Association of variants in angiotensin-converting enzyme and endothelin-1 genes with phototherapy in cutaneous T-cell lymphoma

V. Vaškù, J. A. Vaškù, M. Pávková Goldbergová and A. Vaškù

ABSTRACT

Background. The cutaneous T-cell lymphoma (CTCL) is a disease characterised by cutaneous infiltrates of malignant, clonally expanded T-cells. Because individual genetic determination of angiogenetic and antioxidant properties of blood vessels could be partly responsible for phototherapy in CTCL patients, three polymorphisms in angiotensin converting enzyme and endothelin-1 genes were determined.

Methods. 77 patients with CTCL, diagnosed and treated at the First Dermatological Clinic of St. Ann's Faculty Hospital Brno (46 men and 31 women, median age 62, range 26-80 years) were compared to a control non-CTCL group of the similar age and gender distribution (n=203: 137 men and 66 women, median age 54, range 27-86 years) with negative family history of severe skin diseases and without signs of malignancy. Diagnosis of CTCL was verified according to the clinical picture and histologically. The genotype distributions and allelic frequencies between CTCL with phototherapy and those without phototherapy were compared.

Results. Significant differences were found in genotype distributions of insertion/deletion (I/D) ACE polymorphism between CTCL patients treated with phototherapy and those treated without it. Heterozygote ID was more frequent in the group treated with phototherapy (25/13 vs. 12/27, OR=4.33, 95% confidential interval 1.67-11.24, P=0.02). The 4A4A variant of -3A/-4A EDN1 is more frequent in patients treated with phototherapy (8/30 vs. 1/38, OR=10.13, 95% confidential interval 1.20-85.55, P=0.01). The GA and AA genotypes of G8002A EDN1 polymorphism are more frequent in CTCL patients treated with phototherapy compared to those without it (15/23 vs. 7/32, OR=2.98, 95% confidential interval 1.05-8.48, P=0.03).

Discussion. Some polymorphic variants in ACE and EDN1 genes (a heterozygote I/D ACE, a homozygote -4A-4A in -3A /-4A EDN1 and genotypes GA and GG in G8002A EDN1) seem to carry an advantage for phototherapy effectiveness in patients with CTCL.

KEY WORDS

I/D ACE, endothelin-1-gene polymorphism, mycosis fungoides

Introduction

Cutaneous T-cell lymphoma (CTCL) is a disease characterised by cutaneous infiltrates of malignant, clonally expanded T-cells. The progression of tumors is largely dependent on their vascularization. The expression of specific genes that alter the balance between

Table 1. Insertion/deletion polymorphism in angiotensin converting enzyme (I/D ACE) in patients with cutaneous T cell lymphoma (CTCL) versus controls.

	I/D ACE						
Genotypes and alleles	**II**	**ID**	**DD**	**Pg**	**I**	**D**	**Pa**
CTCL group (n=77)	19 (24%)	37 (48%)	21 (27%)	*0.818	0.487	0.513	*0.649
CTCL treated with UVA (PUVA+SUP) (n=38)	8(21%)	25(66%)	5(13%)	**0.005	0.539	0.461	**0.199
CTCL treated without UVA (n=39)	11(28%)	12(31%)	16(41%)		0.436	0.564	
Control group (n=203)	43(21%)	103(51%)	57(28%)		0.466	0.534	

Pg = probability of difference in genotype distribution
Pa = probability of difference in allelic frequency
* - a difference between CTCL and control group
**-a difference between CTCL with and without UVA therapy

pro- and anti-angiogenic molecules produced by the tumor cells themselves and by cells from the host microenvironment, is described by the term angiogenic switch. This event is critical, as it results in vascularization of a primary tumor and its growth and metastatic spreading. Besides stromal cells, lymphocytes and monocytes/macrophages that infiltrate the tumor also play a major role in regulating angiogenesis. T cells may favor angiogenesis by producing endothelial growth factors and by releasing metalloproteinases (MMPs) that participate in capillary formation. Monocytes/macrophages produce direct and indirect inducers of angiogenesis as well as angiogenic inhibitors, such as angiostatin, inhibitory chemokines, and thrombospondin (1).

The vascular endothelium is considered to be an integral part of the in vivo immune system. Cultured human endothelial cells have the capacity to present antigens to T cells and act as co-stimulators that lead to effective T cell activation. These activities raise the possibility that venular endothelial cells (ECs), at sites of delayed hypersensitivity reactions, could be the primary antigen-presenting cell to circulating memory T cells (2). Human ECs display class I and II MHC-peptide complexes on their surface and come in regular contact with circulating T cells. It has been proposed that they present microbial antigens to memory T cells as a mechanism of immune surveillance. Activated T cells, in turn, provide both soluble and contact-dependant signals to modulate normal EC functions, including formation and remodelling of blood vessels, regulation of blood flow, regulation of blood fluidity, maintenance of permselectivity, recruitment of inflammatory leukocytes, and antigen presentation leading to activation of T cells. T cell interactions with vascular EC are thus bidirectional and link the immune and circula-

Table 2. Polymorphism -3A/-4A in endothelin-1 gene (EDN1) - patients with cutaneous T cell lymphoma (CTCL) versus controls.

	-3A/-4A EDN1						
Genotypes and alleles	**3A3A**	**3A4A**	**4A4A**	**Pg**	**3A**	**4A**	**Pa**
CTCL group (n=77)	34 (44%)	34 (44%)	9 (12%)	*0.588	0.727	0.273	*0.653
CTCL treated with UVA (PUVA+SUP) (n=38)	16(42%)	14(37%)	8(21%)	**0.04	0.605	0.395	**0.139
CTCL treated without UVA (n=39)	18(46%)	20(51%)	1(3%)		0.718	0.282	
Control group (n=203)	90(44%)	97(48%)	16(8%)		0.682	0.317	

Pg = probability of difference in genotype distribution
Pa = probability of difference in allelic frequency
* - a difference between CTCL and control group
**-a difference between CTCL with and without UVA therapy

Table 3. Polymorphism G8002A in enodthelin-1 gene (EDN1) – patients with cutaneous T cell lymphoma (CTCL) versus controls.

Genotypes and alleles	G8002A EDN1						
	GG	GA	AA	Pg	G	A	Pa
CTCL group (n=77)	55 (71%)	19 (25%)	3 (4%)	*0.290	0.838	0.162	*0.234
CTCL UVA treated (PUVA+SUP) (n=38)	23(61%)	13(34%)	2(5%)	**0.112	0.776	0.224	**0.04
CTCL treated without UVA (n=39)	32(82%)	6(16%)	1(2%)		0.897	0.103	
Control group (n=203)	126(63%)	70(34%)	7(3%)		0.793	0.207	

Pg = probability of difference in genotype distribution
Pa = probability of difference in allelic frequency
* - a difference between CTCL and control group
**-a difference between CTCL with and without UVA therapy

tory system (3).

Dermal microvascular endothelial cells form a continuous lining that normally bars blood-borne T lymphocytes from entering the skin, but as part of the response to foreign antigen, dermal ECs undergo alterations in their surface proteins so as to provide signals to circulating T cells that lead to their activation and recruitment. Several observations suggest that human dermal microvascular ECs may help to initiate cutaneous immune reactions by presentation of cognate antigens to circulating T memory cells. On contact with activated T cells or with their secreted products (cytokines), dermal ECs themselves become activated, increasing their capacity to recruit memory and effector T cell populations in an antigen-independent manner (4).

Phototherapy has been utilized for decades in the treatment of various dermatologic conditions, including CTCL. The efficacy of broadband ultraviolet B (UVB) is limited to the patch stage, while psoralen and ultraviolet A (PUVA) is capable of clearing plaques and, sometimes, the early stages of tumors. Phototherapy can also be a useful adjunct to other kinds of treatment such as interferons, retinoids and electron beam therapy (5).

Ultraviolet (UV) A-1 (340-400 nm) radiation is highly effective in inducing apoptosis in skin-infiltrating T cells and thereby exerts beneficial effects in patients with T cell-mediated skin diseases. The malignant CD4+ T cells isolated from a patient with adult T cell leukemia and Sezary's syndrome as well as malignant T cell lines exhibited a significantly higher susceptibility than normal CD4+ T cells toward UVA-1 radiation-induced apoptosis at 4 h (early apoptosis) and at 24 h (late apoptosis) after exposure. UVA-1 radiation-induced T cell apoptosis is initiated through the generation of singlet oxygen. This is in agreement with the present observation that stimulation of unirradiated cells with a singlet oxygen-generating system induced apoptosis in malignant cells to a greater extent than in normal cells. The caspase inhibitor Z-VADfmk decreased interferon-gamma stimulation, which is known to upregulate caspase levels including caspase-3, increased the sensitivity of T cells toward UVA-1 radiation-induced apoptosis. Furthermore, malignant T cells had significantly higher procaspase-3 levels when compared with normal cells. These studies indicate that the susceptibility of human T cells toward UVA-1 radiation-induced apoptosis is related to the availability of caspases such as caspase-3 and that strategies directed at upregulating caspase levels will increase the efficacy of UVA-1 phototherapy (6).

The membrane dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) as a transmembrane protease is supposed to participate in diseases in which angiogenesis is involved (7). The expression of ACE was found to enhance presentation of certain endogenously synthesized peptides to major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. The protease seems to influence the processing of endogenously synthesized antigens (8). The presence of ACE in circulating mononuclear cells raises the possibility that it may play a role in modulating the thymic humoral factor-gamma 2, an immunoregulatory octapeptide important for T-lymphocyte regulation (9). Captopril has been shown to inhibit Fas-induced apoptosis in human activated peripheral T-cells, as an orally active inhibitor of ACE (10). The same drug was found to decrease T-lymphocyte ACE activity in a concentration dependent manner in vitro (11). On the other hand, captopril decreases antioxidant defences in human endothelial cells by decreasing activities of glulathione peroxidase as well as of superoxide dismutase (12).

Angiotensin II (AT II) as a product of ACE action causes significant arteriolar leukocyte adhesion of mononuclear cells. Using function-blocking monoclonal antibodies

against different rat cell adhesion molecules this effect was proved to be dependent on P-selectin and beta (2)-integrin. In postcapillary venules, AT II also induces leukocyte infiltration on endothelium in vivo at physiologically relevant doses (13). AT II was found to be an inducer of apoptosis itself in well-differentiated cell types (14). Transactivation of receptors for ATII (AT1 receptors) was observed in Reed-Steinberg cells (15).

Under physiological conditions, ACE is able to deactivate bradykinin. The peptide has multiple pathophysiological functions such as induction of vascular permeability and mitogenesis, release of mediators such as nitric oxide in inflammatory and cancer tissue. Interestingly, the expression of bradykinin receptors has been proven in human cancer cells (16).

Endothelins, including three 21-amino acid peptides ET-1, ET-2 and ET-3, are potent vasoconstrictors, and involved in the pathophysiology of different malignancies. ET-1 is a relevant growth factor in several tumor types including carcinoma of the prostate, ovary, colon, cervix, breast, kidney, lung, colon, central nervous system tumors as well as melanoma, Kaposi's sarcoma and bone metastasis. ET-1 participates in a wide range of cancer relevant processes, such as cell proliferation, inhibition of apoptosis, matrix remodeling, bone deposition, and metastases (17). ETs are also angiogenic factors. Endothelial cell mitogenesis is mediated by ET_BR, while vascular smooth muscle cells and pericyte mitogenesis is mediated predominantly or solely by the ET_AR. ET-1 modulates various stages of neovascularization, including endothelial cell proliferation, migration, invasion, protease production, tube formation and stimulates neovascularization *in vivo* (18). Endothelin-1 is known to modulate melanogenesis of human melanocytes and to participate in the responses to ultraviolet radiation. Human melanocytes express receptors for endothelin-1 (ET B). Treatment of melanocytes immediately after exposure to UV radiation enables them to overcome G1 growth arrest (19).

Because ACE and EDN1 genes can be associated with both angiogenetic and antioxidant properties of the blood vessels during CTCL progression, polymorphic variants of them may advantage and/or disadvantage UV radiation therapy during CTCL. For this reason we studied the possible associations of certain genetic polymorphisms of the skin that were due to UV radiation therapy.

Methods

Subjects:

In this study, 77 patients with CTCL (stage I=35, stage II=28, stage III=14), diagnosed and treated at the First Dermatological Clinic of St. Ann's Faculty Hospital Brno (46 men and 31 women, median age 62, range 26-80 years) were compared to a control non-CTCL group of the similar age and gender distribution (n=203, 137 men and 66 women, median age 54, range 27-86 years) with a negative family history of severe skin diseases and without signs of malignancy.

Diagnosis of CTCL was verified histologically and by immunophenotyping. The patients were treated topically (steroids in 40 cases, cytostatics in 1 case, steroids + cytostatics in 1 case, tar in 1 case), systemically (steroids in 1 case, interferon alpha in 4 cases, retinoids in 7 cases, interferon alpha + retinoids in 1 case, steroids in 1 case) and/or by systemic phototherapy (PUVA in 29 cases, UVB in 9 cases). At the DNA sampling time, all CTCL patients were in partial or complete remission of the disease.

The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno (no. 64/93, 1993). Signed informed consent was obtained from each subject.

Genotyping:

Genomic DNA was isolated from peripheral leukocytes by a standard technique using Proteinase K.

Genotyping of the insertion/deletion polymorphism in angiotensin I-converting enzyme (I/D ACE) was performed according to Rigat et al., 1992 (20) with a subsequent verification of the DD genotype by the method of Shanmugam et al., 1993 (21). Using gel electrophoresis, the polymerase chain reaction (PCR) products were distinguished as a 190 bp fragment in the absence and a 490 bp fragment in the presence of the insertion (genotypes described as II-490 bps, ID-490+190 bps, DD-190 bps).

The G (8002) A polymorphism in intron 4 in endothelin-1 gene was detected according to our protocol. PCR product (primers 5´-CAA ACC GAT GTC CTC TGT A-3 and 5´-ACC AAA CAC ATT TCC CTA TT-3´) was further analyzed by restriction analysis with *Taq* I (T2CGA). On gel electrophoresis with ethidium bromide, three genotypes GG: 150 and 208 bp; GA: 358, 150, and 208 bp; and AA: 358 bp were identified. The -3A/-4A polymorphisms (-138 insertion/deletion) in the gene coding for endothelin-1 were analyzed by the PCR method (primer 5´-GCT GCT TTT CTC CCC GTT AA-3´ and 5´-CAA GCC ACA AAC AGC AGA GA-3´) using the restriction enzyme *Bsi*YI (CCNNNNN2NNGG). The genotypes were determined as -4A/-4A: 195 bp; -4A/-3A: 176, 195, 19 bp; and -3A/-3A: 176 and 19 bp (22).

Statistics:

The differences in genotype and allelic distributions between patients and control subjects as well as consistency of genotype distribution with Hardy-Weinberg equilibrium were tested using the chi^2 test.

The risk of genotypes was evaluated using odds ratio test (OR) with a 95% confidential interval. The

significances of OR as well as heterozygote/homozygote ratio were calculated using Fisher's exact test.

Holm's test for correction of multiple comparisons (Pcorr) was used when appropriate.

Results

No significant differences were found in genotype distributions and /or allelic frequencies between CTCL and non-CTCL subjects in any of the three evaluated polymorphisms.

On the other hand, significant differences were found in genotype distributions of I/D ACE polymorphism between patients treated with phototherapy and those treated without it (Pg=0.005, Tab.1). Heterozygote ID was more frequent in the group treated with phototherapy (25/13 vs. 12/27, OR=4.33, 95% confidential interval 1.67-11.24, P=0.02.). The power of the test is 82% at the P=0.05 (EpiInfo,Version 6.0) for the numbers of included persons. Thus, the heterozygote ID seems to carry an advantage for phototherapy effectiveness in patients with CTCL.

Similarly, a difference in genotype distribution of -3A/-4A EDN1 between CTCL treated with and without phototherapy was observed (Pg=0.04, Tab. 2). The 4A4A variant of -3A/-4A EDN1 is more frequent in patients treated with phototherapy (8/30 vs. 1/38, OR=10.13, 95% confidential interval 1.20-85.55, P=0.01).

Next, a difference in allelic frequencies of G8002A EDN1 polymorphism between CTCL patients with phototherapy and without it was proved (Pa=0.04, Tab. 3). The GA and AA genotypes are more frequent in patients treated with phototherapy compared to those without it (15/23 vs. 7/32, OR=2.98, 95% confidential interval 1.05-8.48, P=0.03).

And last, the 4A4A EDN1 genotype was found in combination with only the GG genotype of G8002A EDN1 genotype, both in patients with phototherapy and without it. The associated genotype 4A4AGG was more frequent in patients treated with phototherapy than in patients without it (8/30 vs. 1/38, OR=10.13, 95% confidential interval 1.20-85.55, P=0.01, Pcorr=0.06). A significant difference in 4A4AGG frequency between phototherapy treated CTCL patients and control subjects was found (8/30 vs. 10/193, OR=5.15, 95% confidential interval 1.88-14.00, P=0.002, Pcorr=0.02).

Discussion

This is the first study identifying ACE (17q23) and EDN-1 (6p24-p23) advantageous polymorphic variants for successful phototherapy in CTCL patients though no significant association with CTCL was proved. Previously, allelic loss was found to be present in 45% of patients with mycosis fungoides and in 67% of patients with Sézary syndrome. In mycosis fungoides, allelic loss was found in 10% of patients with mycosis fungoides and was present on 9p, 10q, 1p and 17p. In Sézary syndrome, high rates of loss of heterozygosity were detected on 9p, 17p and 2p (23).

T-lymphocytes were proved to contain high levels of ACE, approx. 28 times more per cell than monocytes. No activity was detected in B-lymphocytes. The ACE levels of T-lymphocytes vary widely between individuals but were highly reproducible and influenced by I/D polymorphism of the ACE gene. T-lymphocyte levels of ACE are significantly higher in subjects who were homozygote for the deletion (genotype DD) than in the other subjects. Thus, ACE is expressed in T-lymphocytes and the level of ACE expression in cells synthesizing the enzyme is genetically determined (24). Effects of ACE inhibitors such as captopril and enalapril on T-lymphocyte proliferation were confirmed. These effects are unrelated to their ability to inhibit angiotensin-converting enzyme and perturbation of the bioactive peptides such as angiotensin II and bradykinin (25).

The transcriptional up regulation of vascular endothelial growth factor (VEGF) has been linked to a critical mediator of hypoxia signalling, the hypoxic inducible factor-1α (HIF-1α). ET-1 promotes VEGF production through HIF-1α and this mechanism might be responsible for increasing tumor angiogenesis. Degradation of HIF-1α was in fact reduced in ET-1-treated ovarian carcinoma cells under both hypoxic and normoxic conditions. After ET-1 stimulation, HIF-1α protein levels increase in the cells, the HIF-1 transcription complex is formed and binds to the HRE binding site. Therefore, ET-1-induced HIF-1 accumulation activates all the signals necessary for a complete HIF-1 response (26). The HIF-1α-mediated transcription of VEGF by ET-1 under normoxic conditions points to a general mechanism through which oncogenes and growth factors might upregulate VEGF, and could synergize with hypoxia during tumor growth. In tumor cells, ET-1 might be upregulated by hypoxia and could promote angiogenesis by increasing VEGF production through an HIF-1α-dependent mechanism. Thus, under hypoxic conditions, ET-1 potentiates hypoxia stimulus by amplifying HIF-1α stability and VEGF production (27).

Although no significant association of polymorphic variant of ACE and EDN1 genes with CTCL were proved, some genotypes (a heterozygote ID in I/D ACE, a homozygote -4A-4A in -3A/-4A EDN1 and genotypes GA and GG in G8002A EDN1) seem to carry an advantage for phototherapy effectiveness in patients with CTCL.

Acknowledgement

The study was supported by the project CEZ J07/98:141100002 from the Ministry of Education, Youth and Physical Education of the Czech Republic.

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AUTHORS' ADDRESSES

Vladimír Vaškù MD, CSc, Associate Professor, 1st Department of Dermatology, St. Ann´s Faculty Hospital, Pekarská 53, 656 91 Brno, Czech Republic, e-mail: vladimir.vasku@fnusa.cz
Julie Anna Vaškù (medical student), Institute of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Komenského nám. 2, 662 43 Brno, Czech Republic
Monika Pávková Goldbergová PhD, same address
Anna Vaškù MD, CSc, Professor, same address