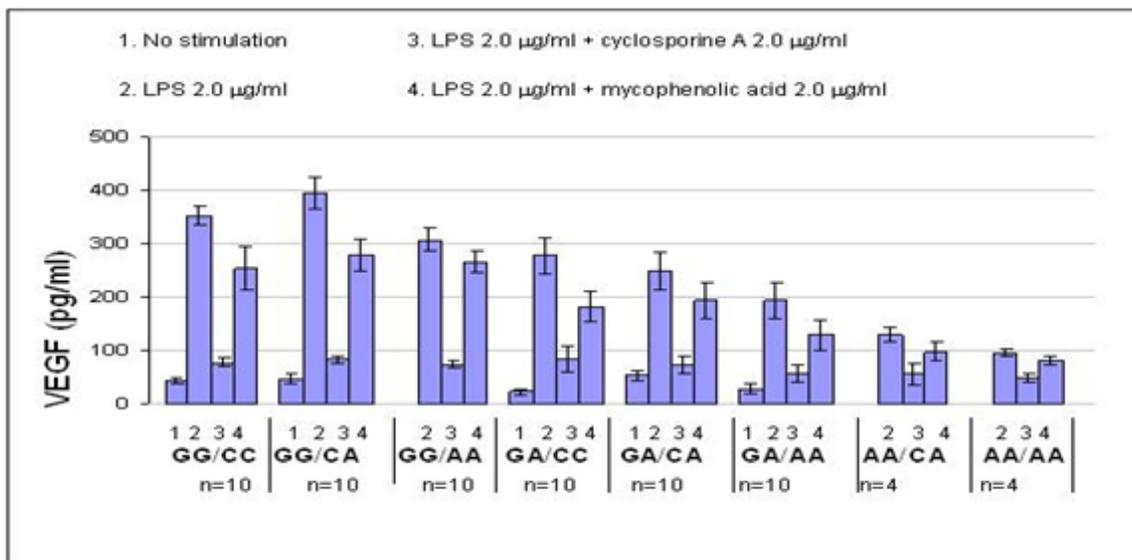


Figure 1. suppressive effect of cyclosporine A and mycophenolic acid on VEGF production in PBMCs from healthy individuals with different VEGF genotype combinations Of VEGF - 1154 G and -2578 C genotypes

VEGF production was significantly suppressed by cyclosporine A not only in the individuals with AA -1154/CA -2578 genotypes ($p=0.0224$), AA -1154/AA -2578 ($p=0.0027$) but also in other VEGF genotype combinations ($p<0.0001$). In addition, significant suppressive effects of mycophenolic acid on LPS-stimulated PBMCs were also observed for individuals with GG -1154/CC -2578 ($p=0.0341$), GG -1154/CA -2578 ($p=0.0153$) and GA -1154/CC -2578 ($p=0.0376$) VEGF genotype combinations.

4. The relationship between VEGF genotype and suppressive effects of rapamycin and methylprednisolone on VEGF production by LPS-stimulated PBMCs. The suppressive effect of rapamycin and methylprednisolone on VEGF level was calculated at day four, for individuals with different VEGF haplotypes as the mean of VEGF production \pm SEM (Fig. 2).

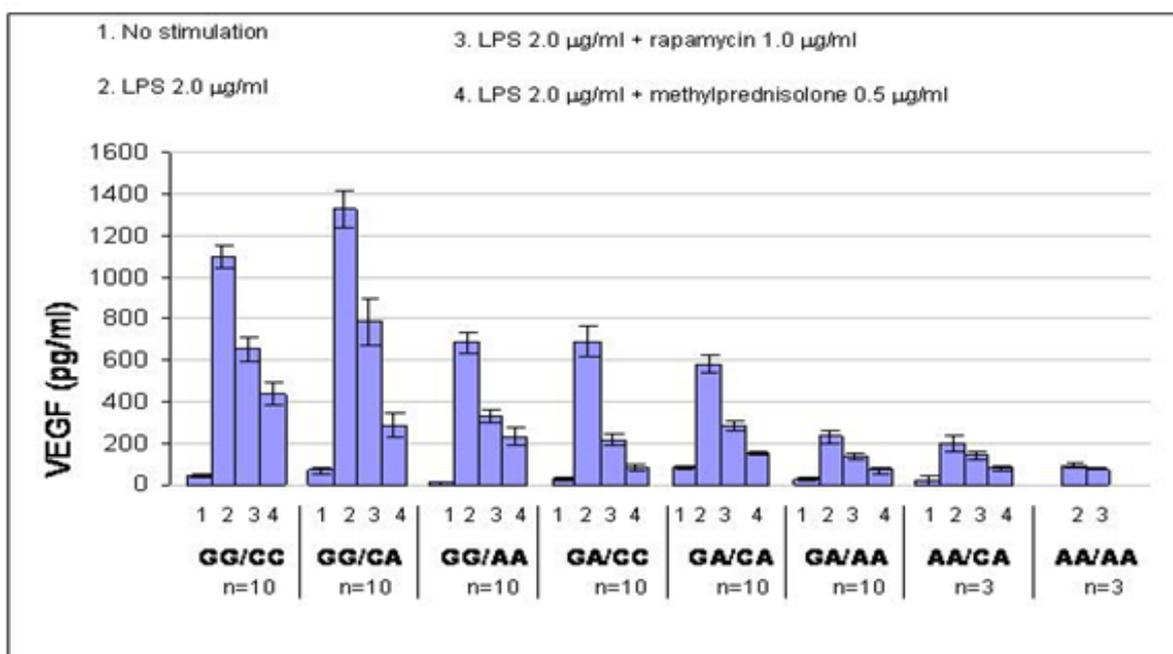


Figure 2. suppressive effect of rapamycin and methylprednisolone on VEGF production in PBMCs from healthy individuals with different combinations of VEGF -1154 G and -2578 C genotypes

Results of this experiment showed that VEGF was significantly suppressed by rapamycin in individuals having combinations of VEGF GG -1154/CC -2578, GG -1154/CA -2578, GG -1154/AA -2578, GA -1154/CC -2578, GA -1154/CA -2578 genotypes with p values less than 0.0001 and also in GA -1154/AA -2578 people with p value of 0.0135. However, no significant difference was observed for the suppressive effect of rapamycin on LPS-induced VEGF production in individuals with AA -1154/CA -2578 and AA -1154/AA -2578. Similar experiments were performed on the effects of methylprednisolone on VEGF suppression in the above-mentioned VEGF genotype combinations. VEGF production was significantly suppressed not only in the

individuals with AA -1154/CA -2578 ($p=0.0333$) but also in other VEGF genotype combinations ($p<0.0001$). Although the VEGF level was suppressed by methylprednisolone, it was not dependent on the combination of VEGF genotypes.

Taken together, results of the association study on the influence of immunosuppressive drugs on VEGF production in relation to the combinations of VEGF -1154 G and -2578 C genotypes showed that although VEGF level was significantly suppressed by cyclosporine A and methylprednisolone, it was not dependent on VEGF genotypes. However, the suppressive effect of mycophenolic acid was just observed in individuals with GG -1154/CC -2578, GG -1154/CA -2578 and GA -1154/CC -2578 haplotypes. Additionally, VEGF was significantly suppressed in all LPS-stimulated PBMCs after treatment with rapamycin except in those having AA -1154/CA -2578 and AA -1154/AA -2578 VEGF genotype combinations.

DISCUSSION

Our results on the influence of immunosuppressive drugs on VEGF production in relation to the combinations of VEGF -1154 G and -2578 C genotypes showed an association between GG -1154/CC -2578, GG -1154/CA -2578 as high producers and GA -1154/CC -2578 as an intermediate producer of VEGF. We also demonstrate the suppressive effects of mycophenolic acid on such genotypes. Additionally, our results showed that VEGF production was significantly suppressed in all individuals except those with AA -1154/CA -2578 and AA -1154/AA -2578 VEGF genotype combinations, after treatment of LPS-stimulated PBMCs with rapamycin.

It is well recognized that different transplant recipients respond in different ways to immunosuppressive medication. In fact, the inter-individual variations are greater than the intra-individual variations and pharmacokinetics of immunosuppressive drug response is determined by inheritance (14). Results of a recent published study regarding the population pharmacokinetics of cyclosporine in kidney and heart transplant recipients and the influence of ethnicity and genetic polymorphisms in the MDR-1, CYP3A4, and CYP3A5 genes, showing patients carrying a CYP3A4*1B variant allele demonstrated a significantly higher oral cyclosporine clearance compared with the patients homozygous for CYP3A4*1 (15). Result of another study, illustrating the large inter-individual variation of tacrolimus dose requirement as influenced by the metabolic activity of CYP3A5 (16). Another study on the liver transplant recipients treated with tacrolimus demonstrated that patients with a strongly expressed multidrug resistance-1 (MDR1) gene required higher tacrolimus doses to achieve therapeutic trough concentrations (17).

Additionally, multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients (18). Moreover, results of a recent study revealed that MMF treatment might be effective in preventing chronic renal rejection only in recipients with an IL-10 high producer genotype (19).

Employing this knowledge, it seems that although the suppressive effect of mycophenolic acid and rapamycin causes suppression of VEGF production, it is quite likely to depend on the genotype of the individual. Therefore, our results might be useful for the improvement in individualizing immunosuppressive therapy based on the recipient's genetic profile including single genes, such as VEGF, as well as gene-to-

gene interactions, and can be viewed as a support to the traditional therapeutic drug monitoring. In fact, results of our experiments showed that different dosages of rapamycin and mycophenolic acid might be administered for the suppression of VEGF in individuals with certain VEGF genotypes. Additionally, these findings are important because they show the effect of VEGF gene polymorphisms rather than MDR-1 and TPMT as conventional genes or CYP3A4, and CYP3A5 (20) as new candidate genes on the suppressive effects of rapamycin and mycophenolic acid.

The important point to be considered is that the range of blood drug concentration in clinical practice is significantly lower than the invitro drug concentrations used in this study. In this experiment the cells were kept in constant high concentration of the immunosuppressive drugs (e.g. 0,5, 1,0 i 2,0 µg/ml for cyclosporine), while in clinical practice the patient is under that range of concentration (C max) only twice a day i.e. 2-3 hours after the oral dose and then the concentration declines to a minimum C, when the next dose is given. To minimize the differences between the conditions in clinical practice and the in vitro conditions, we also tested lower doses of the immunosuppressive drugs, but we could not observe any suppressive effects on VEGF production (data not shown). Therefore it is plausible to see different results from in vitro experiments and the clinical practice. However, what is important about our findings is the relationship between the suppressive effects of MMF and Rapamycin on the VEGF production and the inheritance of some VEGF genotypes. This may lead us to study such effects on patients receiving immunosuppressive drugs in clinical practice.

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