Differentially-expressed genes identified by suppression subtractive hybridization in the bone marrow hematopoietic stem cells of patients with psoriasis

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Received July 6, 2013; Accepted February 24, 2014

DOI: 10.3892/mmr.2014.2203

Abstract. Psoriasis is a T cell-mediated, chronic, relapsing and inflammatory cutaneous disorder. The dysfunctional activity of T cells in patients with psoriasis is attributed to bone marrow hematopoietic stem cells (BMHSCs). To understand the pathogenic roles of BMHSCs in psoriasis, a differential gene expression analysis was performed using suppression subtractive hybridization of the BMHSCs from a patient with psoriasis and a healthy control. Using a cDNA array dot blot screening to screen 600 genes from forward- and reverse-subtracted cDNA libraries, 17 differentially-expressed sequence tags (ESTs) were identified. The genes within the ESTs were observed to be the homologs of genes that are involved in various cellular processes, including hormone signaling, RNA catabolism, protein ADP DNA base melting, transcriptional regulation, cell cycle regulation and metabolism. CD45, which was overexpressed in the psoriatic BMHSCs, was further analyzed using relative quantitative polymerase chain reaction. In addition, the levels of CD45 in the peripheral blood cells (PBCs) of the patients with psoriasis were markedly increased and closely associated with disease severity. An abnormality of hematopoietic progenitor cells, e.g., CD45 overexpression, may be transferred to PBCs via hematopoiesis, and may account for the psoriasis-inducing properties of activated T cells.

Introduction
Psoriasis is a T cell-mediated, chronic, inflammatory and hyperproliferative cutaneous disorder that affects ~2% of the general population. Activated T cells are found in psoriatic plaques and in the circulation, and have been shown to set off a series of cellular and molecular reactions resulting in the creation of psoriatic lesions (1-3). Reagents that inhibit T-cell activation or function, and that have been shown to have efficacy in the treatment of psoriasis include specific immunosuppressive agents, immunomodulatory drugs, fusion proteins that block T-cell activation or the anergizing of T cells, cytokines and biologics that inhibit T-cell migration (4-8). However, recurrence or exacerbation often occurs following disease resolution when treatment with these agents is suddenly halted, which indicates that T-cell activation is not effectively blocked. It is believed that pathogenic T cells are activated in the periphery by a variety of infectious and non-infectious exogenous and endogenous factors (1). However, there is evidence that intrinsic factors play more important roles than extrinsic factors in the pathogenesis of psoriasis (9,10). These intrinsic factors may be involved in spontaneous T-cell activation or proliferation, the regulation of cytokine production pathways, hematopoietic cell development, and T-cell development in the thymus (11).

In humans, bone marrow-derived T-cell progenitors express cluster of differentiation (CD)34 and CD45 (12). These hematopoietic progenitor cells from the bone marrow travel through the blood to seed the thymus, attracted by chemokines, including chemokine (C-C motif) ligand 25 (13). The CD4/CD8-double-negative (DN) population contains the early thymic progenitor cells in the thymus, and the DN1 population is home to the earliest thymocyte progenitors at the corticomedullary junction. The outward migration of these cells to the cortex in response to specific chemokines, including chemokine (C-X-C motif) ligand 12, is accompanied by their differentiation into DN2 and DN3 cells (14). Differentiation into DN4 cells and subsequently, into CD4/CD8-double-positive (DP) cells, occurs along with the inward migration of the cells from the cortex to the medulla, where selection and maturation into
CD4 or CD8 single-positive (SP) T cells takes place. Having escaped negative selection, the positively-selected thymocytes mature into naive T cells and leave the thymus to enter the peripheral circulation (15).

Several observations indicate a pathogenic role for bone marrow hematopoietic stem cells (BMHSCs) in psoriasis. Firstly, Zhang et al. reported that in vitro-differentiated T cells from bone marrow CD34+ progenitor cells of patients with psoriasis are functionally similar to the circulating T cells of these patients. The differentiated T cells from the studied patients with psoriasis showed higher proliferation and a marked capacity to secrete Th1 cytokines in response to streptococcal superantigen, and to induce the overexpression of C-myc and Ki67, but not B-cell lymphoma-extra large (Bcl-XL), in keratinocytes (16). Secondly, a study of high-proliferative potential colony-forming cells (HPP-CFCs) isolated from patients with psoriasis revealed that the bone marrow of patients with psoriasis is pathogenic by its very nature, and is deficient for bone marrow hematopoietic cells (17). Thirdly, runt-related transcription factor 1 (RUNXI), with a restricted expression pattern, is essential for hematopoietic cell development (18). Furthermore, the genetic defect that lies between the sodium-hydrogen antiporter 3 regulator 1 and N-acetyltransferase 9 genes on chromosome 17q25 and which results in the loss of a RUNXI binding site may be involved in psoriasis (19). Finally, allogeneic bone marrow transplantation (BMT) provides evidence to support the hematopoietic basis of psoriasis susceptibility. Reconstituting T-cell populations by allogeneic BMT into psoriatic individuals results in long-term remission or amelioration of the disease (20,21). By contrast, BMT from psoriatic donors into patients with no history of psoriasis results in development of psoriasis (22). Therefore, bone marrow hematopoietic precursor cells with abnormal expression of specific genes are believed to be responsible for the T cell-related immune dysregulation in psoriasis.

To test this hypothesis, suppression subtractive hybridization (SSH) was performed on the BMHSCs of a patient with psoriasis and a healthy control. In the current study, 17 differentially-expressed sequence tags (ESTs), including the CD45 gene, were obtained from the forward- and reverse-SSH libraries. Since CD45 is known to be an important regulator of signal transduction in the process of T-cell development and activation (23), the CD45 expression levels in the bone marrow-derived hematopoietic progenitors and peripheral blood cells (PBCs) of 20 patients with psoriasis and 10 healthy subjects were analyzed by quantitative polymerase chain reaction (qPCR) and flow cytometry.

Materials and methods

Participants. A total of 20 patients with psoriasis vulgaris (16 males and 4 females; median age, 36.8 years; range, 20-58 years) participated in the study. Eligible patients were those diagnosed with plaque psoriasis lasting for at least 6 months, a family history of psoriasis, at least 10% of the total body surface area affected, and a mean psoriasis area and severity index (PASI) score of 16.87±1.26 [mean ± standard deviation (SD)]. The control group consisted of 10 age- and sex-matched healthy individuals. None of the patients or control subjects had received topical or oral medications in the 6 months prior to the study. Among the subjects, the bone marrow samples of a female patient with psoriasis and a healthy control were used to construct a subtractive cDNA library. Bone marrow and peripheral blood samples were collected from all participants and used to check the CD45 expression levels in the BMHSCs and PBCs. This study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. All participants were fully aware of the purpose of the study and provided written informed consent.

Isolation of CD34+ cells from bone marrow. From each subject, 10 ml of bone marrow aspirate was drawn into tubes containing sodium heparin. Bone marrow mononuclear cells (BMMNCs) were isolated from bone marrow aspirates by density gradient centrifugation in Ficoll1077 (Sigma Chemical Co., St. Louis, MO, USA) followed by lysis of the remaining erythrocytes with 0.15 M Tris-ammonium chloride. CD34+ cells were isolated from BMMNCs with magnetic beads conjugated to anti-human CD34 antibodies and anti-idiotype antibodies (Dynal Biotech, Milwaukee, Wisconsin, USA) according to the manufacturer's instructions. The purity of the CD34 cells and the percentage of remaining CD1a CD3 cells were evaluated by flow cytometry with fluorescein isothiocyanate (FITC)-labeled anti-CD34 antibody, phycoerythrin (PE)-conjugated anti-CD1a antibody and FITC-conjugated anti-CD3 antibody (BD Pharmingen, Tokyo, Japan).

Subtractive cDNA library construction. Total RNA was extracted with the TRIzol Reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA), and mRNA was purified with the Dynabeads mRNA Purification kit (Dynal Biotech, Oslo, Norway), according to the manufacturers' instructions. cDNA was synthesized and amplified using the SMART PCR cDNA synthesis kit (BD Biosciences-Clontech, Palo Alto, CA, USA). In the current study, 2 mg total RNA was used to generate each cDNA population for use in the subtraction procedure. The manufacturer's recommendations were used throughout the cDNA synthesis procedure. Differentially-expressed genes were identified using the Clontech PCR-Select cDNA Subtraction kit (BD Biosciences-Clontech). In brief, the SMART cDNA of the patient and healthy control were subjected to RsaI digestion, adaptor ligation, two rounds of subtractive hybridization and subsequent PCR amplifications. The forward-subtracted cDNA, i.e., cDNA from the patient as the tester and cDNA from the healthy control as the driver, and the reverse-subtracted cDNA, i.e., cDNA from the healthy control as the tester and cDNA from yeast as the driver, were inserted into the pMD-T18 vector (Takara, Tokyo, Japan) and transformed into Escherichia coli DH5a. The forward- and reverse-subtracted cDNA plasmid libraries were constructed and individual colonies that showed the presence of inserted DNA were randomly picked and analyzed.

Screening for differentially-expressed genes by cDNA array dot blot screening. From the two subtracted cDNA libraries, 600 recombinant bacterial clones were picked by blue-white selection on plates. The white colonies were incubated at 37°C.
for 12 h in Eppendorf tubes in 1 ml Luria-Bertani (LB) broth that contained 50 µg/ml ampicillin. Each bacterium in the LB culture was amplified in a 50 µl PCR system using the nested PCR primers: 1, 5'-TGGAGCGCGCCGCCGCGCTTTGGG-3'; and 2R, 5'-AGCGTGGTCGCCGAGCTTTGG-3'. PCR was performed as follows: 95°C for 2 min, 35 cycles of 95°C for 30 sec, 62°C for 45 sec and 72°C for 1 min, and 72°C for 6 min. The PCR product was confirmed on a 2% agarose gel and the remainder was used for differential screening.

In total, 571 clones with cDNA inserts from two subtractive libraries were selected randomly for cDNA array dot blot screening. Briefly, the PCR product (5 µl) was denatured with an equal volume of 0.5 M NaOH, and 1.5 µl of this mixture was spotted onto two identical nitrocellulose membranes (Roche, Basel, Switzerland). The two identical blots were UV cross-linked and hybridized with digoxigenin (DIG)-labeled forward- and reverse-subtracted cDNA probes, which were prepared using DIG High Prime DNA Labeling and Detection Starter kit II (Roche). Hybrids were detected using an alkaline phosphatase-conjugated anti-DIG antibody (1:1,000 dilution). The chemiluminescent signals were generated by treating the membranes with 1% CSPD, and recorded on an X-ray film (Fuji Biomax MR film; Fujifilm, Minato, Tokyo, Japan).

Sequencing and sequence analysis. In total, 144 clones identified by differential screening of the two SSH libraries were sequenced with the universal M13 sequencing primer using an automatic DNA sequencer (ABI Applied Biosystems Model 3730). All inserted sequences were queried for similarity in the National Center for Biotechnology Information (NCBI) database using the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST).

Relative quantification of CD45 mRNA expression in BMHSCs by qPCR. The CD45 EST was selected for further analysis by qPCR. The primers and TaqMan probe for the target gene were designed using Primer Select in DNASTAR software (Lasergene, Madison, WI, USA) and are listed in Table I. The levels of RNA (2 µg of each) were assayed in the BMHSCs of 20 patients and 10 healthy controls using the SYBR ExScript RT-PCR kit (Takara). The amplification conditions were optimized for the ABI PRISM-7500 instrument (Applied Biosystems). The cycling conditions using TaqMan probe detection were 95°C for 2 min and 40 cycles of 95°C for 10 sec, 61°C for 10 sec and 72°C for 40 sec. The β-actin gene was selected as the endogenous control. Relative quantification of target gene expression was evaluated using the comparative cycle threshold (CT) method, as previously described (24). The ΔCT value was determined by subtracting the target CT of each sample from its respective β-actin CT value. Calculation of ΔΔCT involved using the healthy control sample ΔCT value as an arbitrary constant to subtract from the ΔCT values of the patient sample. Differences in the expression of target genes were determined by calculating 2^-ΔΔCT.

Flow cytometric analysis of CD45 expression in PBCs. For immunofluorescence staining, 1x10^6 cells were incubated with PerCP-CD45 monoclonal antibodies for 20 min at 4°C. Following two washes with phosphate-buffered saline that contained 5% fetal calf serum, the labeled cells were analyzed by FACSCalibur using the CELLQuest software (all BD Biosciences-Clontech).

Statistical analysis. Data are expressed as the mean ±SD, unless indicated otherwise. Comparisons of patients with psoriasis and healthy control subjects were performed with an unmatched t-test. Spearman's non-parameter correlation analysis was used to examine the correlation between disease severity and CD45 expression levels in the BMHSCs and PBCs. P<0.05 was considered to indicate a statistically significant difference.

Results

Generation and identification of differentially-expressed cDNA fragments. Using nested PCR primers 1 and 2R, 300 recombinant bacteria clones were amplified from the forward-SSH library, and 300 clones were amplified from the reverse-SSH library. Only clones that had cDNA inserts that carried adaptor 1 and adaptor 2R on the two ends were amplified. The inserts ranged in size between 200 and 1,000 bp, as assessed by agarose gel electrophoresis. Overall, 284/300 clones from the forward-SSH library and 287/300 clones from the reverse-SSH library contained inserted fragments, and these were subjected to further analysis. The remaining clones were false-positives. From the two SSH libraries, 571 clones with cDNA insert sizes that ranged in size between 200 and 1,000 bp were selected to be undergo cDNA array dot blot screening. Of these clones, 74 positive clones from the forward-SSH library showed marked signals using the forward-subtracted probe compared with using the reverse-subtracted probe, and 70 clones from the reverse-SSH library showed marked signals with the reverse-subtracted probe compared with the forward-subtracted probe. These 144 positive clones were selected for further sequencing.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers and Taqman probe</th>
<th>Primers for qPCR (5'-3')</th>
<th>Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>F</td>
<td>ctcgccctgaggtgcaaa</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>tgcgaagctggtcctcact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Taqman probe</td>
<td>Fam-aaaccagtcgatccaaacctca	amra</td>
<td></td>
</tr>
</tbody>
</table>
Sequence analysis of differentially-expressed genes. A total of 144 clones were sequenced and compared with NCBI proteins using BLASTX. Significant similarities to known genes were identified for 9/74 clones (Table II) in the forward-SSH library and 8/70 clones (Table III) in the reverse-SSH library; the remaining clones showed low-level similarity to known genes or represented novel genes. The clones that showed marked similarity to known proteins were linked to: i) Metabolism; ii) cell wall biogenesis and remodeling; iii) signal transduction; and iv) stress. Other clones had weaker similarity with known proteins or represented hypothetical proteins; their roles were not confirmed.
Marked expression of CD45 in the BMHSCs of a patient with psoriasis.

To confirm the reliability of SHH and dot blot screening, the expression levels of CD45 were examined in the BMHSCs of the 20 patients with psoriasis and the 10 healthy control subjects using relative qPCR. Following amplification, the CT, ΔΔCT, ΔΔCT and 2^−ΔΔCT values were calculated (Table IV). Notably, the expression level of CD45 was 11.55-fold higher in the BMHSCs of the patients with psoriasis compared with the healthy controls (P<0.001; Fig. 1).

Table IV. Relative abundance of CD45 gene in HSCs, as determined by qPCR.

<table>
<thead>
<tr>
<th>Group</th>
<th>CD45, CT</th>
<th>β-actin, CT</th>
<th>ΔCT</th>
<th>ΔΔCT</th>
<th>2−ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>19.06±0.58</td>
<td>21.40±0.66</td>
<td>-2.28±1.13</td>
<td>-3.53±1.31</td>
<td>11.55^a</td>
</tr>
<tr>
<td>Normal</td>
<td>21.42±0.53</td>
<td>20.17±0.20</td>
<td>0.25±0.53</td>
<td>0.00±0.53</td>
<td>1</td>
</tr>
</tbody>
</table>

^aP<0.001 vs. normal control. qPCR, quantitative polymerase chain reaction; HSCs, hematopoietic stem cells; CD45, cluster of differentiation 45.

High expression level of CD45 in the PBCs of patients with psoriasis. As shown in Fig. 2, the expression level of CD45 in the PBCs was markedly higher in the patients with psoriasis (73.17±2.14%) compared with the healthy control subjects (58.81±3.40%) (P<0.05).

Correlation between disease severity and CD45 expression levels in BMHSCs and PBCs. The CD45 expression levels in the PBCs of the patients with psoriasis were significantly correlated with their PASI scores (r=0.615; P=0.004), whereas the CD45 expression levels in the BMHSCs were not correlated with the PASI scores (r=0.321; P=0.168) (Figs. 3 and 4).

Discussion

Investigations of differential gene expression in the BMHSCs of patients with psoriasis and healthy controls may lead to the
Of the 17 differentially-expressed genes detected in the BMHSCs of the patient with psoriasis and a healthy control in the present investigation, clone 1067-0526, which includes the CD45 EST, was focused upon. CD45, a leukocyte-common antigen, is a transmembrane protein tyrosine phosphatase that is specifically expressed on all nucleated hematopoietic cells, from stem cells to memory cells. In humans, bone marrow-derived T-cell progenitors have been shown to express CD34 and CD45 (12), and CD45 is an important regulator of signal transduction at multiple stages of T-cell development from the early precursors (23). The expression of CD45 in hematopoietic cells is essential for early thymocyte development, and mice and humans lacking CD45 expression are severely immunodeficient (25). In CD45-deficient mice, there is partial blockade of DN1 and DN3 progression and an almost complete blockage of DP development to SP cells, attributed to an increased threshold of T-cell receptor (TCR)αβ, pre-TCR and TCR signaling, respectively. A major reduction in the numbers of mature SP T cells is consequently found in the periphery of CD45-deficient mice (23,26). In the present study, CD45 showed the highest repeat frequency among the pre-TCR and TCR signaling, respectively. A major reduction in the numbers of mature SP T cells is consequently found in the periphery of CD45-deficient mice (23,26). In the present study, CD45 showed the highest repeat frequency among the forward-SSH sequences, and was expressed 11.55-fold higher in the BMHSCs of the patients with psoriasis compared with those of the controls. Previous studies have indicated that the BMHSCs from patients with chronic plaque-type psoriasis differentiate into mature T cells with normal percentages of SP cells, albeit with increased proliferative activity (16). Therefore, the overexpressed CD45 in psoriatic BMHSCs is hypothesized to act as a positive regulator of signals delivered via TCR expression during early T-cell development, so that the proliferative ability, but not the absolute number of mature SP T cells is markedly increased, further involving the pathogenesis of psoriasis.

CD45 expression on mature hemocytes is critical for the regulation of immune function, as it functions positively to regulate T-lymphocyte activation (27). An early study demonstrated that a CD45-deficient CD4+ T-cell clone lost reactivity to its specific antigen and the ability to proliferate in response to a mitogenic signal and anti-CD3 cross-linking, while it retained the ability to proliferate in response to interleukin 2 (28). This implied that CD45 was important in transducing the signal initiated by antigen binding to the TCR. Subsequent studies confirmed this observation. A CD8+CD45-deficient T-cell clone that lost the abilities to cytolyze its appropriate target cells and to produce cytokines in response to the antigen was observed (29). However, transfection with CD45 cDNA restored the activation response to TCR ligation (30). In addition, CD45 may regulate cytokine production and responses to cytokines in other hematopoietic cells. Previous studies have shown that CD45 is required for interferon (IFN)-α and IFN-γ production by dendritic cells and NK cells following stimulation via the immunoglobulin Fc or major histocompatibility complex-binding receptors (31,32).

Compared with the healthy controls, the expression levels of CD45 in the PBCs of the patients with psoriasis were markedly higher in the current study. The CD45 levels in the PBCs were observed to be closely correlated with the PASI, which is indicative of psoriasis severity. Dawes et al produced transgenic mice that expressed an altered level of CD45 and found that the total level of CD45 expressed was crucial for normal TCR signaling, lymphocyte proliferation and the production of cytokines, including IFN-γ and tumor necrosis factor (TNF)-α (33). Therefore, it is likely that increased CD45 levels in PBCs are responsible for the abnormal immune state of patients with psoriasis, which includes increased lymphocyte proliferation activity and high levels of IFN-γ and TNF-α production, which further exacerbate psoriasis.

Taken together, the current data indicate that the abnormalities of hematopoietic progenitor cells that result in the overexpression of CD45 may lead to similar abnormalities in the PBCs via hematopoiesis. The increased level of CD45 in PBCs, particularly on T lymphocytes, may account for the psoriatic features, including the increased proliferative activity and elevated secretion of Th1 cytokines.

In the present study, increased levels of IFN-γ were observed in the BMHSCs of patients with psoriasis, which is in accordance with the changes in IFN-γ previously observed in the sera of patients with psoriasis (34). It is generally hypothesized that serum IFN-γ plays an important role in the pathogenesis of psoriasis (35). Serum cytokine concentrations are altered by several processes, including the production, deposition, degradation and elimination of these molecules. Furthermore, tissue sources of cytokine production other than circulating T cells may exist. The origin of the IFN-γ in the sera of patients with psoriasis is not clear (36). In future studies of psoriasis, further investigations are required to determine whether the increased level of IFN-γ in BMHSCs contributes to the increased serum IFN-γ level, and whether this elevation is conferred upon peripheral T cells via hematopoiesis.

Other genes that encode proteins that affect hormone signaling, RNA catabolism, protein ADP DNA base melting, transcriptional regulation, cell cycle regulation and metabolism were identified in the present study. However, as there are few relevant studies on these genes in BMHSCs or in relation to psoriasis, it is difficult to assign specific roles to these genes in the development of psoriasis. Future studies that investigate these gene expression changes may be beneficial in characterizing the dysfunctional T cells derived from psoriatic BMHSCs.

While several genes, including p15, p21, RUNX1, HLA-C, and PRKCB were previously found to be overexpressed in the HSCs of patients with psoriasis (37,38), these genes were not identified in the present study. The reasons for this could be that earlier investigations extracted mRNA from different samples than those employed in the present investigation. Given the different geographic origins of each sample, there are likely to be innate differences in the genetic structures of the two subjects, which may be reflected in their protein profiles. In
addition, these differences may reflect a bias in choosing gene clones of interest or technical limitations associated with the methodologies employed.

The present data indicate that the abnormal expression of genes, such as the augmented expression of CD45 in the BMHSCs, may induce the production of pathogenic T lymphocytes that play a role in the progression of psoriasis. The present findings may have substantial implications for our understanding of the pathophysiology of psoriasis and the development of novel treatment strategies.

Acknowledgements

The study was supported by a grant from the National Natural Science Foundation of China (grant nos. 30872271 and 81171507).

References