

Coexpression of CD40 and CD40 Ligand in Cutaneous T-Cell Lymphoma (Mycosis Fungoides)¹

Monique Storz, Karoline Zepfer, Jivko Kamarashev, Reinhard Dummer, Günter Burg, and Andreas C. Häffner²

Department of Dermatology, University Hospital of Zurich, CH-8091 Zurich, Switzerland

Abstract

Microarray analysis is a promising new approach for creating specific expression profiles of multiple genes simultaneously. We quantitatively analyzed differential gene expression patterns in mycosis fungoides-derived clonal T cells and autologous, identically cultured CD4⁺ lymphocytes using microarrays containing 588 cDNA segments from genes relevant to cell signaling, carcinogenesis, and apoptosis. Among other dissimilarities, neoplastic T cells showed coexpression of CD40 (Bp50) and CD40 ligand (gp39, CD154). These results could be corroborated by reverse transcription-PCR, immunohistochemistry, and two-color immunofluorescence staining. Our data suggest that in cutaneous T-cell lymphoma, CD40/CD40 ligand interactions might represent a paracrine loop that is crucial not only in preventing apoptosis or positively regulating growth but also in homing of neoplastic cells to the skin.

Introduction

CD40 receptor is a transmembrane protein and has been clustered as a member of the tumor necrosis factor/nerve growth factor receptor superfamily (1). It has been extensively studied and demonstrated to be present on a variety of cell types ranging from benign B cells, monocytes, dendritic cells, endothelial cells, keratinocytes, fibroblasts, and thymocytes (2–5) to neoplastic B-cell non-Hodgkin's lymphomas and leukemias, Reed-Sternberg cells in Hodgkin's disease, myeloma plasma cells, CTCLs,³ a number of carcinomas (including bladder, breast, and ovarian cancer), epidermal tumors, and melanoma cells (6–11). Low-level CD40 expression is also found on some human T-cell lines and on activated peripheral blood T cells (12). Its counterpart, CD40L, which shares significant sequence homology to tumor necrosis factor, is transiently induced and tightly regulated on the surface of CD4⁺ T cells after their activation (13) but is also weakly expressed on some activated CD8⁺ T cells, basophils, mast cells, eosinophils, natural killer cells, and monocytes (14). A consistent fraction (>40%) of peripheral T-cell non-Hodgkin's lymphomas and CTCLs displaying a CD4⁺/CD8[−] phenotype, along with a subset of T-lineage acute lymphoblastic leukemias with stem cell-like phenotype, constitutively display surface CD40L (15).

The CD40/CD40 L interaction is known to be an important feature of B-cell/T-cell collaboration, leading to T-cell-dependent activation, proliferation, and differentiation of B lymphocytes; immunoglobulin isotype switching; and memory B-cell formation (16). Mutations of the *CD40L* gene have been associated with X-linked hyper-IgM immunodeficiency syndrome, pointing to the critical role of the

CD40/CD40L interaction in the T-cell/B-cell interplay (17). In addition, several groups have implicated CD40 in the regulation of B-cell survival via molecules of the Bcl family (18) and of B-cell apoptosis via Fas (19).

It has been reported that binding of CD40L to its CD40 receptor may activate not only B lymphocytes but T lymphocytes as well and that CD40L can act as a stimulatory molecule for T lymphocytes (20). The fact that neoplastic CD4⁺ T cells constitutively express detectable amounts of surface and/or cytoplasmic CD40L molecules indicates a possible physiological role of this molecule in these neoplasms (21). Further data demonstrated coexpression of CD40 and CD40L in B-cell lymphoma cells, raising the possibility of an autocrine loop that may contribute to the growth regulation of malignant B cells *in vivo* (22) and may potentially exist at the T-cell branch as well.

Comparative microarray analysis is a new and promising approach to establish gene expression profiles that may finally help to delineate the intricate interactions responsible for pathogenesis and clinical phenotype of this particular disease (23, 24).

We performed comparative analyses of clonal lymphocytes derived from MF lesions and their autologous CD4⁺ counterparts originating from peripheral blood by microarray and obtained further confirmation by RT-PCR, immunohistochemistry, and two-color immunofluorescence staining.

Materials and Methods

Cell Culture. All human material was obtained with the patient's informed consent. Lymphocytic cells from a diagnostic MF tumor sample were cultured for 8 weeks in 500 ml of RPMI 1640 supplemented with 100 ml of 10% FCS, 10 ml of fungizone/streptomycin, 10 ml of glutamin, 5 ml of sodium pyruvate (all from Life Technologies, Inc., Grand Island, NY), 100 μ l of human epidermal growth factor (1 μ g), 200 ml of interleukin 2 (10⁶ units/ml), 2 ml of interleukin 4 (50,000 units/ml), 2 ml of granulocyte macrophage colony-stimulating factor (80,000 units/ml), and 400 μ l of basic fibroblast growth factor (250,000 units/ml; all from Becton Dickinson, San Jose, CA). Clonality of the morphologically homogenous tumor cell population was determined by PCR/denaturing gradient gel electrophoresis as described previously (25) and compared with original cryopreserved biopsy material.

Autologous peripheral CD4⁺ T cells were cultured for 3 days under identical conditions to normalize for tissue culture artifacts.

Separation and Activation of Autologous CD4⁺ Cells from Peripheral Blood. CD4 cells were separated by an immunomagnetic procedure using paramagnetic polystyrene beads (Dynal, Lake Success, NY) and a magnetic separation device (Dynal). An antihuman CD4-specific murine IgM antibody (DAKO, Glostrup, Denmark) was conjugated to Dynabeads M-450 (Dyna) by overnight incubation at pH 9.5, washed twice in PBS containing 10% FCS, and incubated under constant slow agitation with peripheral blood mononuclear cells generated by Ficoll gradient enrichment from the patient's blood at 4°C. Purified CD4⁺ T cells were stimulated by exposure to 10 μ g/ml phytohemagglutinin (Life Technologies, Inc.) and harvested on day 3.

RNA Isolation. Total mRNA was extracted from CTCL cell culture and peripheral blood mononuclear cell culture using RNeasy Mini Kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instructions.

RT-PCR. RNA samples (500 ng) were reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) according

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² To whom requests for reprints should be addressed, at Department of Dermatology, University Hospital of Zurich, Gloriastrasse 31, CH-8091 Zurich, Switzerland. Phone: 41-1-255-4049; Fax: 41-1-255-4403; E-mail: ach5@derm.unizh.ch.

³ The abbreviations used are: CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; CD40L, CD40 ligand; RT-PCR, reverse transcription-PCR.

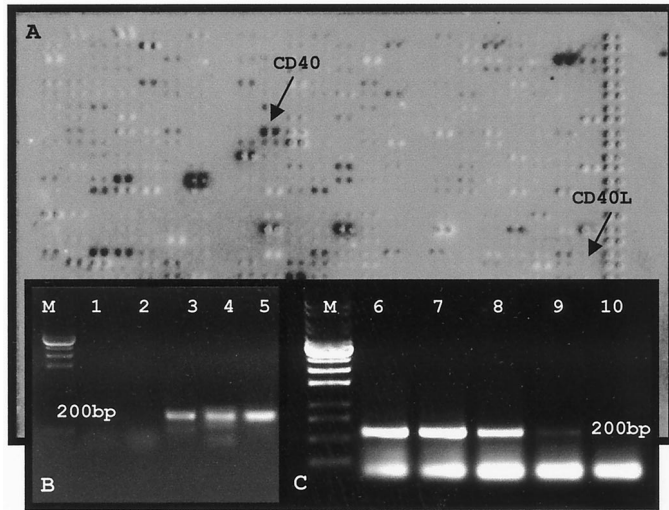


Fig. 1. A, cDNA microarray (Human Cancer; Clontech) showing differentially expressed genes resulting from subtraction of profiles generated from CTCL-derived T cells and autologous CD4+ T lymphocytes. The arrows indicate subtractive hybridization signals to the CD40 gene segment and CD40L sequence (there is no visible signal in the subtractive array because the ligand is expressed in activated CD4+ and CTCL cells). B, RT-PCR for CD40 in patients with MF (Lanes 3–5). Lanes 1 and 2, negative controls; M, marker. C, RT-PCR for CD40L in patients with MF (Lanes 6–9). Negative control, Lane 10.

to the manufacturer's instructions. cDNA was amplified using primer pairs specific for CD40 (sense primer, 5'-CTGTTTGCCATCCTCTTGGT-3'; antisense primer, 5'-CGACTCTCTTGGCCATCCTC-3') and CD40L [sense primer, 5'-ACCAAAGTCTCTCCCGATTC-3'; antisense primer, 5'-GCAAA-AAGTGCTGACCCAAT-3' (both sets of primers were from Microsynth; Balgach Switzerland)]. Cycling conditions were as follows: 35 cycles of 94°C for 30 s; 55°C for 30 s; and 72°C for 30 s. Ten μ l of amplified cDNA were run on a 1.5% agarose gel.

cDNA Microarray. Total mRNA was reverse transcribed using the cDNA Synthesis (CDC) Primer Mix including [α -³²P]dATP into radioactively labeled cDNAs (Atlas TM Pure Total Labeling System; Clontech, Palo Alto, CA). The probes were then hybridized to separate Atlas Arrays according to the manufacturer's instructions (Atlas TM cDNA Expression Array; Clontech).

Immunohistochemistry. Cryosections from 13 patients with MF were fixed in acetone, air dried, and stained for CD40 and CD40L using an alkaline phosphatase-anti-alkaline phosphatase technique (reagents were from DAKO) as described previously (26). The adequacy of the immunohistochemical stainings was always confirmed by positive and negative controls (data not shown). Routine H&E staining was performed on adjacent sections to verify the diagnosis.

Direct Immunofluorescence Two-color Staining. Acetone fixed cryosections were washed with PBS, blocked with protein block serum-free solution (DAKO), and washed again with PBS. One μ l of FITC-conjugated CD40 monoclonal antibody (CALTAG, Burlingame, CA) and 1 μ l of R-phycoerythrin-conjugated CD40L monoclonal antibody (DAKO) were each diluted in 20 μ l of antibody diluent (ChemMate; DAKO) and then deposited on the cryosection slides and incubated for 1 h at 4°C. After three washes in PBS, localization of CD40/CD40L was observed under a fluorescence microscope using the appropriate filters, photographed, and digitally superimposed.

Results and Discussion

The aim of this investigation was to define distinct phenotypic aspects in T lymphocytes derived from MF lesions using microarray analysis and different molecular biological and immunohistochemical methods. We consistently found constitutive expression of CD40 and CD40L in neoplastic T-cell populations. The results of the microarray analysis (Fig. 1) are supported by those of two-color immunofluorescence, showing colocalization of CD40/CD40L (Fig. 2; Table 1). Because RNA was extracted from cultured tumor cells, a contamina-

tion of microarray expression profiles from signals caused by fibroblasts or keratinocytes, which would occur using lesional tissue, can be reasonably excluded.

Previous data suggest that CD40L+ neoplastic T cells may be relevant for the process of selective tumor homing into the skin (13). Especially in the early stages of MF, CD40 antigen presentation by endothelial cells and basal keratinocytes, as well as other epidermal cells, could explain the epidermotropism of the proliferating clonal

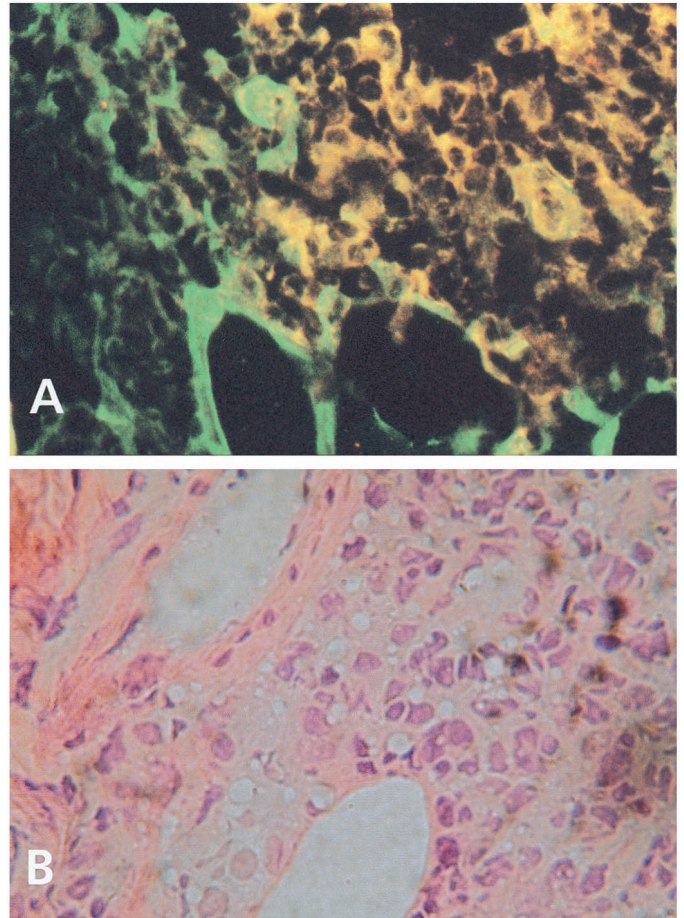


Fig. 2. A, direct immunofluorescence double staining for CD40 (FITC-conjugated antibody, green) and CD40L (R-phycoerythrin-conjugated antibody, red). Cells with overlapping expression stain orange to yellow and demonstrate colocalization of CD40/CD40L. Membrane-anchored CD40 surface antigen on endothelial cells of a capillary vessel is easily detectable by green fluorescence ($\times 40$). B, routine H&E staining was performed on adjacent sections to verify the diagnosis of MF ($\times 40$).

Table 1 Characteristics of patients investigated, including expression intensity of CD40/CD40L staining in immunohistochemistry

No.	Patient	Age (yrs)	Sex	Diagnosis	Stage	CD40	CD40L
1	H.A.	56	M	MF ^a	Ib	+++	+
2	H.W.	52	M	MF	Ia	+++	++
3	H.H.	71	M	MF	Ia	+++	+
4	M.J.	70	M	MF	Ia	++++	++
5	L.D.	59	M	MF	Ib	++++	+
6	B.D.	24	M	MF	Ib	+++	+
7	M.L.	80	M	MF	Ib	+++	+
8	G.W.	57	M	MF	Ib	+++	+
9	D.K.	69	M	MF	Ib	+++	+
10	K.H.	52	F	MF	III	+++	+
11	S.A.	78	F	MF	Ia	+++	++
12	B.B.	52	M	MF	IVb	+++	+
13	G.G.	53	F	MF	Ia	+++	++

^a According to the EORTC classification for primary cutaneous lymphomas, MF is a epidermotropic CTCL characterized by a proliferation of small or medium-sized neoplastic T lymphocytes with cerebriform nuclei (30).

population of CD40L+ T cells (21). Furthermore, CD40L has been described as T-cell growth factor (20). It has also been shown that the CD40/CD40L costimulation pathway allows selective expansion of CD4+ T cells after interaction with CD40-bearing antigen-presenting cells (27). Our findings are consistent with those of previous reports that demonstrated expression of CD40 and its ligand in CTCL and indicated that the CD40/CD40L interaction is likely to play an important role in the control of neoplastic T-cell growth (21). This report is the first to show that CD40/CD40L surface antigens are coexpressed in MF lesional lymphocytes and potentially provide the molecular bridge for direct stimulating contact between adjacent cells. It is intriguing that the T-cell-T cell interaction itself might be responsible for enhancing T-cell survival and regulating malignant growth in the form of autocrine or paracrine loops, analogous to previous results shown in B-cell lymphoma cells (22). In addition, *in vitro* observations of CTCL cell cultures revealed a typical growth behavior with monolayer formation by closely connected cells, in which paracrine mechanisms could play a crucial role. Disruption of this paracrine/autocrine loop might severely impair the survival and growth of neoplastic T cells and thus prove beneficial in the immunotherapy of cutaneous T-cell lymphoma.

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