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EVIDENCE FOR TRYPTOPHAN HYDROXYLASE AND HYDROXY-INDOL-O-METHYL-TRANSFERASE mRNAs IN HUMAN BLOOD PLATELETS

Jacques Champier, Bruno Claustrat°, Roger Besançon,
Cécile Eymin, Charlotte Killer, Anne Jouvet,
Geneviève Chamba and Michelle Fèvre-Montange

INSERM U433, Faculté de Médecine René-Laënnec, 69372 Lyon, France.
° Laboratoire de Radioanalyse, Hôpital Neurologique, 69394 Lyon, France.

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Summary

Human blood platelets were tested for the presence of mRNAs coding for tryptophan hydroxylase (TPOH) and hydroxy-indol-o-methyl-transferase (HIOMT). Total RNA was extracted from platelets (12.9 ± 3.3 mg RNA/100 ml blood, mean ± SEM of 6 preparations) and cDNA synthesized by reverse transcription using random hexamers, oligo-dT or TPOH- or HIOMT-specific primers, designed to amplify a 254 bp fragment for TPOH and a 301 bp fragment for HIOMT. Positive controls were performed using RNA extracted from human normal or tumoral pineal glands. The PCR products were analyzed by gel electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labeled internal probe. When random hexamers, oligo-dT or specific primers were used for reverse transcription, amplification products of the predicted sizes were detectable following electrophoresis in the case of pineal glands and following transfer and hybridization in the case of platelets. These results show TPOH and HIOMT mRNAs to be present in human blood and support the hypothesis that serotonin and melatonin may be synthesized in blood and, more particularly, in platelets.

Key Words: melatonin, serotonin, platelets, tryptophan hydroxylase, hydroxy-indol-o-methyl-transferase, pineal gland

Blood platelets, anucleated cells derived from megacaryocytes, contain only minute amounts of messenger RNAs (mRNAs) capable of being transcribed into protein. The biosynthetic capacities of these cells are poorly understood and controversial (1). Nevertheless, platelets and serotonin neurons are believed to share many common structural and functional similarities, such as their embryological ancestry and the presence of the serotonin transporter system (2-4). Furthermore, platelets accumulate about 99% of the total blood serotonin (5) and a number of serotonin-related metabolic events in platelets show circadian periodicity in humans (6). Despite these similarities to brain neurons, the activity of tryptophan hydroxylase (TPOH) was found to be very low in human platelets (7). Rabbit platelets, which contain highest serotonin levels among all the mammalian species studied (8), are able to convert ^{3}H-serotonin into ^{3}H-melatonin under certain experimental conditions, indicating that

Dr Jacques Champier, INSERM U433, Faculté de Médecine René-Laënnec, 69372 Lyon, France. Fax: (33) 4-78-77-86-12, E-mail: champier@laennec.univ-lyon1.fr

these cells contain the enzymes required for the melatonin pathway, in particular hydroxy-indol-o-methyl transferase (HIOMT) (9). The presence of HIOMT has recently been demonstrated in both rabbit and human blood platelets, suggesting that melatonin may be synthesized from platelet serotonin (10).

Using reverse transcription and the polymerase chain reaction (RT-PCR), we have investigated whether mRNAs encoding TPOH and HIOMT are present in human platelets.

Material and Methods

Platelet preparation

100 ml of blood from healthy volunteer donors was collected in 5 mM EDTA and centrifuged for 20 min at 100 g at room temperature; the supernatant (about 1/3 of the total volume) was then centrifuged for 20 min at 1,200 g and the final pellet stored at -70°C or used immediately. As a control, leucocytes were prepared from 10 ml of total blood collected in 5 mM EDTA.

RNA extraction

a- From platelets and leucocytes

RNA was extracted using the RNAzol procedure based on the method of Chomczinsky and Sacchi (11). RNA pellets were solubilized in 20 ml of sterile water and the solution frozen at -80°C until use.

b- From normal or tumoral pineal gland

About 5 mg of tissue (from normal post-mortem glands or from a pineocytoma (12)) was homogenized with 1 ml of RNAzol in a Dounce (glass-glass) homogenizer (10 up-and-down strokes) and the above process for the extraction of RNA was followed.

Reverse Transcription (RT)

About 1-2 mg of RNA was added to 6 ml of water, incubated for 3 min at 75°C and then placed immediately in ice. 4 ml of buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3) containing 200 mM of each nucleotide (dCTP, dATP, dGTP, dTTP), 1 ml of RNAsine (40 U) and 2 ml of dithiotreitol (10 mM) was then added. The following primers were used (alone or in combination): oligo dT-strands (1 mM), random hexamers (dN6) (2 mM) or specific 3'primers (1 mM). The primer sequences were 5'-AAATTATCTCGTAGATTCACAGAGA-3' (antisense) and 5'-GTTAGTTCCAGGTCACAAGAAACAGTT-3' (antisense) for TPOH and HIOMT, respectively. Sterile water was added to bring the volume to 19 ml, then 1 ml of Moloney Murine Leukemia reverse transcriptase (200 U) was added. Controls without reverse transcriptase were included. The solutions were incubated for 90 min at 42°C, then 80 ml of water was added.

Polymerase Chain Reaction (PCR)

20 ml of cDNA preparation was placed in an Eppendorf tube to which 5 ml of PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 3 mM or 1.5 mM $MgCl_2$) containing 200 mM of the four nucleotides, 1 ml of TPOH or HIOMT 3'primer (0.4 mM) (sequences given above) and 1 ml of TPOH or HIOMT 5'primer (0.4 mM) was added. The 5'primer sequences were 5'-CATTCTTTAGAAAGGCGAAGAGCAAGT-3' (sense) and 5'-CATGACTGGGCAGACGGAAA-3' (sense) for TPOH and HIOMT, respectively. The nucleotide primers were chosen so as to be several hundred bases apart in the mRNA sequences (13-16): 254 bp for TPOH and 301 bp for HIOMT. The 254 bp fragment corresponded to the portion 31-284 of TPOH mRNA and the 301 bp fragment corresponded to the portion 846-1146, which expands from exon 8 to exon 9 (15), of the HIOMT mRNA. The volume was then made to 50 ml by the addition of sterile water and the tubes heated for 7 min at 98°C and 5 min at 72°C, then 0.4 ml of Taq (Thermophilus Aquaticus) Promega enzyme (20 U) was added. After 5 min at 72°C and 1 min at 95°C, the cycling parameters of 1 min at 95°C, 1 min at 60°C and 2 min at 72°C (10 sec extension/cycle) were applied for 30 cycles in a PCR apparatus Bio-Med. After 7 min at 72°C, the products were then either stored at -20°C or used immediately.

Gene primers were also designed for a specific platelet membrane protein (glycoprotein IIb) to give a 424 bp fragment: 5'-CGTGGTCACTCAGGCCGGAGAGCT-3' (sense) and 5'-CCATATACAGTGAGCGCCCACCAG-3' (antisense) and for a glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase: GAPDH) to give a 509 bp fragment: 5'-GGAGATTCAGTGTGGTGG-3' (sense) and 5'-GGCTCTCCAGAACATCATCC-3' (antisense).

The specificity of the primers was analysed by comparing their sequences to various gene sequence databases, using the EMBL Network File Server and the Fasta Program (17).

Electrophoresis

Products underwent electrophoresis on a 2 % agarose gel for 2 hr at 90 V in 0.5X Tris-borate-EDTA buffer, pH 8.3, and the separated DNA visualised by staining with ethidium bromide in the presence of a DNA molecular size standard.

Southern blot

The cDNA was electrotransferred on to a nylon membrane for 30 min at 15 V, then fixed by incubation for 30 sec in 0.4 M NaOH and the nylon washed for 5 min in 6X SSC.

Hybridization

The oligonucleotide internal probes (10 pmoles) were incubated for 30 min at 37°C with g ATP^{32} (3,000 Curie/mmole) in 20 ml of buffer, consisting of 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiotreitol and 10 U T4 polynucleotide kinase, then the labeled probes purified by filtration on a NucTrap (Stratagene) column. Nylon membrane-bound TPOH or HIOMT cDNA was preincubated for 45 min at 42°C with 5 ml of buffer (Denhart, 6X SSC, 25 mM PO_4 buffer, 250 mg/ml salmon sperm DNA and 0.1 % SDS); this was then replaced by 5 ml of the same buffer containing 5.10^6 cpm of labeled TPOH or HIOMT oligonucleotide probe and the membranes incubated for 45 min at 42°C, then washed in 6X SSC, 0.1 % SDS for 15 min.

Results

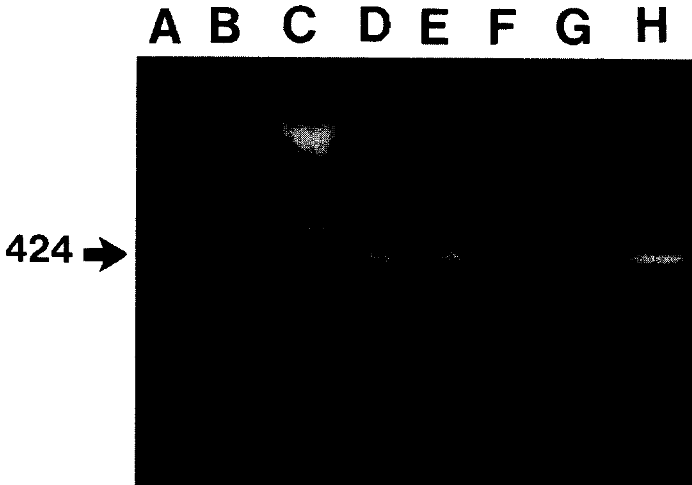

Fig. 1

Evidence for the presence of a specific platelet membrane protein (glycoprotein IIb) mRNA. PCR products were electrophoresed on a 2% agarose gel in the presence of ethidium bromide. Two nucleotide primers were constructed separated by 424 bases pairs in the mRNA sequence. RT-PCR was performed in the presence of:

Lane A: dN6 and 3 mM $MgCl_2$.
Lane B: dN6 and 1.5 mM $MgCl_2$.
Lane C: DNA ladder (100 bp jump).
Lane D: oligo-dT and 3 mM $MgCl_2$.
Lane E: oligo-dT and 1.5 mM $MgCl_2$.
Lane F: 3' primer and 3 mM $MgCl_2$.
Lane G: 3' primer and 1.5 mM $MgCl_2$.
Lane H: cDNA of GPIIb.

The yield of total RNA from human platelets was 12.9 ± 3.3 mg RNA/100 ml blood (mean ± SEM of 6 preparations). As platelets are anucleated cells containing minute amounts of mRNAs, we investigated whether the mRNA coding for a specific platelet membrane protein: glycoprotein IIb (cluster designation 41: CD 41), was present in the RNA preparations. Figure 1 shows that this was the case, regardless of the primer or the $MgCl_2$ concentration (1.5 or 3 mM) used during the reverse transcription. The band was stronger when oligo-dT was used (lanes D and E), indicating that GP IIb mRNA is polyadenylated.

The analysis of TPOH and HIOMT mRNA expression in the normal pineal gland after electrophoresis and ethidium bromide staining is shown in Figure 2. Using dN6, PCR products of 254 bp and 301 bp, corresponding, respectively, to TPOH and HIOMT, were found in the pineal gland (lanes A and C), but not in platelets using dN6 and oligo-dT, either separately or in combination (not shown). Different conditions were tested to optimize the sensitivity of RT-PCR for platelet mRNAs; these included variation of the Mg^{++} concentration from 0.5 to 5 mM, variation of each dNTP concentration from 200 mM to 600 mM, variation of the annealing temperature, variation of the cycle number (20 to 30), with or without an extension time, in the absence or presence of dimethyl sulfoxide (2 to 5 %) and attempts to reamplify from a previous PCR. Fragments of the expected length were sometimes seen when the gene-specific primers were used, but the signals were weak. The quality of mRNA preparations was demonstrated by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Figure 2). A single band was visualised in all the tested samples: normal pineal gland (lane D), platelets (lane E) and leucocytes (lane F).

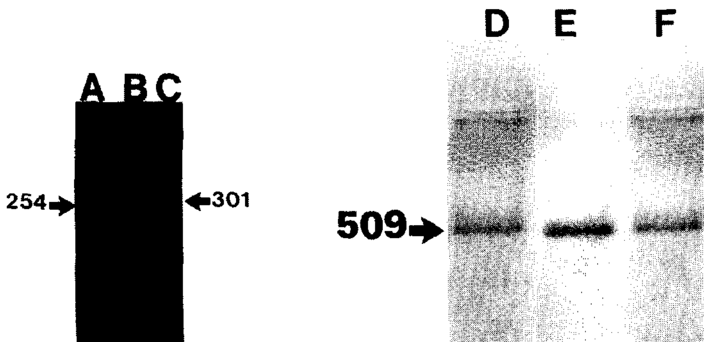

Fig. 2
Analysis of PCR products after electrophoresis (RT using dN6):
Lane A: analysis of TPOH mRNA expression in human normal pineal gland.
Lane B: DNA ladder (100 bp jump).
Lane C: analysis of HIOMT mRNA expression in human normal pineal gland.
Lane D: analysis of GAPDH mRNA expression in human normal pineal gland.
Lane E: analysis of GAPDH mRNA expression in human platelets.
Lane F: analysis of GAPDH mRNA expression in human leucocytes.

Amplification products of the predicted sizes were detected in platelets after transfer and hybridization with a specific internal probe (Figure 3). A single band was seen when the specific 3'TPOH or 3'HIOMT primers (lanes A and E, respectively) were used. Moreover, a weak signal was seen when oligo-dT was used for reverse transcription (lane F), indicating that HIOMT mRNA is present as a polyadenylated form. No amplification products were detected in leucocyte preparation (lanes D and H). No amplification band was seen in the negative controls (lacking transcriptase or amplification enzyme) (results not shown).

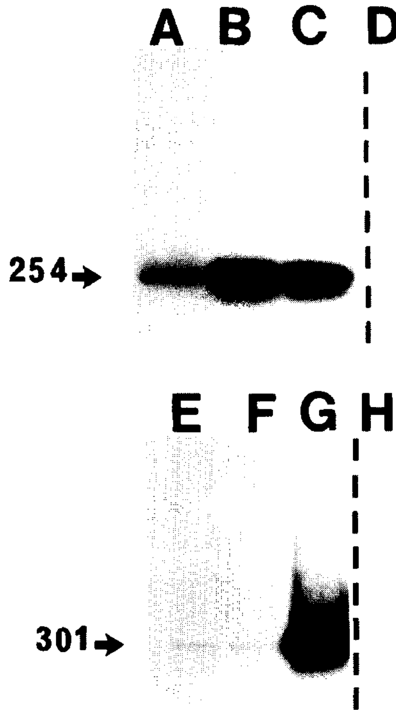

Fig.3
Southern blot analysis of expression of TPOH mRNA (upper panel) and of HIOMT mRNA (lower panel). Exposure time of the films: 3 days.
Lane A: human platelets (RT using 3'TPOH primer).
Lane B: human tumoral pineal gland (RT using dN6).
Lane C: human normal pineal gland (RT using dN6).
Lane D: human leucocytes (RT using dN6).
Lane E: human platelets (RT using 3'HIOMT).
Lane F: human platelets (RT using oligo dT).
Lane G: human normal pineal gland (RT using dN6).
Lane H: human leucocytes (RT using dN6).

Discussion

Low amounts (10 mg per 100 ml blood) of total RNA were obtained from human platelets. Other studies have reported similar yields of from < 1 mg (18) to 20 mg (19) per individual (50 ml of venous blood).

The presence of mRNA coding for GPIIb, a protein specific for the platelet-megakaryocyte membrane (20), indicates that the RNA preparations contained well-preserved platelet mRNAs presenting a polyadenylated sequence. mRNAs coding for TPOH and HIOMT were found to be present in these preparations. It can thus be inferred that human blood cells, and, more particularly, platelets, are able to synthesize both serotonin and melatonin, corroborating the hypothesis of the extrapineal production of melatonin in humans, already suggested by the detection of HIOMT in the human retina (21) and gut (22). Furthermore, the rapid large increase in plasma melatonin levels seen after L-tryptophan infusion into healthy young men during the daytime was suggested to be of extrapineal origin (23). This extrapineal origin was also demonstrated in rats where serotonin contents are very different as compared to human. Indeed, L-tryptophan has been shown to cause a large increase in levels of immunoreactive serum melatonin in pinealectomized rats (24). Many studies have suggested that daytime melatonin levels are essentially unaffected by removal of the

pineal gland and that only the nightime rise is affected, suggesting that melatonin produced in the daytime may result from extrapineal synthesis (25).

The possibility of slight contamination of the platelet preparations by leucocytes (monocytes, neutrophils and lymphocytes) cannot be totally excluded; however, in our conditions, no amplification for TPOH and HIOMT mRNAs was obtained with leucocyte mRNA. Moreover, in higher vertebrates, white blood cells contain low levels of serotonin compared with circulating platelets (25). Also, pharmacological manipulation of the production of indoleamines by cultured human peripheral blood mononuclear leucocytes (75% lymphocytes-25% monocytes) shows that unstimulated cells do not produce melatonin, this being found only in cells stimulated for more than 12 hours by interferon-γ or serotonin (26).

In conclusion, platelet melatonin may play a physiological role, as certain platelet properties (aggregation and thromboxane-B_2 production) show a melatonin-related diurnal variation (6). Further studies on daytime variations in mRNA levels might help to clarify this.

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