Expression of PC3, carboxypeptidase E and enkephalin in human monocyte-derived macrophages as a tool for genetic studies

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Abstract Circulating monocytes in human peripheral blood are readily available, easily obtained, and can be cultured in vitro. Once plated, the monocytes spontaneously differentiate into macrophages. Undifferentiated human monocytes do not express carboxypeptidase E (CPE), prohormone convertase 3 (PC3/PC1) or proenkephalin (ENK), suggesting that gene induction during differentiation results in the expression of these genes. RT-PCR of human monocyte-derived macrophage (HMDM) mRNA showed detectable levels of ENK mRNA at 48 h after plating, followed by PC3 and CPE mRNAs at 72 h. PC3 expression was confirmed by Western blotting in THP-1 cells. Similarities in expression of enzymes involved in the conversion of neuroendocrine precursors, such as proenkephalin, into functionally mature peptides underscores the close association HMDMs may share with the neuroendocrine system. Because of this, HMDMs may prove to be a valuable, non-surgical source of human tissue for clinical genetic diagnosis of some convertase disorders.

Key words: Human monocyte; Prohormone convertase 3; Carboxypeptidase E; Enkephalin; Neuroendocrine; Genetic diagnosis

1. Introduction

The prohormone convertases, PC3/PC1 and PC2 are the preeminent neuroendocrine convertases of the subtilisin-like proprotein convertase (SPC) family and are believed to process a wide variety of neuroendocrine and hormone precursors in the brain and endocrine systems. Both PC2 and PC3 have been shown to be involved in the post-translational processing of proinsulin in the islets of Langerhans [1,2], in the differential processing of proglucagon in the islets versus intestine [3] and proopiomelanocortin in the anterior versus intermediate lobes of the hypophysis [4,5]. Carboxypeptidase E (CPE) is an exopeptidase capable of removing the C-terminal arginine residues remaining after endoproteolytic cleavage of various precursors. Recent studies have directed attention towards syndromes arising from defects in these processing enzymes. The obesity and hyperglycaemia observed in the fat/fat mouse are a consequence of a mutation in the CPE gene [6]. A deficiency in PC3 activity has been proposed to be the causitive agent responsible for a unique form of hyperproinsulinemia in a human subject [7], and may also be related to the elevated proinsulin levels associated with Type 2 diabetes [8]. The reported presence of PC2 in rat polymorphonuclear leukocytes and alveolar macrophages and PC3 in rat alveolar and splenic macrophages [9] led us to hypothesize the existence of these enzymes in human monocyte-derived macrophages (HMDM) and in the human monocytic leukemia cell line THP-1 as well. Accordingly, we have investigated the expression of PC3, PC2, CPE and enkephalin (ENK) mRNAs by RT-PCR in HMDMs and in THP-1 cells and have examined whether these enzymes are induced during normal human monocytic differentiation in vitro. The results indicate that HMDMs may be a previously unexploited source of human tissue for clinical genetic diagnosis of hormonal processing defects in man because of their partial enzymatic similarities with the neuroendocrine system.

2. Materials and methods

2.1. Amplification of PC2, PC3, CPE and ENK

THP-1 mRNA from 1×10^7 cells/time point and HMDM mRNA from 5×10^6 cells/time point was prepared using QuickPrep Micro mRNA purification kit (Pharmacia Biotech). All mRNA obtained were immediately converted into cDNA using Superscript Preamplification System (Life Technologies). RT-PCR reactions employed Elongase (Life Technologies) and HotStart 50 tubes (MβP). Each reaction was denatured at 94°C for 1 min 30 s, amplified 30 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s to 1 min 15 s depending on the target size. Amplification primers used were: PC2 5′-GACCGGTCTTCACGAATCAT and PC2 3′-CCGTAGCCAAAGAGGTGATT; PC3 5′-TGACCTGCACAATGACTGCA and PC3 3′-TGCACTTGGGGACTTCTTTG; CPE 5′-TCAGCAGGATTTACACGGTG and CPE 3′-GCTGTAAGGAACTGCCACTT; ENK 5′-ACATCAACTTCCTGGCTTGC and ENK 3′-CACCATCAACAGTTTCCCAC. PCR reactions were analyzed on a 1% agarose gel, 1× TBE [10].

2.2. Preparation and cycle sequencing of PC3, CPE and ENK DNA

DNA bands for PC3, CPE and ENK were excized from 1% agarose gels, placed in 1.5 ml Ependorff tubes containing 200 μl of sterile water and frozen at −20°C overnight. The tubes were then thawed, the contents transferred to Ultrafree-MC filter units (Millipore) and centrifuged for 10 min, 10K rpm, 25°C. The filtrates were ammonium acetate/ethanol precipitated [11] overnight at −20°C using 1 μl of 10 mg/ml glycogen (Boehringer-Mannheim) as carrier. The DNAs were pelleted by centrifugation for 30 min, 14K rpm, 25°C. The ethanol was aspirated, the pellets were washed 1× with cold 70% ethanol and dried for 2 min in a SpeedVac. Each pellet was dissolved in 10 μl of sterile water. Analysis by cycle sequencing was performed on 5 μl of each sample using SequiTherm (Epicentre Technologies), and ^{33}P-ATP end-labelled internal sequencing primers.

2.3. Isolation of human monocytes from peripheral blood

Blood samples were obtained from normal human volunteers, and monocytes prepared as previously described [12], by drawing one 15 ml Sodium Heparin Vacutainer Tube (A – monocytes) and 1–15 ml SST Gel and Clot Activator Vacutainer Tube (B – human serum). The contents of Tube A were transferred to a 50 ml conical tube and diluted with 25 ml of 0.9% NaCl/1 mM EDTA solution. Four milliliters of Ficoll-Paque Gradient (Pharmacia Biotech) were pipetted into 4–15 ml conical tubes and 10 ml of diluted blood solution layered on top. The tubes were centrifuged at 1500 rpm in a 20°C refrigerated table-top centrifuge, 20 min, without brake, and the serum aspirated from the gradient. White blood cells (WBC) from each of the four

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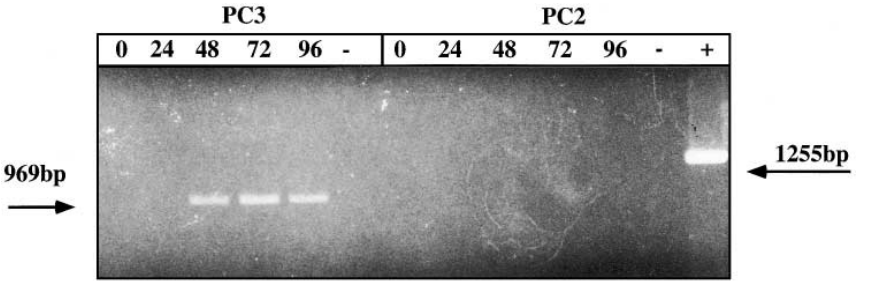

Fig. 1. RT-PCR analysis of PC3 and PC2 mRNA expression in THP-1 cells. Messenger RNA from 1×10^7 THP-1 cells was isolated at 0, 24, 48, 72, and 96 h after treatment with 400 ng/ml PMA (Sigma). The 969 bp band corresponding to PC3 is visible at 48, 72 and 96 h. PC2 is undetectable throughout the time course. Water was used as a negative control. A plasmid containing the complete coding region of PC2 cDNA was used as template for the positive control.

gradients were collected, transferred to 1–50 ml conical tube and the volume brought to 50 ml with 0.9% NaCl solution. The WBCs were centrifuged at 1200 rpm, 8 min, 20°C with brake and the cell pellet washed 1× with 10 ml of RPMI-1640/1% Pen-Strep (Life Technologies). The WBCs were centrifuged at 1200 rpm, 8 min, 20°C with brake, resuspended in 10 ml of RPMI-1640/1% Pen-Strep and counted. WBCs were diluted to 5×10^6 cells/ml with RPMI-1640/1% Pen-Strep/10% NHS (normal human serum). WBCs were plated in 6 cm tissue culture dishes (Corning), gently roughened with a braided wire brush and incubated at 37°C for 1 h to allow the monocytes to adhere to the tissue culture plate surface. The medium was removed and replaced with 5 ml of fresh RPMI-1640/1% Pen-Strep/10% NHS.

2.4. Immunoblotting

Secretory granule enriched fractions were prepared from THP-1 cells by homogenization in 0.275 M sucrose as previously described [13]. Samples were run on a 7.5% SDS-PAGE gel [14] and transferred by Western blotting [15] onto an Immobilon-P membrane (Millipore). The membrane was blocked (5% non-fat dried milk, 1× PBS, 0.2% Tween-20), incubated overnight with a 1:1000 dilution of RS20 PC3 antibody [13], and the immunoreactive bands visualized using ECL (Amersham).

3. Results

3.1. RT-PCR of THP-1 mRNA shows expression of hPC3, hCPE, and hENK

THP-1 cells were obtained directly from suspension, or cultured in media containing PMA, and allowed to differentiate into macrophages for 24, 48, 72 and 96 h. The mRNA was then isolated and subjected to RT-PCR analysis to identify the presence of hPC2 [16], hPC3 [17], hCPE [18] or hENK [19] transcripts. Primer pairs were designed to generate amplified fragments of a unique size (PC2, 1255 bp; CPE, 1102 bp; PC3, 969 bp; and ENK, 752 bp). Amplification of PC2 and PC3 were done simultaneously and run on the same agarose gel. The only band identifiable as PC2 is in the positive control lane (Fig. 1, lane 13). The adjacent PC3 amplification reveals bands at 48, 72, and 96 h (Fig. 1, lanes 3–5). Bands of the appropriate size for CPE (Fig. 2a, lanes 1–5) and ENK (Fig. 2b, lanes 1–5) were observed in all lanes during the 96 h time course.

3.2. RT-PCR of HMDM mRNA shows expression of hPC3, hCPE, and hENK

Human monocytes were plated and grown in culture for 0, 24, 48, 72 and 96 h. HMDM mRNA was isolated for each time point and subjected to RT-PCR analysis to identify the presence of PC2, PC3, CPE, or ENK transcripts. Amplification of PC3 showed the presence of an appropriate size band of 969 bp after 72 and 96 h of differentiation (Fig. 3a, lanes 4 and 5). Southern Blot analysis of the same gel revealed the complete absence of any signal prior to 72 h (Fig. 3b, lanes 1–3). CPE expression is identical to that of PC3 with the first appearance of the CPE transcript at 72 h (Fig. 4a, lane 4). A 752 bp band corresponding to ENK is observable at 48–96 h (Fig. 4b, lanes 3–5).

3.3. Western analysis and immunoprecipitation of PC3 protein in THP-1 cells

A granule rich fraction was prepared from THP-1 cells grown in PMA-treated and PMA/*E. coli* lipopolysaccharide (LPS)-treated media for 96 h. Two different amounts of sample (6 and 12.5 μg) were electrophoresed in adjacent lanes, for both conditions, on an SDS gel and Western blotted using the anti-PC3 antibody RS20. The lanes containing only 6 μg of sample (Fig. 5, lanes 1 and 3) showed the presence of a single 66 kDa band corresponding to the C-terminally processed form of PC3. The lanes containing 12.5 μg of sample (Fig. 5, lanes 2 and 4) showed a very prominent band at 66 kDa in addition to a second, less prominent band at 87 kDa [20]. The higher molecular weight band corresponds to the unprocessed form of PC3. Both forms of PC3 were also identified by pulse-chase labelling and immunoprecipitation in HMDMs (data not shown).

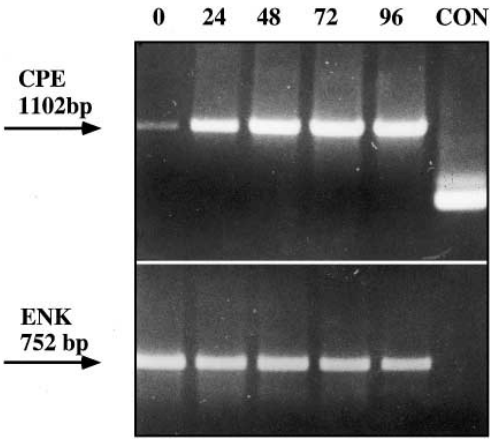

Fig. 2. RT-PCR analysis of CPE and ENK mRNA expression in THP-1 cells. Messenger RNA from 1×10^7 THP-1 cells was isolated at 0, 24, 48, 72, and 96 h after treatment with PMA. a: The 1102 bp amplification product corresponding to CPE is visible at 0 h in untreated THP-1 cells and is markedly increased at 24, 48, 72 and 96 h. b: The 752 bp amplification product corresponding to ENK is prominent at all time points during the 96 h time course.

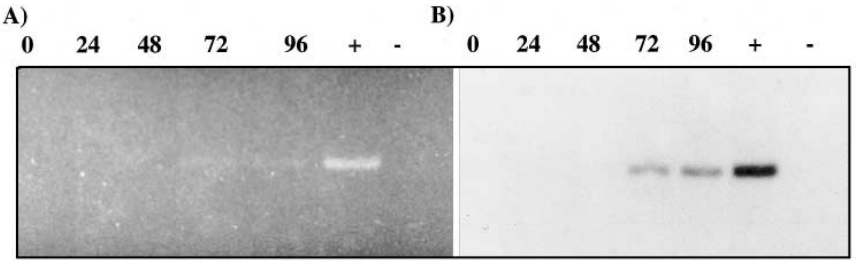

Fig. 3. RT-PCR analysis of PC3 mRNA expression in HMDM. a: cDNA from 0, 24, 48, 72, and 96 h was amplified by PCR. The DNA band corresponding to PC3 can be seen at 72 and 96 h. Lane 6 represents a positive control for PC3 from THP-1 cDNA. Lane 7 is a negative water control. b: The gel was transferred by Southern blotting onto a GeneScreen Plus Membrane. The filter was probed with a ^{33}P end-labelled, 40-mer oligonucleotide corresponding to bases 1468–1508 of the human PC3 DNA sequence.

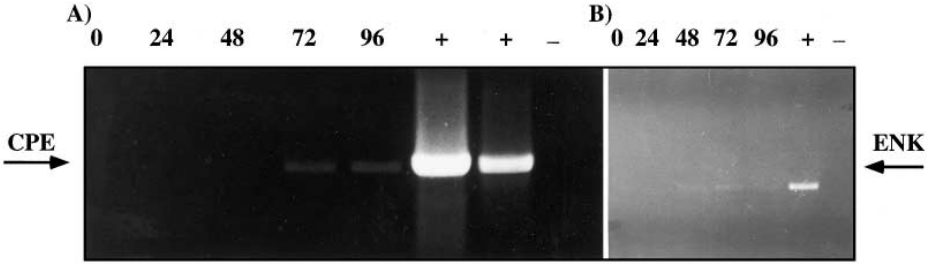

Fig. 4. RT-PCR analysis of CPE and ENK mRNA expression in HMDMs. Messenger RNA was obtained from 5×10^6 HMDMs at 0, 24, 48, 72, and 96 h after plating. a: The DNA band corresponding to CPE can be seen at 72 and 96 h. Lanes 6 and 7 are positive THP-1 controls for CPE. Lane 8 is a negative water control. b: The DNA band to corresponding to ENK can be seen at 48, 72 and 96 h. Lane 6 is a positive control for ENK using cDNA from 96 h THP-1 cells. Lane 7 is a negative water control.

4. Discussion

PC3, CPE and ENK mRNA were detected in differentiated THP-1 cells and differentiated HMDMs by RT-PCR. Expression of CPE, ENK and PC3 mRNA appear to be the result of either gene induction or derepression during differentiation in respect to HMDMs. The transcription of PC3 and CPE mRNA in HMDMs occurs simultaneously, is first evident 72 h after plating and is preceded by ENK mRNA which can be detected by 48 h. Western blot analysis of THP-1 cells confirmed the presence of both the 87 kDa (unprocessed), and the 66 kDa (C-terminally processed) forms of PC3 in the granule fraction. The THP-1 cell line may differ somewhat in the temporal aspects of induction of PC3, CPE, and ENK but was congruous with HMDM mRNA expression with regard to the lack of PC2 expression, adding further evidence that THP-1 cells are a useful model of macrophage differentiation [21].

Single-stranded conformational polymorphism (SSCP) is a powerful method of mutational analysis [22]. A common source of human genomic DNA, which is used as template for SSCP, is from blood samples and can be purified by standard methods [23] or by use of a variety of commercially available kits. SSCP can detect DNA point mutations after amplification of the target DNA with radiolabelled oligonucleotide primers. A shift in electrophoretic mobility on a polyacrylamide-urea gel can be seen following autoradiography. This method is limited by resolution, which is dependent on the size of the DNA fragment(s) used for electrophoresis.

Genetic analysis, for the purpose of medical diagnosis of disorders involving PC3 [7,8], CPE [24] or more complex genes, like the sulfonylurea receptor [25], requires complete knowledge of the genomic organization of the suspected culprit gene so that each exon can be properly amplified. If the genomic organization is unknown, as is the case for hCPE, SSCP becomes technically impossible or is at best a guessing game based on gene organization inferred from that of other species. The use of HMDMs for PC3 and CPE analysis circumvents this problem. The flexibility of the system is an advantage not possessed by conventional SSCP protocols. Any mRNA which is expressed in monocytes or differentiated macrophages can be converted to cDNA and analyzed indirectly by SSCP. There are no intron sequences to avoid, so primers can be designed anywhere along the coding sequence to generate fragments of optimal length for analysis. The cDNA can also be analyzed directly by manual or automated cycle sequencing methods.

It has been proposed that PC3 could be used as a marker for neuroendocrine differentiation [13], because PC3 expression has been confined to representatives of this family of tissues [26,27]. In addition to their medical value as a source

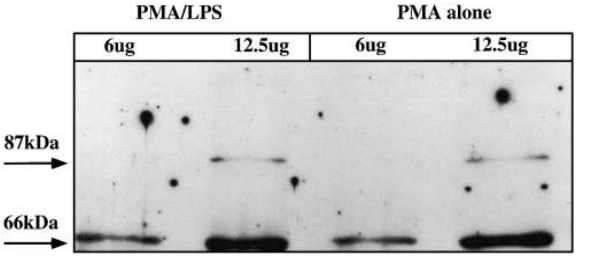

Fig. 5. Detection of PC3 protein in THP-1 cells and HMDMs. Secretory granule enriched cell extracts were prepared from THP-1 cells grown for 96 h in PMA-treated RPMI-1640 media or PMA-treated media supplemented with *E. coli* 055:B5 LPS (Difco) for the final 24 h. Immunoblotting revealed the presence of the 66 kDa form of PC3 visible as the lower band in all four lanes. The 87 kDa form of PC3 is also visible in lanes 2 and 4.

of information for genetic analysis, HMDMs possess additional attributes which enhance their value as an investigatory tool. Transcription of ENK mRNA, immunoreactivity to met-ENK peptide [28], secretion of met-ENK by monocytes and the PC3 marker, suggest an alternate function of HMDMs as a previously unrecognized neuroendocrine source.

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