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Single-Nucleotide Polymorphisms of Vascular Endothelial Growth Factor in Psoriasis of Early Onset

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Vascular endothelial growth factor (VEGF) – a stimulus of angiogenesis – is produced by epidermal keratinocytes, and elevated levels have been found in plaques of psoriasis. Polymorphisms in the VEGF gene regulate production of VEGF. We postulated that patients with psoriasis may have altered systemic expression of VEGF consequent upon programming at the genomic level. We investigated the genetic basis of VEGF expression in patients with type 1 (onset before age 40 y) chronic plaque psoriasis compared to healthy controls and also measured plasma levels of VEGF and its receptors *flt*-1 and *KDR*. Patients with severe disease, and those with onset of psoriasis between the ages of 20 and 40 y showed significantly increased frequency of the +405 CC genotype ($p = 0.04$ and $p = 0.02$) and the C allele ($p = 0.03$ and $p = 0.02$), respectively, compared to healthy controls. Plasma levels of VEGF and *flt*-1 were significantly detectable in patients with psoriasis compared with controls ($p < 0.001$); by contrast, mean plasma levels of *KDR* in psoriatic patients were comparable with controls. These results suggest that alterations in the biology of VEGF may be involved in the pathogenesis of psoriasis. VEGF, *flt*-1, and *KDR* could provide attractive targets for future psoriasis therapy.

Key words: angiogenesis/VEGF/VEGF receptors.
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Psoriasis occurs in 1 to 2% of the population and in early-onset disease, onset before age 40 (Henseler and Christophers, 1985), is genetically predetermined in conjunction with environmental triggers. Several putative and confirmed genetic loci for psoriasis have been reported as a result of genome scanning. The locus of strongest effect appears to be on chromosome 6p.21, in a region telomeric to HLA-C, and has been designated *PSORS* 1 (Trembath *et al*, 1997; Nair *et al*, 2000). The key histologic events in a plaque of psoriasis include abnormal keratinocyte proliferation and differentiation, inflammation, and vascular expansion (Griffiths and Voorhees, 1996; Creamer *et al*, 1997). Microvascular changes within plaques of psoriasis include dilatation, tortuosity, increased permeability, and endothelial cell proliferation within the venous limb of capillaries in the dermal papillae (Braverman and Yen, 1977; Braverman and Sibley, 1982). Vascular proliferation in psoriasis is in part influenced by angiogenic factors produced by the epidermis (Malhotra *et al*, 1989; Barnhill *et al*, 1984); these include vascular endothelial growth factor (VEGF)/vascular permeability factor (Thomas, 1996).

Angiogenesis is a common component of several pathogenic mechanisms including tumor growth and metastasis (Ferrara *et al*, 1992; Schott and Morrow, 1993; Senger *et al*, 1993; Neufeld *et al*, 1994), inflammatory joint disease (Brenchley, 2000), atheroma formation, and psoriasis (Detmar *et al*, 1994). The role of VEGF as a primary angiogenic mediator has been confirmed in experimental models of angiogenesis (Kim *et al*, 1993) and is now under investigation in clinical disease (Ferrara, 1999). To date, the majority of clinical studies have focused on VEGF in relation to tumor angiogenesis and VEGF expression detected by immunostaining in cancer of the breast (Toi *et al*, 1994), colon (Takahashi *et al*, 1995), stomach (Maeda *et al*, 1996), and esophagus (Inoue *et al*, 1997). Recent studies suggest that circulating plasma levels of VEGF are 10 times higher than normal in patients with ovarian carcinoma (Santin *et al*, 1999) and might be associated with extent of tumor angiogenesis in hepatocellular carcinoma (Jinno *et al*, 1998). These results suggest that levels of VEGF in plasma may act as a surrogate marker of pathological angiogenesis. VEGF is one of the most potent angiogenic factors secreted both by tumor cells and tumor-invading macrophages in response to hypoxia and other cytokines (Dolecki and Connolly, 1991; Detmar *et al*, 1997). It is an endothelial cell mitogen and survival factor that binds to specific receptors *flt*-1 (VEGFR1) and kinase insert domain receptor (*KDR*; VEGFR2) thereby triggering signal transduction pathways that mediate vascular angiogenesis and permeability (Neufeld *et al*, 1999).

We have previously demonstrated that VEGF and another angiogenic factor, endothelial cell-stimulating angiogenesis factor, are significantly elevated in plaques of

Abbreviations: PASI, psoriasis areas severity index; SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor.

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psoriasis and that these levels appear to correlate with clinical severity of psoriasis (Bhushan *et al*, 1999). Furthermore, elevated levels of VEGF are present in the plasma of patients with erythrodermic psoriasis (Creamer *et al*, 1996; Creamer *et al*, 2002). The gene for VEGF is located on chromosome 6p.21 (Vincenti *et al*, 1996; Mattei *et al*, 1996), close to *PSORS* 1 (Trembath *et al*, 1997; Nair *et al*, 2000). The VEGF gene is polymorphic with at least 15 single-nucleotide polymorphisms (SNPS) described (Watson *et al*, 2000; Brogan *et al*, 1999; Renner *et al*, 2000). Two of these, a C → T at −460 (Genebank Accession No. RS833061) in the promotor region and a G → C at +405 (Genebank Accession No. RS2010963), in the 5′-untranslated region (5′ UTR) are useful for association studies as they occur at highest frequency in this area of the gene and have been implicated as candidate SNP in other diseases with a putative angiogenic basis (Awata *et al*, 2002).[1–3] A significant correlation has been observed between VEGF protein production and the polymorphism, +405, within the 5′-UTR of the VEGF gene in healthy subjects (Watson *et al*, 2000).

We postulated that patients with psoriasis may have altered systemic expression of VEGF consequent upon programming at the genomic level and that regulation of VEGF protein expression may be related to disease severity. We investigated the genetic basis of VEGF expression in 160 well-characterized patients with early onset, type 1, chronic plaque psoriasis by measuring the frequencies of the VEGF polymorphisms −460 C → T (Genebank Accession No. RS833061) and +405 G → C (Genebank Accession No. RS2010963) and observed whether this correlated with plasma levels of VEGF, VEGF receptors, and clinical severity of disease.

Results

A total of 160 patients with type 1 chronic plaque psoriasis of a range of clinical severities were recruited to the study. Twenty-three patients were then excluded from further analysis on the basis of systemic treatments for psoriasis in the 3 mo before the study and/or a diagnosis of psoriatic arthropathy. The final patient group consisted of 68 men and 69 women, with a median age of 38 y (range, 16–80 y), a median age of psoriasis onset of 18 y (range, 0–39 y), and a median PASI of 12.2 (range, 1.8–32.4). Sixty-five percent (89 of 137) of patients had a family history of psoriasis and 63.5% (87 of 137) of patients had psoriatic nail involvement.

There were no significant differences in genotype frequencies at the −460 or +405 sites when comparing all psoriasis patients (n = 137) with controls (n = 102; Tables I, II). Nevertheless, when patients were subgrouped on the basis of disease severity those with severe disease (defined as PASI ≥ 12; n = 72) showed significantly increased frequency of the +405 CC genotype (chi-square test, p = 0.04) compared to healthy controls (Table I). Overall C allele frequency was also significantly increased—as expected given an increase in the observed frequency of the CC genotype (chi-square test, p = 0.03). Patients with onset of psoriasis between the ages of 20 and 40 y (n = 56) also showed significantly increased frequency of the +405 CC genotype (chi-square test, p = 0.02) and, as a related phenomenon, the C allele (chi-square test, p = 0.02) compared to healthy controls (Table I). In addition, patients with onset of psoriasis between the ages of 20 and 40 y (n = 56) showed significantly reduced frequency of the −460 CC genotype (chi-square test, p = 0.02; Table II).

Comparison of patients with "severe" and "mild/moderate" psoriasis revealed no significant genotype differences. However, when patients with psoriasis onset at less than 20 y of age were compared with patients with psoriasis onset after 20 y of age there was significantly reduced frequency of the –460 CC genotype for the older onset group (chi-square test, p = 0.01) and significantly increased frequency of the TC genotype for the older onset group (chi-square test, p = 0.03; Table II), with no significant differences in observed genotype frequencies for the +405 polymorphism (Table I). Fifty-eight percent of the psoriasis patient group were Cw6-positive, and no linkage disequilibrium was observed between either the +405 or the −460 VEGF polymorphisms and HLA-Cw6 (chi-square test).

Levels of VEGF, *flt*-1, and *KDR* in plasma (Webb *et al*, 1998) were analyzed in 135 patients; two further patients were excluded on the basis of concurrent use of COX-1 and COX-2 nonsteroidal anti-inflammatory drugs that can inhibit angiogenesis by blocking VEGF-induced signal transduction (Jones *et al*, 1999; Brenchley, 2000). Expression of VEGF protein and *flt*-1 receptor in the plasma of healthy volunteers was uncommon with only 24 and 11%, respectively, having measurable levels. In patients with psoriasis (n = 135), 98% had measurable expression of VEGF and 100% had detectable *flt*-1 in plasma which was significant (chi-square test, p < 0.001). The mean levels of VEGF in the plasma of all psoriasis patients and all normal controls were 43.03 (SD, 52.35; range, 0–462) and 14.6 pg per mL (range, 0–115), respectively. The mean plasma level of *flt*-1 in all psoriatic patients was 31.1 pg per mL (SD, 13.0; range, 9–74), and the mean plasma level of *flt*-1 in all normal controls 5.2 pg per mL (range, 0–73.6). In contrast, plasma levels of KDR were equally detectable in patients and controls with a mean plasma level of 8762 pg per mL (SD, 1759; range, 4,650–14,480) in all patients with psoriasis and 9577 pg per mL (range, 6,635–13,553) in controls.

Plasma levels of VEGF, *flt*-1, and *KDR* did not correlate with psoriasis severity. Nevertheless, there was a weak but significant correlation between plasma *flt*-1 and *KDR* receptor levels in all patients with psoriasis (r = 0.2, p = 0.02, one-tailed). There were no significant correlations between plasma levels of VEGF and *flt*-1 or VEGF and *KDR* levels, respectively; we have shown previously (Webb *et al*, 1998) that *flt*-1 masks the detection of VEGF in plasma samples and could result in "underestimation" of the true level of circulating VEGF.

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[2]Summers AM, Brenchley PEC, Morgan L, Baker PN: Association of VEGF +405 GG polymorphism with pre-eclampsia [abstract]. J Soc Gynecol Invest 9:80, 2002

[3]Summers AM, Ralph SA, Short CD, Brenchley PEC: VEGF −460 CC genotype is associated with the progression towards end stage renal disease [abstract]. J Am Soc Nephrol 13:260, 2002

Table I. Observed frequencies for the +405 VEGF gene polymorphism within a population of type 1 psoriasis patients and healthy controls[a]

	Controls (n = 102)	Psoriasis (n = 137)	Mild/moderate psoriasis (n = 65)	Severe psoriasis (n = 72)	Psoriasis onset before age 20 (n = 81)	Psoriasis onset between age 20 and 40 (n = 56)
Genotype						
GG	47 (46)	50 (36)	26 (40)	24 (33)	32 (40)	18 (32)
GC	48 (47)	67 (49)	32 (49)	35 (49)	41 (51)	26 (46)
CC	7 (7)	20 (15)	7 (11)	13 (18)[b]	8 (10)	12 (21)[c]
Allele						
G	142 (69.6)	167 (60.9)	84 (64.6)	83 (57.6)	105 (64.8)	62 (55.4)
C	62 (30.4)	107 (39.1)	46 (35.4)	61 (42.3)[d]	57 (35.2)	50 (46.6)[c]

[a]Data are presented as number (%). There is significantly increased frequency of the CC genotype and the C allele for patients with severe psoriasis (column 1 vs. column 4) and psoriasis onset between age 20 and age 40 compared to healthy controls (column 1 vs. column 6).
[b]$p = 0.04$.
[c]$p = 0.02$.
[d]$p = 0.03$.

Table II. Observed frequencies for the –460 VEGF gene polymorphism within a population of type 1 psoriasis patients (n = 56) and healthy controls (n = 101)[a]

	Controls (n = 101)	Psoriasis (n = 137)	Mild/moderate psoriasis (n = 65)	Severe psoriasis (n = 72)	Psoriasis onset before age 20 (n = 81)	Psoriasis onset between age 20 and 40 (n = 56)
Genotype						
CC	21 (20.8)	21 (15)	7 (11)	14 (19)	18 (22)	3 (5)[b]
CT	60 (59.4)	74 (54)	39 (60)	35 (49)	37 (46)	37 (66)[c]
TT	20 (19.8)	42 (31)	19 (29)	23 (32)	26 (32)	16 (29)
Allele						
C	102 (50.5)	116 (42.3)	53 (40.8)	63 (43.8)	73 (45.1)	43 (38.4)
T	100 (49.5)	158 (57.7)	77 (59.2)	81 (56.2)	89 (54.9)	69 (61.6)

[a]Data are presented as number (%). There is significantly reduced frequency of the CC genotype for patients with psoriasis onset between age 20 and age 40 compared to healthy controls (column 1 vs. column 6) and significantly decreased frequency of the CC genotype with significantly increased frequency of the CT genotype for patients with psoriasis onset between age 20 and age 40 compared to those with onset before age 20 (column 5 vs. column 6).
[b]$p = 0.02$; $p = 0.01$.
[c]$p = 0.03$.

Patients with psoriasis and the −460 CC genotype had significantly lower plasma levels of *flt*-1 ($p = 0.005$; mean, 24.0 ± 9.4) than psoriasis patients with a TC or TT genotype at the −460 locus.

Discussion

We have found a significant association of the VEGF +405 CC genotype and C allele for severe psoriasis and onset between 20 and 40 y of age. In addition, plasma levels of VEGF and *flt*-1 are increased in patients with psoriasis, although levels of *KDR* are no different from controls.

Cutaneous angiogenesis is important for a variety of physiologic processes, for example, in the anagen stage of the hair cycle (Mecklenburg *et al*, 2000). However, a sustained and significant increase in new blood vessel formation can be seen in cutaneous disease; for instance, angiogenesis occurs in primary and metastatic cutaneous malignant melanoma (Hubler and Wolf, 1976; Srivastava *et al*, 1988; Marcoval *et al*, 1997) rosacea, and psoriasis (Creamer and Barker, 1995). Epidermal keratinocytes appear to be the main source of agonists for physiologic and pathologic cutaneous angiogenesis (Malhotra *et al*, 1989). VEGF, a permeability-enhancing factor in the early stages of angiogenesis and an endothelial-cell-specific mitogen, appears to be both constitutively expressed in skin and upregulated in conditions where active neovascularization is occurring (Brown *et al*, 1992; Ballaun *et al*, 1995; Weninger *et al*, 1996).

VEGF is over expressed by keratinocytes in the clinically involved and the uninvolved skin of patients with stable, chronic plaque psoriasis (Detmar *et al*, 1994). Previously it has been shown that plasma levels of VEGF are elevated in patients with erythroderma, compared with normal control subjects (Creamer *et al*, 1996). In chronic plaque psoriasis we have previously reported a correlation between levels of VEGF in plaques of psoriasis, PASI, and levels of VEGF in peripheral blood (Bhushan *et al*, 1999). Patients with generalized pustular psoriasis or a psoriasis PASI of greater than 30 have been reported as having significantly higher levels of plasma VEGF compared with patients with a PASI

of less than 30 and that generalized pustular psoriasis is accompanied by pathologic proteinuria (Creamer *et al*, 2002) indicative of enhanced vascular permeability. Nevertheless, in our patient group, we found no correlation between clinical severity of disease and levels of VEGF in plasma, although virtually all our psoriasis patients had elevated levels of VEGF compared with a normal control population. We observed a lower mean plasma VEGF level in patients with psoriasis than that published previously (Creamer *et al*, 2002). This discrepancy could have occurred owing to: (1) the difference in choice of subtypes of psoriasis, that is, chronic plaque psoriasis in the current study versus pustular or erythrodermic psoriasis; (2) a difference in spectrum of psoriasis severity; and (3) our exclusion of patients with psoriatic arthropathy on the basis of reported elevations in plasma VEGF levels in inflammatory arthritis. We did not address whether conventional psoriasis treatments influence plasma levels of either VEGF or its receptors, although it has been reported (Creamer *et al*, 2002) that patients with moderate/severe or severe psoriasis have plasma VEGF levels which are significantly increased during relapse of psoriasis compared with remission.

As we learn more about the immunogenetics of psoriasis it is apparent, with seven psoriasis susceptibility loci so far described, that psoriasis is polygenic (Elder *et al*, 2001). There are, perhaps, several genotypically different but phenotypically similar forms of chronic plaque psoriasis. These subsets may be characterized by different triggers, disease severity and age of onset. For instance, a promoter polymorphism in the gene encoding TNF-α is associated with early-onset (at or before age 40) psoriasis in men (Reich *et al*, 2002). The $+405$ CC genotype in patients with psoriasis would appear to confer a tendency to develop severe disease with increased susceptibility to disease onset between the ages of 20 and 40 y. Furthermore, in view of the relative cytogenetic proximity of the VEGF gene with the MHC the possibility that the observed associations may be due to linkage disequilibrium between the VEGF polymorphisms and HLA-Cw6 was investigated—there was no linkage disequilibrium observed between either of the VEGF polymorphisms studied and HLA-Cw6. The $+405$ G $\rightarrow$ C SNP may therefore be a significant factor in the control of disease severity and phenotypic expression in psoriasis. In the future, it is possible that determination of VEGF gene polymorphisms may be used as a prognostic factor for psoriasis severity.

It is possible that the $+405$ G $\rightarrow$ C SNP may exert significant functional control over VEGF expression in psoriasis. However, no significant association was found between either the $+405$ or the -460 genotype and plasma levels of VEGF in our patients. This may be due to the primary assay used being affected by circulating soluble receptor. We have previously demonstrated that in VEGF-specific ELISA assays, addition of *flt*-1 to serum containing a high concentration of VEGF reduced the detected level with a clear dose response, that is, nondetection of VEGF-*flt*-1 complexes (Webb *et al*, 1998). Alternative analyses such as Western blotting would probably be unlikely to be sensitive enough for detection of receptor or protein. Further study is required to explain whether genetic variability within the VEGF gene has any measurable impact on VEGF protein expression. It is possible that the -460 C/$+405$ C haplotype, which is very rare in the normal population (1 in 230 chromosomes analyzed; Watson *et al*, 2000), could represent a disease-associated haplotype in psoriasis and correlate with VEGF plasma levels. One of the other SNPS, through tight linkage disequilibrium, may be responsible for the observed association with psoriasis and we are therefore further investigating this area of the VEGF gene.

Flt-1 is a receptor tyrosine kinase which is expressed in two forms (Kendall and Thomas, 1993; Kendall *et al*, 1996): a full-length, membrane-bound receptor capable of transducing signal, and a truncated, soluble receptor capable of sequestering ligand or dimerizing with full-length receptor and preventing signal transduction. *KDR* is also a receptor tyrosine kinase—both receptors are almost exclusively expressed within endothelial cells and preferentially within proliferating endothelium. VEGF, *flt*-1, and *KDR* represent a regulatory system essential for both normal and pathologic angiogenesis. High levels of *flt*-1 reportedly occur in the plasma during pregnancy and in patients with essential hypertension (Clark *et al*, 1998; He *et al*, 1999; Belgore *et al*, 2001a, b). Significantly lower levels have been observed in the plasma of patients with cardiovascular disease and in smokers (Belgore *et al*, 2001a, b; Belgore *et al*, 2000). *Flt*-1 has a greater affinity for VEGF than *KDR*, yet *KDR* is phosphorylated approximately 10-fold more efficiently upon ligand binding. Observations suggest that *flt*-1 functions to limit VEGF/*KDR*-mediated angiogenesis (Waltenberger *et al*, 1994; Fong *et al*, 1995; Shalaby *et al*, 1995; Hiratsuka *et al*, 1998; Bussolati *et al*, 2001). There is evidence, however, that the tyrosine kinase domain of *flt*-1 does play an angiogenic role. Induced *flt*-1/*KDR* heterodimers can transduce signal (Huang *et al*, 2001). VEGF-induced nitric oxide release appears to be mediated by *flt*-1, and this nitric oxide release in turn acts as a molecular switch, inhibiting *KDR*-mediated proliferation and affecting redifferentiation of endothelial cells into capillary-like structures (Bussolati *et al*, 2001). In our study *flt*-1 levels were significantly elevated in patients with psoriasis compared with normal controls; by contrast mean plasma levels of *KDR* in psoriatics were comparable with controls. Nevertheless, there was a significant correlation between plasma *flt*-1 and *KDR* levels in psoriatic patients. In contrast to soluble *flt*-1, which is an alternatively spliced cellular product, circulating soluble *KDR* is thought to derive from shedding of the receptor from endothelial cell membrane into the circulation. Thus, circulating levels of both VEGF receptors could be regarded as surrogate markers of endothelial cell activity—with increased endothelial cell turnover likely to result in shedding of membrane-bound receptor into the plasma. Overexpression of *flt*-1 and *KDR* receptors in dermal microvascular endothelium has been reported in psoriasis (Detmar *et al*, 1994), healing skin wounds (Peters *et al*, 1993), and delayed hypersensitivity reactions (Brown *et al*, 1995). It is possible that differential receptor expression may modulate the biologic effects of VEGF in psoriasis via transmission of different angiogenic signals to the skin. It has only recently become possible to measure levels of *KDR* in plasma and to our knowledge the current study is the first to report on soluble *KDR* levels for any disease.

A number of antiangiogenesis approaches are being assessed for the therapy of solid cancers (Brekken *et al*, 2000); the results of such studies might produce therapeutic options for angiogenesis-associated skin disease. Currently a phase II study of bevacizumab (an anti-VEGF monoclonal antibody) and interferon-α2b is in progress for the treatment of metastatic malignant melanoma (Chen *et al*, 2001). There is particular interest in developing immune-based therapeutic approaches that block VEGF function and/or signaling in angiogenesis either by downregulation of VEGF expression or by interaction with high-affinity receptors on endothelial cells (Aiello *et al*, 1995; Presta *et al*, 1997; Arora *et al*, 1999; Fong *et al*, 1999; Schlaeppi and Wood, 1999). Recent progress made in the crystallization of protein kinases has confirmed the ATP-binding domain of tyrosine kinases as an attractive target for drug design. Two new compounds for oral administration, currently in clinical trials, have been developed which are potent inhibitors of both VEGF receptor kinases with relative selectivity for KDR receptors—PTK787/ZK222584 and ZD6474 (Hennequin *et al*, 2002; Wood, 2000; Traxler *et al*, 2001). Blockade of the VEGF/VEGF receptor system has reported utility for VEGF-dependent pathologic neovascularization and may represent a future novel, selective approach for the therapy of psoriasis.

A greater understanding of VEGF gene expression in psoriasis may lead to better genotypic characterization of severe disease and allow a pharmacogenetic-based management approach and could result in the development of new anti-VEGF therapies for psoriasis.

Materials and Methods

Patients Venous blood samples were collected from 102 normal controls and 160 patients with type 1 chronic plaque psoriasis, with a range of clinical severities, recruited from the Psoriasis Clinic at Hope Hospital (Manchester, UK). Patients were excluded from the study if they had psoriatic arthropathy, were on systemic treatment for psoriasis, or were using COX-1 or COX-2 nonsteroidal anti-inflammatory drugs that can inhibit angiogenesis by blocking VEGF-induced signal transduction. Psoriasis severity was assessed by the psoriasis area severity index (PASI; Fredriksson and Pettersson, 1978); a PASI $\geqslant$12 was designated as "severe" psoriasis. Venous blood was collected into ethylenediaminetetraacetic acid blood tubes for DNA extraction and also for plasma collection. Centrifugation of blood samples and separation and collection of plasma were performed within 2 h of venepuncture. Plasma, red cell pellets, and whole-blood samples were stored at -80°C until required. The study adhered to the Declaration of Helsinki Guidelines and was approved by the Salford and Trafford local research ethics committee and all subjects gave written, informed consent.

DNA preparation Genomic DNA was isolated from whole blood and red cell pellets using the QIAamp DNA blood midi kit (Qiagen Ltd UK, Crawley, West Sussex, UK). Briefly, samples of whole blood or red cell pellets were mixed with protease and incubated at 70°C for 10 min. After being further mixed with absolute ethanol, the samples were loaded into QIAamp Midi columns and centrifuge filtered, allowing DNA to remain within the filter but permitting residual waste to pass through. After two washing steps, the entrapped DNA was eluted from the filter with distilled water. Quantification of DNA concentration and purity was performed using a spectrophotometer (Cecil Series II, Cecil Instruments, Cambridge, UK).

PCR sequencing PCR was carried out using previously described primers (Watson *et al*, 2000) known to amplify the target polymorphic sequences at positions $+405$ (Genebank Accession No. RS2010963) and –460 (Genebank Accession No. RS833061) in the VEGF gene. The primers (5′ to 3′) used were forward TGTGCGTGTGGGGTTGAGCG and reverse TACCTGCGGA-CAGGGCCTGA for the –460 polymorphism and forward ATT-TATTTTTGCTTGCCATT and reverse GTCTGTCTGTCTGTCCGTCA for the $+405$ polymorphism. This generated PCR products of 304 and 175 bp, respectively. PCR amplification was performed in a total reaction volume of 20 μL with 100 ng of DNA, PCR buffer—16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.0), 85 μg per mL bovine serum albumin, 0.5 μM of each primer, 10% dimethyl sulfoxide, 0.75 mM of each deoxynucleoside triphosphate, and 0.5 units *Taq* polymerase, with varying concentrations of Mg, 5 mM for the –460 polymorphism and 4 mM for the $+405$ polymorphism. A geneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) was used. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 3 min followed by 32 cycles for the -460 polymorphism at 94°C for 1 min (denaturation), 60°C for 1 min (annealing step), and 72°C for 1 min (extension) and 32 cycles for the $+405$ polymorphism at 94°C for 1 min (denaturation), 55°C for 1 min (annealing step), and 72°C for 1 min (extension) with a final extension step for both polymorphisms at 72°C for 5 min. For both polymorphisms, the presence of PCR product was assessed by electrophoresing 5 μL of PCR product and 2 μL of loading buffer with a 100-bp DNA ladder (Life Technologies, Paisley, UK) in a 2% agarose gel stained with ethidium bromide.

Restriction fragment length polymorphism typing PCR product (15 μL) was digested with the appropriate restriction enzyme, B*st*UI (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire, UK) for the -460 polymorphism and B*sm*FI (New England Biolabs) for the $+405$ polymorphism overnight at the recommended temperature (60°C for the -460 polymorphism and 65°C for the $+405$ polymorphism) and the digested DNA fragments were analyzed on 3% 1 $\times$ TBE agarose gels (Fig 1).

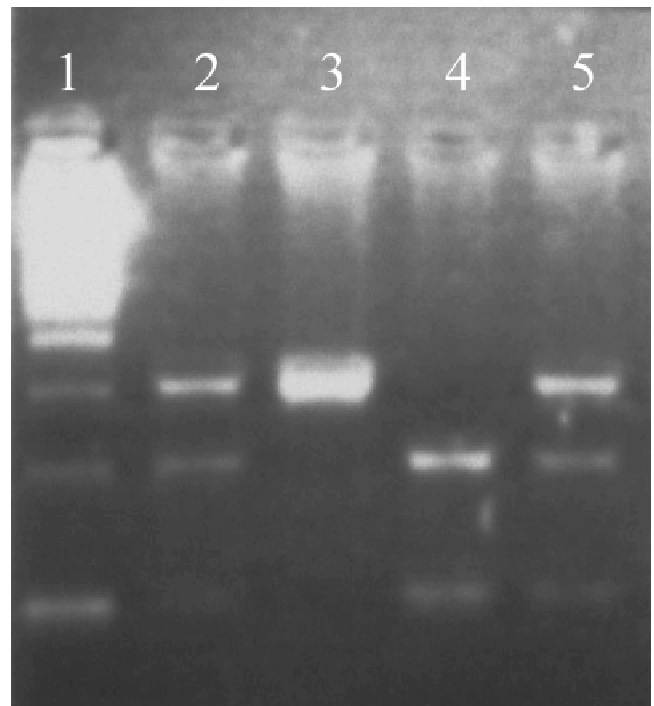

Figure 1
PCR-restriction fragment length polymorphism analysis of the VEGF gene $+405$ polymorphism. *Lane 1*, a 100-bp DNA ladder; *lane 3*, CC genotype; *lane 4*, GG genotype; *lanes 2* and *5*, CG genotype.

HLA-Cw6 polymerase chain reaction sequence-specific typing The Cw6 genotyping was performed by PCR sequence-specific typing. Primers, sense TACTACAACCAGAGCGAGGA and antisense GGTCGCAGCCATACATCCA, specific for Cw6 were used, generating a PCR product of 297 bp. The primers were used at a final concentration of 1.0 μM. A pair of control primers C1P1 and C1P2, sense GCCTTCCCAACCATTCCCTTA and antisense GTCCATGTCCTTCCTGAAGCA, amplifying a 1060-bp fragment of the human growth hormone gene (GH2) were added to each Cw6 primer mix to identify failed PCR reactions. The control primers were used at a final concentration of 0.1 μM. Plate preparation, PCR conditions, and visualization/interpretation of results were as previously described by our laboratory for short-form HLA-DP typing (Davidson and Poulton, 2001).

Determination of plasma levels of VEGF, *flt*-1, and KDR Plasma levels of VEGF protein and the receptors *flt*-1 and *KDR* were measured using a specific Quantikine ELISA (R & D Systems, Abingdon, UK). Briefly, plasma samples and standards were added in assay diluent to precoated wells and incubated for 2 h at room temperature. After a standard washing step, anti-VEGF, anti-*flt*-1, or an anti-*KDR* conjugate was added to each well and incubated for 2 h at room temperature. After an additional washing step, substrate solution was added to each well for 25 to 30 min, after which a "stop" solution was added and the plate read on a ThermoMax plate reader (Molecular Devices, Sunnyvale CA) at 450 nm. VEGF, *flt*-1, and *KDR* standards were used to ascertain interplate variability.

Statistical analysis The chi-square test was used to compare differences in the observed genotype frequencies and in the detectability of plasma VEGF/*flt*-1/*KDR* levels in the patient and normal populations. The chi-square test was also used to investigate linkage between the +405 or −460 VEGF polymorphisms and HLA-Cw6 for patients with psoriasis. One-way ANOVA was used to assess the relationship between plasma levels of VEGF/*flt*-1/*KDR* and genotype. Graphical methods and correlations were used to assess the relationships between plasma VEGF/*flt*-1/*KDR* levels; $p < 0.05$ was considered statistically significant.

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