Aberrant Expression of T-Plastin in Sezary Cells

Ming-wan Su, Irene Dorocicz, Wieslawa H. Dragowska, Vincent Ho, Gang Li, Nicolas Voss, Randy Gascoyne, and Youwen Zhou

ABSTRACT

Mycosis fungoides (MF) and its leukemic variant, Sezary syndrome (SS), are the most common cutaneous T-cell lymphomas, with a combined incidence of 0.36 of 100,000 person-years. Although thought to be closely related to mature T-helper cells, the true nature of the cancer cells in MF/SS is unknown. In addition, there is no known specific marker for MF/SS cancer cells, which can result in difficulties in the diagnosis and treatment. To identify MF/SS-specific markers, Sezary cancer cells were analyzed with a global genomic screening tool, the modified representational difference analysis. It was discovered that unlike T-helper cells from healthy individuals or patients with nonmalignant dermatoses, Sezary cells from most patients with Sezary syndrome aberrantly expressed T-plastin mRNA and protein. This is the first time T-plastin protein, a cytoplasmic protein regulating actin assembly and cellular motility, has been detected in the hematopoietic cells. Therefore, T-plastin has the potential to be a Sezary cell-specific marker valuable for diagnostic and treatment of Sezary syndrome.

INTRODUCTION

MF and SS are the most common CTCLs, with a combined incidence of 0.36 of 100,000 person-years (1). They account for 2–3% of all lymphomas and >70% of all CTCLs. Although MF primarily affects the skin, in advanced stages, it can enter the blood and the lymph nodes, becoming leukemic. SS is an uncommon, leukemic form of MF, accounting for ~10% of all MF cases. Clinically, SS patients have erythroderma with two additional features: lymphadenopathy (enlarged lymph nodes) and tumor cells (the Sezary cells) in the peripheral blood. Although most MF patients follow an indolent course, with a life span approaching normal, SS has a poor prognosis with 10-year survival of 10–20% (2–4). Therefore, the development of Sezary cells denotes a poor prognosis.

MF cells are atypical T cells that most closely resemble mature memory T-helper cells in the skin (CD2+, CD3+, CD4+, CD5+, CD45RO+, CD8−, and CLA+; Ref. 2). Their atypical features include abnormal morphology and biological behavior. In morphology, these cells are 10–50% larger than normal T-helper cells and have hyperchromatic nuclei as seen under light microscopy. Under electronmicroscopy, the nuclei appear hyperconvoluted, having many grooves and lobules. In biological behavior, the MF cells have abnormal epidermal tropism (residing in the epidermis, unlike normal T-helper cells), frequent loss of certain cell surface antigens such as CD7 (5–7) and CD26 (8), monoclonal rearrangement of the TCR gene (9–13), and aberrant but nonuniform chromosomal abnormalities (14–19). Loss of heterozygosity at the 10q23.3 locus was found in some cases of MF, implicating PTEN tumor suppressor gene abnormality might be involved (19). However, this is not likely to be applicable for all cases because most patients’ MF cells do not have this deletion. Because viral particles or genetic sequences were found by some researchers in MF cells or their culture medium, retroviral infection or activation was suggested to be pathogenically important (20–27). However, the viral etiology has not been substantiated by other studies (28–30).

Sezary cells are closely related to and in many cases derived from cutaneous MF cells. They share many of the morphological and biological abnormalities with the MF cells, including nuclear atypia, mature T-cell-like immune phenotype, frequent TCR gene rearrangement, and chromosomal rearrangements, including PTEN gene abnormalities in some cases (2). However, there are several important differences. These include more advanced nuclear atypia, with more pronounced hyperconvolution that is visible under light microscopy, loss of the CLA skin-homing antigen, gaining the ability to circulate in the peripheral blood, and secretion of TH2 cytokines (31–34), instead of TH1 cytokines of cutaneous MF cells. The full scope of the relationship between Sezary cells and MF cells is not clear.

Several research groups have reported the identification of molecular markers specific for Sezary cells or MF cancer cells such as CD40, CD40 ligand (35), p140/KIR3DL2 (36), and SC5 (36, 37). However, the potential for these molecules as diagnostic markers is limited because they can also be found on some normal cells present in the peripheral blood of non-MF and non-SS patients. For example, CD40 and CD40 ligand are widely present in several types of blood cells, especially activated T cells (38, 39). The p140/KIR3DL2 is a natural killer cell receptor and normally is expressed in NK cells and CD8+ T cells (36). Finally, although Sezary patients contain a higher percentage of SC5+ CD4+ T cells (16–83%, with a median of 32%), normal individuals also have significant number of CD4+ T cells expressing this molecule (3–12%, with a median of 11%). In addition, SC5 is normally present in a number of other cell types in the peripheral blood, including CD45RO+ subsets of CD4+ and CD8+ T cells, natural killer cells, monocytes, and B lymphocytes (36, 37). Therefore, they are not truly specific for Sezary cells.

To better understand the molecular characteristics of Sezary cells and to clarify their relationship to MF cancer cells and normal T-helper cells, it is necessary to identify genes that are specifically or preferentially expressed in Sezary cells. In this article, a modified procedure of RDA was used to identify one such gene, encoding the T-isofrom of the human plastin. This cytoplasmic protein was aberrantly expressed in Sezary cells of most Sezary patients but not in normal T cells or other cells in the peripheral blood of non-SS patients.

MATERIALS AND METHODS

Patients. Nine patients with SS were recruited from the Skin Lymphoma Clinic of British Columbia Cancer Agency with informed consent. They all had erythroderma, lymphadenopathy, and >5% lymphocytes in peripheral
haptenized peptide was injected into two rabbits (terminus). After conjugation with a protein (keyhole limpet hemocyanin), the (T-plastin, L-plastin, and I-plastin; Ref. 41, 42). The NH2-terminal 11 amino were purified by negative selection from the peripheral blood with monoclonal collected from the brachial vein into EDTA-containing tubes. Sezary cells Fifty to 100 ml of peripheral blood were recruited. benign dermatoses such as psoriasis and atopic dermatitis were also recruited. CD7 type-based methods, we only included patients with Sezary cells that had CD7− immune phenotype. As controls, 37 subjects with no skin disease or benign dermatoses such as psoriasis and atopic dermatitis were also recruited. Purification of Sezary Cells and Normal T-Helper Cells from Peripheral Blood and Flow Cytometry. Fifty to 100 ml of peripheral blood were collected from the brachial vein into EDTA-containing tubes. Sezary cells were purified by negative selection from the peripheral blood with monoclonal antibodies directed against the granulocytes. B cells, CD5+ T cells, and CD7+ T cells using custom designed Rosette Sep kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). For purification of normal CD4+ T-helper cells from the peripheral blood of non-Sezary subjects, similar procedures were used, except that the CD7 antibody was omitted. The purity of the resultant Sezary cells and nonmalignant CD4+ T cells was verified by flow cytometry using phycoerythrin-conjugated anti-CD7 monoclonal antibody and fluorescein-conjugated anti-CD4 antibody (Beckman Coulter, Miami, FL).

MRDA on mRNA from Sezary Cells and Normal CD4+ T Cells. mRNA from Sezary cells and CD4+ T cells from healthy individuals were purified using Fasttract RNA isolation Kit (Invitrogen, Carlsbad, CA). After reverse transcription, the cDNA was digested with Sau3AI and used for RDA (40). A modified protocol of RDA was used. The modifications included elimination of restriction digestion of the driver amplicons, elimination of gel electrophoresis fractionation of the tester representations, addition of single-step spin column (Amersham Biosciences) purification of the tester and driver amplicons for the removal of excessive primers, and purification of the RDA primers (Life Technologies, Inc.) with high-pressure liquid chromatography. Two-way RDA was carried out, with Sezary cells cDNA and normal T-cell cDNA each serving as tester and driver in two parallel RDA reactions. For each round of RDA reaction, 0.4 μg of tester and 40 μg of driver materials were used. After three rounds of RDA, each with 28 cycles of amplifications, distinctive bands identified after agarose gel electrophoresis were cloned into pBluescript sequencing vector (Stratagene, La Jolla, CA) and their sequence determined. RT-PCR was used to confirm the transcript levels of the sequences identified. The primers and the expected length of the products are listed in Table 1.

Antibody Preparation. Unique regions of the T-plastin gene were identified by pair wise comparison between three isoforms of human plasmins (T-plastin, L-plastin, and I-plastin; Ref. 41, 42). The NH2-terminal 11 amino acid fragment was unique for T-plastin and an oligo peptide corresponding to this region was synthesized (TP-1: MATTQISKDEC, AA 1 to 11 of the NH2 terminus). After conjugation with a protein (keyhole limpet hemocyanin), the haptenated peptide was injected into two rabbits (α Diagnostic International). The sera were collected at 7, 9, and 12 weeks. The titers were determined using ELISA as well as Western blot against recombinant T-plastin produced in bacterial cells as described previously (42).

Detection of T-Plastin Protein in Purified Sezary Cells and Control T Lymphocytes. One to 5 million Sezary cells from Sezary patients and equivalent numbers of normal T-helper cells from healthy individuals were extracted to isolate total cellular protein using the tris lysis procedure and used for Western blot analysis according to standard protocol (43). The above-produced polyclonal T-plastin antibody was used for the detection of T-plastin protein. As a positive control, human T-plastin produced in Escherichia coli using the pET 21D vector (Novogen) was used. To serve as reference for antibody specificity, the human L-plastin and I-plastin expressed similarly were also analyzed using the same antibody. The construction of expression vectors for the plastin expression was essentially the same as reported by Lin et al. (42). Healthy volunteers’ T-cell extracts were used as negative controls.

Statistic. The levels of expression of the T-plastin transcripts from Sezary cells and non-Sezary CD4+ T cells were analyzed using Student t test.

RESULTS

Sezary Patients and Sezary Cells. Nine SS patients were identified from the database of the British Columbia Cancer Agency Skin Lymphoma Clinic and recruited for this study with informed consent. These patients consisted of 5 men and 4 women with ages ranging from 50 to 80 years (mean, 67.5 years). Three patients had preexisting diagnosis of MF, although the other 6 did not. They all had typical SS presentations with erythroderma, lymphadenopathy, and Sezary cell number >5% in the peripheral blood (5−98%). They all had phenotypic loss of CD7 antigen. Two patients had received chlorambucil chemotherapy, although 7 patients did not. Six patients had TCR gene clonality in the peripheral blood or in skin biopsies, with the other 3 patients being negative for clonality.

For isolation of the Sezary cells or the CD4+ T cells from the peripheral blood, between 50 and 100 ml of venous blood were obtained from each patient or volunteer. More than 50 million of Sezary cells and CD4+ T cells can be obtained from a single such blood donation. After cell purification as described in “Materials and Methods,” the purity of the Sezary cells and normal T lymphocytes was examined with fluorescence-activated cell sorting analysis using monoclonal anti-CD4 and anti-CD7 antibodies (Fig. 1). Over 90% purity by immune phenotyping can be consistently obtained.

Identification of Candidate Sezary-Specific Genes with MRDA. After three rounds of MRDA using Sezary cDNA as the tester and the normal T cells as the driver, three distinct bands can be visualized on agarose gel electrophoresis (Fig. 2). These bands were then subcloned into pBluescript SK sequencing vector and their nucleotide sequences determined. The GenBank database was searched to determine the identity of these fragments. One was found to be the CD45 gene (44). Another fragment corresponded to a region on chromosome 6 that was
not known to contain a transcribed gene thus far (unpublished result). The third fragment is identified as the T-plastin gene, which encodes a cytoplasmic protein involved in the assembly of actin intermediary filaments that are required for cell migration and maintenance of cellular architecture (42).

Specific Expression of T-Plastin in Sezary Cells. To determine the levels of transcription of the three candidate genes, RT-PCR was performed using primers located outside the positions corresponding to the fragments isolated as a result of RDA. These primers were designed to minimize potential cross contamination (Table 1). The results showed that CD45 mRNA was similarly expressed in both Sezary cells and normal T-helper cells. The level of transcripts from the unknown gene (MW5) was variable among the Sezary cells and the non-Sezary T cells tested. The T-plastin gene was detected in significant levels only in Sezary cells (Fig. 3). We therefore chose to focus on T-plastin for subsequent studies.

To quantify the level of T-plastin expression, semiquantitative analyses on 9 Sezary patients and 37 non-Sezary patients were performed. As shown in Fig. 4, Sezary cells on average express 20 times more T-plastin transcripts as compared with normal T-helper cells or T-helper cells from patients with non-Sezary, benign skin conditions such as psoriasis, rosacea, alopecia areata, drug hypersensitivity syndrome, and atopic dermatitis. This difference is statistically significant ($P < 0.001$). To determine whether the difference between Sezary patients and non-Sezary patients can be demonstrated directly from unfractinated whole blood, RT-PCR was performed on mRNA isolated from peripheral blood from 1 Sezary patient and 1 non-Sezary patient. As shown in Fig. 5, T-plastin transcripts can be readily detected from the Sezary whole blood but not from the normal volunteer’s blood.

To determine whether the T-plastin transcripts were associated with protein expression, Western blot analyses was performed using a polyclonal antibody generated from synthetic oligopeptides corresponding to the NH$_2$-terminal 11 amino acids (see “Methods and Materials”). As can be seen in Fig. 6, a signal was detected from a Sezary cell preparation, whereas normal T-helper cell preparation from a healthy individual did not express this protein. This expression was specific to the T-plastin gene because L-plastin and I-plastin expressed in E. coli did not react with the antibody, unlike the T-plastin.

Differential Expression of the Three Isoforms of Plastins. The plastins represent a family of cytoplasmic proteins that bind and modulate the actin intermediate filaments. To determine whether aberrant expression of the plastins was only limited to the T-plastin, RT-PCR was carried out for all three plastin isoforms (T-plastin, I-plastin, and L-plastin). As shown in Fig. 7, L-plastin levels were the same in all samples tested, Sezary or non-Sezary. I-Plastin was not found in any of the samples tested. In contrast, T-plastin was only found in the Sezary cells, whereas none of the other T cells showed detectable levels of the T-plastin expression.

DISCUSSION

To date, little is known about the molecular nature of the cancer cells involved in SS and MF. Because it is difficult to isolate large...
numbers of MF cells from skin biopsies, whereas the Sezary cells are readily purified from patients’ peripheral blood, the Sezary cells were chosen to serve as the study material. Once unique molecular features are identified for the Sezary cells, subsequent experiments can be performed to determine whether the cancer cells of MF and other CTCLs also share these features. Here, we report that aberrant mRNA and protein expression of the T-plastin gene only occurs in Sezary cells, not in normal T-helper cells or whole blood from healthy volunteers and patients with non-Sezary skin diseases.

T-plastin is a member of a highly conserved cytoplasmic protein family that has three members in the human genome, T-plastin, I-plastin, and L-plastin. The estimated molecular weight for each plastin is \( M_r \) 68,000 (627 amino acids). There are 67% of amino acid identity among the three plastin proteins (45, 46). The plastins regulate cell structure and motility by binding to cellular actins. Although thought to have similar functions, the distribution of the plastins is highly isoform specific. L-plastins are highly expressed in all leukocytes and up-regulated in many malignancies, including T-cell and B-cell malignancies (47–49). However, we did not observe any significant differences between Sezary and non-Sezary T cells. The I-plastin is restricted to the intestine and the kidney tissues (42, 50). This is confirmed in our study in that none of the Sezary or non-Sezary T cells showed expression of I-plastin. The significance of this observation is unknown. The T-plastin is normally absent in hematological cell types but widely distributed at low levels in other tissue types and nonhematopoietic malignancies (50). Our results suggest that T-plastin is the only one of the plastin protein family to be differentially expressed between Sezary cells and normal T cells from the peripheral blood.

The difference in mRNA levels of T-plastin between Sezary cells and non-Sezary cells is striking (20-fold) and statistically significant (\( t \) test, \( P < 0.001 \)). Furthermore, the difference between Sezary cell and non-Sezary cell T-plastin mRNA is so large that the aberrances are still evident by comparative analysis of the whole-blood total RNA from Sezary and non-Sezary patients (Fig. 5). Recently, T-plastin mRNA was detected in 78% of SS patients and in no controls using unseparated peripheral blood mononuclear cells (51). This significant difference was noted despite large variations in the number of Sezary cells (5–99%) present in the peripheral blood mononuclear cell preparations. Both our RNA and protein data on purified Sezary cells now demonstrates that disease-specific T-plastin expression is restricted to the Sezary cells among the hematopoietic cell types. This conclusion is also consistent with previous reports that among all tissues/cells surveyed, T-plastin was shown to be absent from all hematopoietic cell types or whole blood studied and from malignant B-cell and T-cell lines studied (Sezary cells were not studied in these studies; Ref. 52).

Previously, several other groups have reported molecular characterization of the Sezary/MF cells (8, 36, 37, 53–56). Loss of several cell surface antigens in Sezary cells and MF cells, including CD7 and CD26, has been reported previously (53, 54). However, no specific SS or MF cancer cell markers have been described that consistently distinguish malignant cells in SS or MF from other malignancies of the blood or from their normal counterparts such as the T-helper cells. Recently, Nikolova et al. (36, 37) described two cell surface antigens (p140/KIR3DL2 and SC5) that were preferentially expressed in Sezary cells. However, in activated T cells, expression of these antigens
was also found. In addition, many normal cell types such as the CD8+ T cells and natural killer cells also express these antigens. Therefore, the diagnostic value of these antigens remains unclear. The T-plastin expression in most Sezary cells and absence of its expression in any of the other hematopoietic cell types or whole blood surveyed thus far suggests that T-plastin may prove to be more specific than the previously reported markers.

The relationship between Sezary cells and MF cells has not been clearly defined. It is generally known that at least in some cases of SS, the Sezary cells are a direct derivative of the MF cells in MF stage. This conclusion is supported by the observation that some patients have antecedent diagnosis of MF and that the Sezary cells in the Sezary stage and the MF cells have the same cytogenetic abnormalities such as the rearrangement of the same chromosomal locations. However, in other cases, there is no identifiable MF stage before the development of the SS. It is noted that 3 of the 8 T-plastin-positive patients had preexisting diagnosis of MF, whereas the other 3 did not. Therefore, aberrant T-plastin expression does not seem to be dependent on antecedent MF disease status of the Sezary patients.

The pathogenic significance of T-plastin protein in the development of Sezary cells remains to be determined. T-plastin protein functions in the formation of actin bundles that are required for cell locomotion and maintenance of the cellular architecture. Cultured fibroblasts that overexpressed T-plastin showed increased mobility and altered cellular architecture (with more cytoplasmic pseudopods formed; Ref. 41). Sezary cells are known to contain cytoplasmic abnormalities (more abundant cytoplasm) and unique nuclear structures (57). It is possible that T-plastin is contributory to these structural abnormalities. Sezary cells also express high levels of L-plastin, the dominant plastin isoform in hematopoietic cells. All plasmins are thought to have similar functions in cell locomotion and maintenance of cellular architecture. However, there may be important functional differences between L- and T-plasmins, and their functions are not interchangeable. In cultured LLC-PK1 cells (polarized epithelial cell line), T-plastin expression induced shape change of the microvilli, whereas L-plastin failed to do so (41). The relative contribution of T- and L-plastin isoforms to the cellular function of Sezary cells is unclear.

The mechanism of aberrant T-plastin expression in Sezary cells is unknown. Previous investigations indicate that at least two mechanisms were implicated in the suppression of T-plastin expression in leukocytes (52). One is binding of an upstream T-plastin enhancer sequence element located in the promoter region of the T-plastin gene. This region was not bound in cells (such as fibrosarcoma and breast cancer cell lines) that express the T-plastin gene (52). The other is presence of methylation of CpG islands in the T-plastin gene promoter region of leukocytes but not T-plastin positive, nonleukocyte-cell cultures (52). It remains to be determined if the aberrant expression of the T-plastin is attributable to a change of the enhancer elements (such as sequence mutation or methylation), or to the changes of the transcription factors required for T-plastin expression, or both. Additional experiments are needed to clarify these possibilities.

In summary, we describe the discovery of aberrant T-plastin mRNA and protein expression in Sezary cells. The full biological and clinical value of this finding remains to be clearly defined. Future experiments are in progress to fully define the mechanism, biological function, and clinical value of aberrant T-plastin expression in Sezary cells and to determine whether related T-cell lymphomas such as MF also share T-plastin abnormalities.

ACKNOWLEDGMENTS

We thank Drs. Harvey Lui, Jan P. Dutz, David McLean and Mark Pittelkow for invaluable discussions regarding this research.

REFERENCES


