Journal of Korean Association
of Cancer Prevention
2003; 8(3): 195-200

Identification of Human Peptidylglycine Alpha-amidating Monooxygenase (PAM) in Psoriasis by DDRT-PCR

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Psoriasis is a chronic relapsing and angiogenic skin disease characterized by variable clinical features. However, not only pathogenetic process but also etiological factors remain to be proven. In order to identify genes differentially expressed in psoriasis, we performed mRNA differential display analysis using total RNA extracted from normal and psoriatic primary cultured cells. Of the differentially expressed genes from psoriasis, some of cDNA fragments were cloned and sequenced. The expression patterns of these clones from psoriatic primary cells were confirmed by reverse northern blot analysis. Up-regulated gene from lesional keratinocytes of psoriasis was identified as human peptidylglycine alpha-amidating monooxygenase (PAM). Up-regulated expression of PAM in psoriasis may contribute to pathogenesis of psoriasis.

Key Words: Psoriasis, PAM, Differential display analysis

INTRODUCTION

Psoriasis is a common, chronic skin disease characterized by recurrent erythematous skin plaques that exhibit epidermal hyperplasia,[1,2] a variable inflammatory cell infiltrate, and angioproliferation.[3] Recent etiological studies for psoriasis concentrated on molecular mediators of inflammation or keratinocyte growth. However, the fundamental pathogenic mechanism and the key molecules that are causative for psoriasis have not yet been identified. To investigate underlying cause of psoriasis, we have used the technique of mRNA differential display,[4] which is able to detect genes differentially expressed between normal keratinocytes and lesional keratinocytes of psoriasis.

This differential display strategy enabled us to identify genes that were up-regulated in lesional keratinocytes of psoriasis as compared with normal keratinocytes. This study demonstrated that peptidylglycine alpha-amidating monooxydase (PAM) is overexpressed in psoriasis.

It has been reported that neuropeptides such as substance P, known to be present in unmyelinated dermal nerve endings,[5,6] might be of pathogenic importance in psoriasis.[7] On the other hand, an important step in the maturation of as many as one-

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Received : 2003년 7월 20일, Accepted : 2003년 9월 1일

half of known peptide hormones and growth factors is alpha-amidation at their COOH terminus.[8)]

In this paper, we found that peptidylglycine alpha-amidating monooxydase (PAM), the only enzyme complex catalyzing alpha-amidation, is overexpressed in psoriasis.

MATERIALS AND METHODS

1) Cell Culture primary cell culture

The tissue specimens were taken from the psoriatic lesions on the trunk of patients who had not been treated at least for 6 weeks. The specimens were immediately placed in holding medium consisting of Eagle's balanced salt solution containing penicillin/streptomycin. Fresh epidermal single-cell suspensions of the tissues were treated with dispase (dispase grade II, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 16 to 20 hr at 4°C to be separated the epidermis smoothly from the dermis using forceps. The epidermis was then placed in phosphate-buffered saline (PBS) solution containing 0.05% trypsin (Gibco, Gaithersburg, MD) for 20 minute at 37°C. The epidermis was gently agitated with a pipette to separate the cells. After centrifugation at 1,000 rpm for 5 minute, the epidermal cells were gathered to make the cell suspensions. An appropriate number of the epidermal cells were seeded onto culture flask. The culture medium was keratinocyte basal medium (KBM; Clonetics Corp., San Diego, CA) containing 5×10^{-7} M hydrocortisone, 5 ng/ml epidermal growth factor, 30 μg/ml bovine pituitary extract, 5 μg/ml insulin, 50 μg/ml gentamycin, 50 ng/ml amphotericin B. The culture was maintained in a humidified atmosphere of 5% CO2 in air at 37°C. Generally, after 2 or 3 days small clusters of adherent cells were apparent and the KBM was changed every 2 or 3 days with the subsequent appearance of progressively larger colonies of keratinocytes.

2) RNA isolation

Total RNA to be used for Reverse Northern blot analysis was isolated according to the single step guanidium thiocyanate-phenol-chloroform extraction procedure using Trizol (Gibco/BRL) according to the manufacturer's instructions. For differential display analysis, possible DNA contamination was removed by treating the obtained RNA with RNase-free DNase I for 30 min at 37°C. After phenol/chloroform (3/1) extraction and ethanol precipitation, RNA was resuspended in DEPC-treated H_2O.

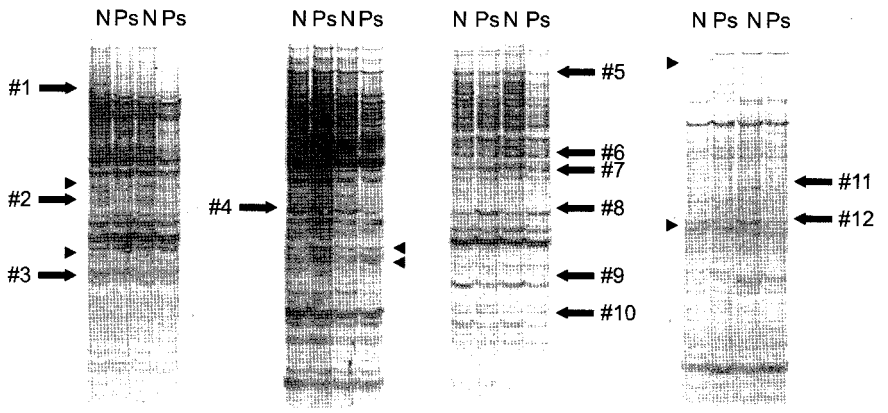

Fig. 1. Differentially expressed genes in psoriasis detected by Differential display analysis. Each samples loaded twice in the same gel to compare intensity of bands. Arrows indicate the clone to be confirmed by Reverse northern and arrow heads indicate false positive clones. N; normal keratinocytes, Ps; lesional keratinocytes of psoriasis.

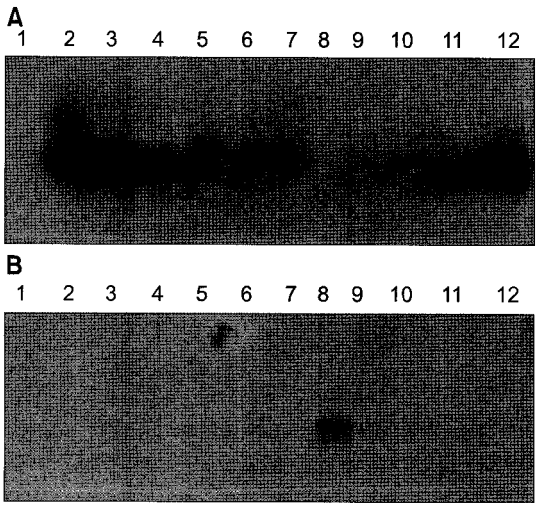

Fig. 2. Differentially expressed genes in psoriasis confirmed by Reverse northern. Reverse northern analysis was performed as described under "Materials and Methods" using total RNA (5 μg) isolated from normal keratinocytes or lesional keratinocytes of psoriasis. cDNA from (A) normal keratinocytes and (B) lesional keratinocytes of psoriais was used as probe.

3) Differential display analysis

Differential display was carried out using RNAmap kit (Genhunter Corp., Brookline, MA). DNA-free total RNA (0.4 μg) was reverse-transcribed using $T_{12}MA$, $T_{12}MT$, $T_{12}MG$, and $T_{12}MC$ primer where M represent a degenerated mixture of dA, dG, dC. 1/10 of reverse transcribed mixture was used directly for PCR amplification in the presence of α-S^{35} ATP (1,200 Ci/mmole, Amersham, Arlington, IL) using Taq polymerase (Amplitaq, Perkin Elmer, CA, U.S.A). The 20 different primer sets used for PCR amplification were the combination of five 5'-AP (arbitrary primer) with the four 3'-$T_{12}MN$ primer that were used for initial cDNA synthesis. The cycle parameters were as follows: 94°C for 30s, 40°C for 2 min, 72°C for 30 s for 40 cycles, followed by 72°C for 5 min. The amplified cDNAs were separated on a 6% DNA sequencing gel and autoradiographed with X-ray film. To determine cDNA size, partial sequencing of the DNA from bacteriophage M13mp18 by the chain-termination method was

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Query: 232  gactgtgtacattggagatgctcataccaacaccgtgtggaagttcaccttgactgagaa 291
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2052 gactgtgtacattggagatgctcataccaacaccgtgtggaagttcaccttgactgagaa 2111

Query: 292  attggaacatcgatcagttaaaaaggctggcattgaggtccaggaaatcaaagaagccga 351
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2112 attggaacatcgatcagttaaaaaggctggcattgaggtccaggaaatcaaagaagccga 2171

Query: 352  ggcagttgttgaaaccaaaatggagaacaaacccacctcctcagaattgcagaagatgca 411
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2172 ggcagttgttgaaaccaaaatggagaacaaacccacctcctcagaattgcagaagatgca 2231

Query: 412  agagaaacagaaactgatcaaagagccaggctcgggagtgcctgntgttctcattac 468
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2232 agagaaacagaaactgatcaaagagccaggctcgggagtgcctgttgttctcattac 2288
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B

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Query: 229  FDCVHWRCSYQHRVEVHLD*EIGTSIS*KGWH*GPGNQR 345
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Sbjct: 2450 WDCVHWRCSYQHRVEVHLD*EIGTSIS*KGWH*GPGNQR 2566
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Fig. 3. Nucleotide and deduced amino acid sequence of clone #8. (A) Comparison of deduced nucleotide sequence and (B) amino acid sequence of clone #8 with Homo sapiens peptidylglycine alpha-amidating monooxygenase.

performed on the same gel as used in the differential display.

4) Recovery and reamplification of cDNAs

Differentially displayed cDNAs were cut from the dried sequencing gel, and the DNA was recovered and reamplified, using the same primer set and PCR conditions as used in the differential display except for dNTP concentrations of 20 μM and no radioisotope. The amplified cDNA was purified with Quiaquick spin columns according to the manufacturer's conditions (Quiagen, Hilden, Germany).

Cloning and sequecing of cDNAs. The reamplified cDNA bands were cloned into plasmid pGEM-T vector using pGEM-T vector system (Promega, Madison, WI) according to the manufacture's instructions. Plasmid DNA was purified using a Wizard Plus Miniprep Kit (Promega Corporation). Plasmid DNA sequencing of cloned cDNAs with either T7 or SP6 primer was carried out using the Sequenase version 2.0 DNA sequencing kit (United States. Biochemicals, Cleveland, OH) following the manufacture's instructions or using the ABI PRISMTM Dye Terminator cycle sequencing ready reaction kit protocol with the Automated DNA sequencer (Perkin Elmer ABI PRISMTM model). Comparison of DNA homology and Protein homology with GenBank and the EMBL data base was performed using BLAST and FASTA.

5) Reverse Northern blot analysis

Reamplified bands were resolved on 1.5% agarose gel, applied to nylon membranes (Zeta-Probe membrane; Bio-Rad). 5 μg of total RNA from the lesional keratinocytes of psoriasis or normal keratinocytes were reverse transcribed as described above, except oligo $(dT)_{12\text{-}18}$ was used as a primer. Radioactive DNA probe was produced from the cDNA reaction by the Rediprime DNA labelling system (Amersham). Probes were added to each filter and hybridized in (0.5 M sodium phosphate/7% SDS) overnight at 65°C. Filters were washed in 20 mM sodium phosphate/0.1% SDS at 65°C for 15 min and exposed for 1 day.

RESULTS and DISCUSSION

To identify genes whose expression was altered in pathogenetic progression of psoriasis, we performed mRNA differential display using total RNAs extracted from lesional keratinocytes of psoriasis and normal keratinocytes. A total of 5 arbitrary 10-mer primers were used in combination with all 4 $T_{12}MN$ primers. Utilizing the application of 20 different primer sets, our results yielded 3 up-regulated and 15 down-regulated cDNA fragments from lesional keratinocytes of psoriasis compared with normal keratinocytes (Fig. 1).

All of these were successfully recovered from the dried gel, reamplified, and used as probes for reverse Northern blotting. Among them, 12 bands were observed to be differentially expressed by the reverse Northern blotting (Fig. 2). These 12 cDNAs were cloned into the vector pGEM-T and then sequenced in both directions. Surprisingly, we found that the nucleotide sequence of clone #8 was completely matched to based 2,052~2,288 of human DNA sequence from human peptidylglycine alpha-amidating monooxydase (PAM) (Genbank accession no. BT007419). In contrast, other clones were matched to Escherichia coli genome sequences. It might be contaminated in the step of transformation for sequencing. Finally, the #12 which is down-regulated in psoriasis is novel gene as they have no significant homology in Genbank.

Many important peptides, such as neuropeptide Y, vasoactive intestinal peptide, galanin, gastrin- releasing peptide, thyrotropin-releasing hormone and substance P[9,10] are all alpha-amidated at their COOH terminus, a structural modification that is essential for their biological activity.[10] The only enzyme complex catalyzing this key posttranslational modification has been identified as the PAM.

In psoriasis, substance P and vasoactive intestinal

peptide are increased.[11] Recently it has been reported that substance P enhances the production of interferon-induced protein of 10 kDa production, which is increased in psoriasis, by human keratinocytes in synergy with interferon-gamma, it increases inflammation in psoriasis.[12]

PAM consists of two enzymes acting sequentially to convert peptidylglycine substrate into alpha- amidated products and glyoxylate. The first enzymatic step is carried out by the PHM (peptidylglycine alpha-hydroylating monooxygenase) which, in the presence of ascorbate, copper, and molecular oxygen, produces an alpha-hydroxylated intermediary product.[13~15] The subsequent step is then performed by the PAL (also peptidyl amidoglycolate lyase or PGL), which catalyzes the synthesis of the final alpha-amidated peptide and glyoxylate.[16,17]

Several human PAM cDNA have been cloned and primary transcript is on chromosome 5q14-5q21.[18,19] In this paper, we identified that PAM gene which overexpressed in psoriasis had 99% homology with transcript variant 1, sequence based on 2,746~2,982 and transcript variant 3, sequence based on 2,425~2,661 of PAM mRNA.

PAM proteins undergo tissue-specific endoproteolytic cleavage, yielding both soluble and membrane-associated PHM and PAL.[20,21] A search of protein database using BLASTAX indicated that the amino acid sequence of #8 clone was completely matched to amino acid 2,450~2,566 of pancreatic PAM-3, which have region of transmembrane domain (Genbank accession no. S75037) (Fig. 3).

In summary, we have demonstrated the utility of mRNA differential display as a molecular screen for differentially regulated genes in the pathogenesis of psoriasis; genes of known and unknown function have been identified. Further investigations about these genes differentially expressed in psoriasis will helpful to make the gene profile which provide much information of gene regulation in psoriasis. Furthermore, elucidating the overexpression of PAM in psoriasis will be important for understanding the process of psoriasis progression and also for application the basic knowledge of psoriasis to clinical and therapeutic fields.

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