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TRP-1 expression correlates with eumelanogenesis in human pigment cells in culture

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We have investigated the relationship in human cultured normal and malignant melanocytes between the accumulation of mRNAs encoding tyrosinase and tyrosinase-related protein-1 (TRP-1), the activity of tyrosinase and the presence of melanin. Tyrosinase mRNA correlates with tyrosinase activity and with the presence of pheomelanin, eumelanin or both melanin types. In contrast TRP-1 mRNA is only detectable in cells containing eumelanin, which suggests a role for TRP-1 in the eumelanin synthesis pathway.

Brown; Tyrosinase; Melanogenesis; Human

1. INTRODUCTION

Epidermal melanocytes are highly differentiated cells which are derived from the neural crest, and responsible for basal skin color and tanning. They produce two types of melanin: eumelanins (brown or black), and pheomelanins (red or yellow). The key enzyme in the biosynthesis of both pigments is tyrosinase (EC 1.14.8.1), which catalyses the hydroxylation of tyrosine to DOPA and the oxidation of DOPA to dopaquinone [1]. Recently, two melanocyte-specific cDNAs have been cloned, which were both candidates for encoding tyrosinase [2,3]. One of the genes proved to be tyrosinase, and has now been mapped to the *c* locus on mouse chromosome 7 [3] and to human chromosome 11 (q14-q21). The other is assigned to the *brown* (*b*) locus on mouse chromosome 4 [4] and to human chromosome 9 [5]. The protein encoded by this gene is now termed tyrosinase-related protein 1 (TRP1) or Brown. Human and mouse TRP1 are 93% identical and they differ in length by ten amino acids at the carboxyl terminus [6]. The enzymatic role of TRP1 is not clear, and different enzymatic functions have been postulated: dopachome tautomerase [4], DHI conversion factor [9], melanosomal catalase [10] or even another tyrosinase [11]. In mouse, it has been shown that TRP1 is required for the formation of black rather than brown eumelanin [7]. Slominski et al. reported a high abundance of the protein TRP1 in a pigmented (eumelanic) hamster melanoma but a low one in a pheomelanotic cell line [8]. Recently, Vijayasaradhi et al. [12] have demonstrated that a human melanoma antigen known as gp75 is identical to TRP1/Brown. Interestingly, the presence of gp75 has been associated with visible pigmentation of human melanoma cells [13]. In the present work, we have examined in more detail the relationship between tyrosinase and TRP1/Brown mRNA accumulation, tyrosinase activity and the presence of eumelanins or pheomelanins.

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Abbreviations: TRP-1, tyrosinase related protein; DOPA, 3,4-dihydroxyphenylalanine; DHI, dihydroxyindole; AHP, aminohydroxyphenylalanine; PTCA, pyrrole-2,3,5-tricarboxylic acid; SSC, sodium citrate solution; SDS, sodium dodecyl sulfate; TPA, tetradecanoylphorbol-13 acetate; IBMX, isobutyl methylxanthine; gp75, glycoprotein 75.

2. MATERIALS AND METHODS

2.1. *Cells*

All melanoma cell lines (HBL, SCL, LND1, DOR, BEU, IGR3) studied were cultured in Ham F10 medium supplemented with 10% foetal calf serum and 1% penicillin–streptomycin solution (10,000 U/ml–10,000 μg/ml), 1% kanamycin solution (10,000 μg/ml), and 1% glutamine (200 mM) as previously described [14]. Normal melanocytes were cultured in Ham F10 medium as previously described by Smit et al. [15]. DOR, BEU cell lines were originally a gift from Dr. J.F. Dore (INSERM, Lyon) and IGR3 were provided by Dr. C. Aubert (Marseille). SCCI is a human squamous cell carcinoma (of the floor of the mouth) used as a control.

2.2. *Tyrosine hydroxylase assay*

Tyrosinase was extracted from 8.5×10^6 cells in 1 ml HBSS, after homogenisation and sonication. Crude extracts were divided into two lots: one was used to estimate the melanin content and its type (AHP and PTCA) [16] and the total protein content of this extract as described below. The other was treated with NP40 (1% final concentra-

tion), centrifuged at 10,000 × *g* for 10 min and the supernatant was used to measure tyrosine hydroxylase activity, as described by Jara et al. [16]. One unit of tyrosine hydroxylase was defined as the amount of enzyme that catalyses the hydroxylation of 1 μmol L-tyrosine per min. In each experiment, results were normalised according to the protein content of the cell extract, determined by the method described by Lowry et al. [17].

2.3. *Quantitative analysis of eumelanin and pheomelanin*

The concentrations of eumelanin and pheomelanin in the cell extracts were determined by HPLC according to the method described by Ito and Fujita [18]. Permanganate oxidation of eumelanin to pyrrole-2,3,5-tricarboxylic acid (PTCA) and hydriodic acid hydrolysis of pheomelanin to aminohydroxyphenylalanine (AHP) were used to estimate pigment content.

2.4. *Northern blot*

Northern blots were performed as described by Wathelet et al. [19]. cDNAs corresponding to human tyrosinase and TRP1 genes (clones BBTY-1, and gp 8, a gift from B. Bouchard, Memorial Sloan-Kettering Cancer Center, New York, USA) were radiolabelled with [^{32}P]dCTP using a commercial Multiprime DNA labelling kit (Amersham). To confirm that equal quantities of RNA were efficiently transferred to nylon membranes the Northern blots were re-probed with a 28S rRNA probe (data not shown).

2.5. *PCR*

The published sequences of mouse and human tyrosinase [3,7] and TRP-1 [2,6] and mouse TRP-2 [20] were examined to identify peptide motifs in common to all five. Degenerate oligonucleotide primers were designed, based on these motifs, which should allow simultaneous amplification by the polymerase chain reaction of all three cDNA sequences. The primer sequences are:

5′-GCACTCGAGAYGAY(C/A)GIGAGARIINTGGCC-3′

which will prime from any cDNA encoding the peptide DDREXWP and is flanked by an *Xho*I site; and:

5′-TAGGAGCTC(T/G)RTTRTGICCIATNGGNGC-3′

which will prime the complementary strand of any cDNA encoding the peptide APIGHNR, and is flanked by an *Sst*I site. (R = G + A, Y = C + T, I = inosine, N = all four bases, bases in parentheses are both included). Synthesis of double-stranded cDNA used a kit from Boehringer Mannheim according to their instructions, using random hexamer primers, and 1 μg total cellular RNA as substrate. 10% of the product was used in a polymerase chain reaction as previously described [20] with 3 μg/ml final primer concentration. The reaction was denatured at 94°C for 2 min, then cycled 5 times through 92°C, 1 min, 42°C, 1 min and 72°C, 2.5 min, followed by 30 cycles of 92°C, 1 min, 48°C, 1 min, 72°C, 2.5 min. The reaction products were analysed by restriction endonuclease digestion and gel electrophoresis.

3. RESULTS AND DISCUSSION

Table I shows the melanogenic phenotypes of a number of normal melanocytes and melanoma cells in culture. Detectable tyrosinase activity (tyrosine hydroxylase) appears to be associated with the presence of melanin regardless of the type of melanin as determined by HPLC analysis. Our results also show that a clear pigmentation of the cells is observed only when eumelanins are detectable. Even when large amounts of pheomelanin alone are present there is no visible pigmentation (as seen with the LND1 and BEU cell lines).

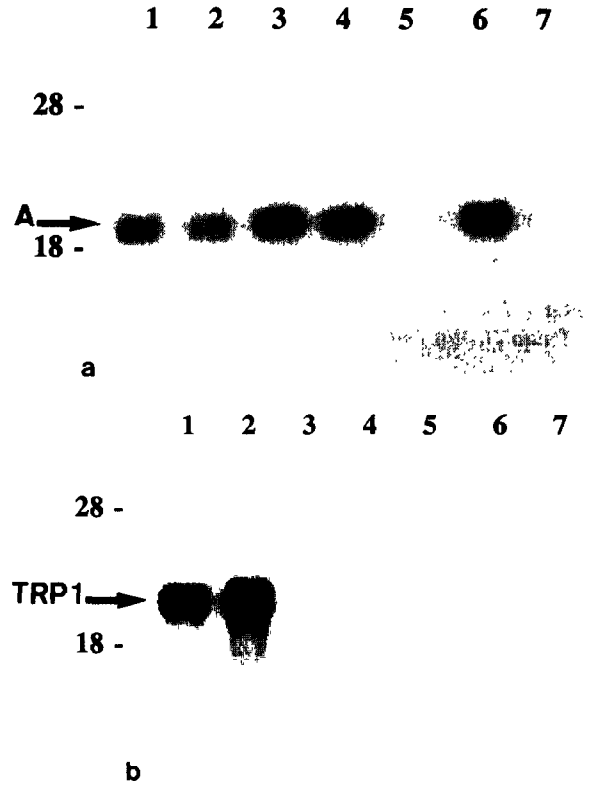

Fig. 1. Northern blot analysis of RNA from melanoma cell lines. Total RNA (5 μg) extracted from the melanoma cell line was analysed by Northern blot with either the tyrosinase (A) probe (Fig. 1a) or TRP-1 probe (Fig. 1b). The probe used is indicated by the arrow on the left (A = tyrosinase, TRP-1, tyrosinase related protein 1). 1, SCL; 2, HBL; 3, LND1; 4, BEU; 5, IGR3; 6, DOR; 7, SCC1. Lanes 1–6 correspond to human melanoma cells RNAs. SCC1 is a human lingual carcinoma used as control.

On the other hand, it should be noted that both types of melanin are present in some of the cell lines tested (HBL) and in normal melanocytes. It also appears that the melanin content of normal melanocytes is much higher than that of melanoma cells. However, although the normal melanocytes and the melanoma cell lines were grown in the same tyrosine (10 μM) containing medium, the growth of the normal melanocytes additionally requires the presence of TPA (30 nM), cholera toxin (2 nM) and IBMX (0.1 mM). It thus remains to be seen if one or several of these agents is responsible for the high melanin content. It may also be that the slower growth rate of these cells allows a higher accumulation of intracellular melanin.

To investigate any relationship between tyrosinase and TRP1 gene expression and their role in melanogenesis, we analyzed the accumulation of the transcription products of these two genes by Northern blotting (Fig. 1) and by PCR amplification (Fig. 2). The human TRP-1 cDNA sequence contains a *Bam*HI site, absent from tyrosinase, which will cut the amplified cDNA into two fragments of 550 and 510 bp. Fig. 2 (PCR) shows that the diagnostic TRP-1 *Bam*HI cleavage doublet was

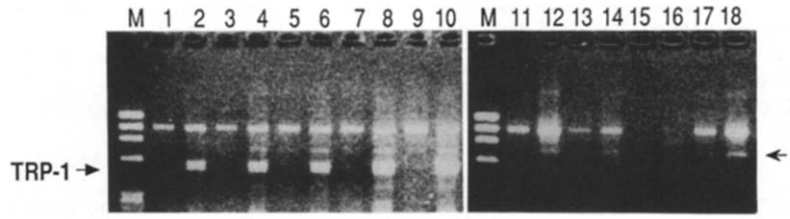

Fig. 2. Agarose gel electrophoresis of PCR-amplified cDNAs. Odd numbered tracks are 10 μl undigested PCR products, even numbered tracks are 25 μl product digested with *Bam*HI. M = ΦX174 *Hae*III marker. (1,2) MNGHM; (3,4) MNWC; (5,6) MNKB; (7,8) SCL; (9,10) HBL; (11,12) LND1; (13,14) BEU; (15,16) IGR3; (17,18) DOR. SCC1 was used as a human non-melanocytic control (data not shown). The arrow on the left indicates the diagnostic TRP-1 *Bam*HI doublet. The arrow on the right indicates a faint PCR product present in all tracks except 15 and 16 which hybridises to the tyrosinase probe and is probably a spliced variant.

present in a subset of cell RNAs, including the two shown by Northern blotting (Fig. 1) and detailed in Table I. Blotting of these gels and probing with a tyrosinase cDNA probe showed that in all except cell line IGR3, tyrosinase sequences were amplified (data not shown) again in agreement with Northern blot data. In normal and malignant melanocytes, tyrosinase mRNA appears to be associated with detectable tyrosinase activity and the presence of pheomelanin, eumelanin or both (Table I). However, TRP1 mRNA accumulation is only found in the five cell lines (SCL, HBL, MNGHM, MNWC, MNKB) containing eumelanins. This finding strongly supports the idea of an involvement of this protein in eumelanogenesis. Mouse genetics data also supports this hypothesis. The yellow pigmentation of mutant mice which only produce pheomelanin such as *lethal yellow* (*Ay*) or *extension* (*e*) is not further affected by the *brown* (TRP-1) mutation, which only modifies the colour of eumelanin-producing mice.

In conclusion, our results show that visible pigmentation is correlated with eumelanin content and that in both normal and malignant human melanocytes, tyrosinase mRNA accumulation is associated with both detectable tyrosinase activity and measurable melanin content. In addition, we also show that TRP1 mRNA is only detectable in eumelanin-containing cells. It suggests that in human melanocytes, TRP1 is involved only in eumelanin and not in pheomelanin synthesis. The role that TRP-1 is playing remains to be defined.

Table I

Phenotypic and genotypic parameters measured in melanoma and normal melanocyte cells in culture

Cell line	Phenotypic expression				Type	Genotypic expression			
	Tyrosine hydroxylase (μU/mg)	PTCA (ng/10^6 cells)	AHP (ng/10^6 cells)	Visible pigment		Tyrosinase		TRP1	
						NB	PCR	NB	PCR
Melanoma									
IGR3	<5	<1	<10	−	Amelanotic	−	−	−	−
DOR	66 ± 35	<1	11 ± 3	−	Weakly pheomelanotic	+	+	−	−
LND1	1,408 ± 497	<1	458 ± 327	−	Pheomelanotic	+	+	−	−
BEU	242 ± 32	<1	373 ± 172	−	Pheomelanotic	+	+	−	−
HBL	277 ± 155	14 ± 3	103 ± 43	+	Mixed type	+	+	+	+
SCL	200 ± 10	43 ± 16	181 ± 46	++	Mixed type	+	+	+	+
Normal melanocytes									
MNGHM	654 ± 11	136.5 ± 3.5	1,144 ± 206	+++	Mixed type	ND	+	ND	+
MNWC	1,057 ± 103	40.5 ± 4	1,315 ± 191	+++	Mixed type	ND	+	ND	+
NHMKB	904 ± 18	178.5 ± 32	1,675 ± 77	+++	Mixed type	ND	+	ND	+
Human carcinoma									
SSC1	<5	<1	<10	−	Amelanotic	−	ND	−	ND

The pigmentation was visually estimated by intensity of brown or black pigment in each cell pellet: −, absent; +, moderate; ++, high; +++, very high. The classification of melanogenesis type is based on the following criteria: Eumelanic, PTCA > 5 ng/10^6 cells and AHP > 10 ng/10^6 cells; Pheomelanic, AHP > 10 ng/10^6 cells and PTCA < 1 ng/10^6 cells; Amelanotic, PTCA < 1 ng/10^6 cells; AHP < 10 ng/10^6 cells; Mixed type, contain both pheomelanin (AHP > 10 ng/10^6 cells) and eumelanin (PTCA > 1 ng/10^6 cells); NB, detection by Northern blot: +, detected; −, below detection; ND, not done; PCR, detection by polymerase chain reaction.

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