Cultured Human Melanocytes Express the Intermediate Filament Vimentin

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Human melanocytes are neural crest–derived cells that synthesize the pigment melanin. These cells migrate from a central location to the dermal-epidermal junction early in gestation and situate themselves between keratinocytes of the basal layer of the epidermis, extending thin dendritic processes upwards into the epidermis. *In vitro,* neonatal melanocytes can assume a variety of morphologies, depending on the culture conditions. Using standard immunofluorescent, immunoblotting, and Northern blotting techniques, we investigated the expression of intermediate filament proteins and demonstrate here that cultured human melanocytes express vimentin gene and protein under a variety of culture conditions. Vimentin is a 57-kD intermediate filament protein synthesized primarily by cells of mesenchymal origin. It is transcribed as a single-messenger RNA species of 2.0 kb and the human gene is located on chromosome 10. As a member of the intermediate filament family of proteins, we suggest that vimentin is an important component of the cytoskeleton of neonatal, human melanocytes. Key words: Vimentin/melanocytes/cytoskeleton. *J Invest Dermatol 101:383–386, 1993*

Human melanocytes are pigment cells that migrate to the dermal-epidermal junction of the skin around the sixth week of gestation [1]. The melanocytes locate themselves between basal keratinocytes and extend dendritic processes upward into the epidermis. Melanin is then synthesized, packaged in granules known as melanosomes, and delivered to neighboring keratinocytes to provide a protective, pigmentary shield against the carcinogenic effects of sunlight.

Very little is known about melanocyte structure and function. Recent advances have allowed for large-scale propagation of these cells in either a serum-free or serum-containing medium [2], using crude bovine hypothalamic extract [3], crude cell extracts [4], or basic fibroblast growth factor (bFGF)[5,6] as mitogens. Nerve growth factor (NGF), a survival factor for many neural crest cells, is not a mitogen for human melanocytes despite the fact that melanocytes, under certain conditions, express NGF receptors [7]. Melanocytes assume a tripolar or stellate morphology in the presence of cholera toxin and serum, or a dendritic, neuron-like morphology in the presence of the phorbol ester 12-tetradecanoate 13-acetate (TPA). In fact, the neuronal morphology of melanocytes is associated with high levels of NGF receptor expression [7], though the role of NGF and its receptor in the normal biology of the melanocyte remains elusive.

The cytoskeleton of all eukaryotic cells is made up of tubulin-containing microtubules, actin-containing microfilaments, and 10-nm intermediate filaments. Intermediate filaments are expressed in a tissue-specific manner, with vimentin expressed most commonly in cells of mesenchymal origin, desmin in muscle cells, the three neurofilament proteins in neurons, glial fibrillary protein in glial cells (GFAP), and keratins in epithelial cells [8]. Vimentin is a 57-kD protein expressed primarily by cells of mesenchymal origin [9,10]. The human gene is located on chromosome 10 and is transcribed as a single-messenger RNA species of 2.0 kb [11].

Using immunocytochemistry as well as Western and Northern blotting techniques, we demonstrate here that cultured human melanocytes express the intermediate filament vimentin in both the stellate as well as the neuronal form. These data suggest that vimentin is an important component of the cytoskeleton of neonatal human melanocytes.

MATERIALS AND METHODS

Tissue Culture and Cell Lines Pure melanocyte cultures were either grown from neonatal foreskins, as described [7], or obtained from Clonetics Corporation, San Diego, CA and maintained at 37°C in 8% CO_2/92% air. Growth medium for all experiments was MCDB 153 (Irvine Scientific) supplemented with 10 ng/ml epidermal growth factor (Santa Ana, CA), 1 nM triiodothyronine, 10 μg each of transferrin, insulin per ml, 1 nM cholera toxin, and a dialyzed bovine pituitary extract (100 μg/ml) known to contain growth-promoting activity for human melanocytes. For the experiments requiring stimulation, TPA at 50 ng/ml was added to the medium to generate the "neuronal" morphology. All melanocytes were used prior to passage 3, except the Northern analysis, which required expanding the cells to passage 5. PC-12 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), and induced to differentiate with NGF (50 ng/ml) and cholera toxin (1 nM).

Indirect Immunofluorescence Melanocytes were seeded on rat-tail-collagen–treated coated plastic slides and grown for 3 d. Slides were rinsed in phosphate-buffered saline (PBS), and fixed in 4% (vol/vol) paraformaldehyde for 30 min. The first antibody used was either the mouse monoclonal V-9 [12] used at a dilution of 1:750 or an irrelevant mouse monoclonal antibody at a similar dilution. The second antibody used was a fluorescein-tagged goat anti-mouse IgG (Organon Teknika-Cappel, West Chester, PA). The cells were examined and photographed with a Nikon inverted microscope (Diaphot-TMD) and camera (Microflex UFX-II).

Immunoblotting For protein analysis, a confluent 100-mm dish of cells was rinsed twice with PBS, and 100 μl of 2× sample buffer (20% w/v glycerol, 0.125M Tris, 4% sodium dodecyl sulfate, pH 6.8) was added to the

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Abbreviations: GFAP, glial cells.

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dish. The cells were scraped and the extracted proteins quantitated using a Bio-Rad kit. A total of 10 μg melanocyte protein was reduced, denatured, and separated by 7.5% polyacrylamide gel electrophoresis, then electrophoretically tranferred to nitrocellulose using a transblot apparatus (Bio-Rad overnight) at 4°C and 60 V in Tris-glycine buffer with 20% (vol/vol) methanol. The antibody clone V-9 was obtained from ICN. Biomedicals, Inc., Costa Mesa, CA. Antigens on the nitrocellulose blot were incubated with the V-9 anti-vimentin antibody or an irrelevant antibody at a dilution of 1:750. Specific binding was detected by immunoperoxidase staining of the nitrocellulose blot.

RNA Isolation and Northern Analysis Melanocytes were grown to 80–90% confluence and then stimulated with TPA or left unstimulated. The cells were washed once with PBS, lysed at 4°C in a pH 7.8 solution containing 20 mM Tris, 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.65% NP-40, and allowed to stand for 5 min. The lysate was collected and the nuceli were removed by centrifugation. To this supernatant, 1/100 volume of 0.5 M ethylenediaminetetraacetic acid, 1/100 volume of 5.0 M NaCl, and 3.5/100 volume of 20% sodium dodecyl sulfate were sequentially added. After gentle mixing, two phenol/chloroform (1:1 volume) extractions were performed, followed by one chloroform extraction. The aqueous phase was precipitated overnight at −70°C with 3.0 M sodium acetate and ethanol. RNA was quantitated spectrophotometrically and quantitations were reaffirmed visually on a 1% agarose gel. Forty-microgram samples of total RNA were size fractioned on a 1% agarose-formaldehyde gel and transferred to nylon membrane. RNA was fixed onto the membrane by exposure to ultraviolet light. The cDNA insert was oligo-labeled [13] to a specific activity of 2×10^8 cpm/μg DNA and denatured by boiling. Pre-hybridization and hybridization conditions were as described [7]. The blots were then dried and autoradiography performed at −70°C with Kodak XRP film. The vimentin cDNA used for these studies was obtained from the American Type Culture Collection (ATCC 59160) and is a 1.1-kilobase piece inserted into the *Eco RI* site of pUC18. The human cDNA for tyrosinase (pMEL 34) was a kind gift of Dr. Byoung S. Kwon, Indiana University [14].

RESULTS

Immunofluorescence Melanocytes were grown in standard complete MCDB 153 with and without TPA 50 ng/ml for 48 h, then fixed and prepared for staining. Examination of Fig 1*A* demonstrates positive vimentin staining in a filamentous pattern in cells grown without TPA. When stimulated with TPA, the cells still retained positive staining with the anti-vimentin antibody (Fig 1*B*).

Immunoblotting Immunocytochemical studies demonstrated the presence of vimentin staining in dendritic cells found in the basal cell of the epidermis and presumed to be melanocytes [15–17]. Subsequently, vimentin has been demonstrated in epidermal melanocytes by electron microscopy [18]. Recent studies demonstrated that what was initially thought to be vimentin in PC-12 cells [19] is not vimentin, but an intermediate filament known as peripherin [20]. These results are understandable, as both proteins have a molecular weight of 57 kD. We needed to ascertain whether the protein we had detected by immunofluorescence was vimentin. PC-12 cells are known to be peripherin positive but vimentin negative [21], so we used these cells as a control for antibody cross reactivity. Western blotting studies, using the anti-vimentin V-9 antibody, clearly recognize a single band of 57 kD in cultured human fibroblasts (Fig 2, *lane 1*) and melanocytes (Fig 2, *lane 3*), which is absent in the PC-12 cells (Fig 2, *lane 2*). The data demonstrate that the V-9 monoclonal antibody recognizes vimentin and not peripherin, and that this protein is expressed by neonatal human melanocytes.

Northern Analysis Because of the immunofluorescent immunoblotting studies, we wondered if the 2.0-kb vimentin and mRNA was expressed by human melanocytes. Northern blotting studies were performed on total cellular RNA from human epidermal melanocytes grown in the presence of cholera toxin and 10% serum, as well as TPA-treated melanocytes. Examination of the autoradiograph (Fig 3*A*) demonstrates high levels of the 2.0-kb vimentin mRNA expression in both the unstimulated *(lane 1)* and TPA-treated cells *(lane 2)*.

Dermal fibroblasts can contaminate human melanocyte cultures, although the cholera toxin and calcium levels we employ in our serum-free culture system tend to decrease this. Tyrosinase is the rate-limiting enzyme in melanin biosynthesis and is not present in human fibroblasts. To demonstrate that our cells were mostly melanocytes, we performed Northern analysis of equivalent amounts of total cellular RNA (40 μg) from the same experiment as described above and probed with the cDNA for human tyrosinase [14]. Examination of this autoradiograph (Fig 3*B*) demonstrates high levels of the 2.4-kb tyrosinase messenger RNA in both unstimulated *(lane 1)* and TPA-stimulated cells *(lane 2)*. The tyrosinase autoradiograph was exposed to XRP film for the same amount of time as the vimen-

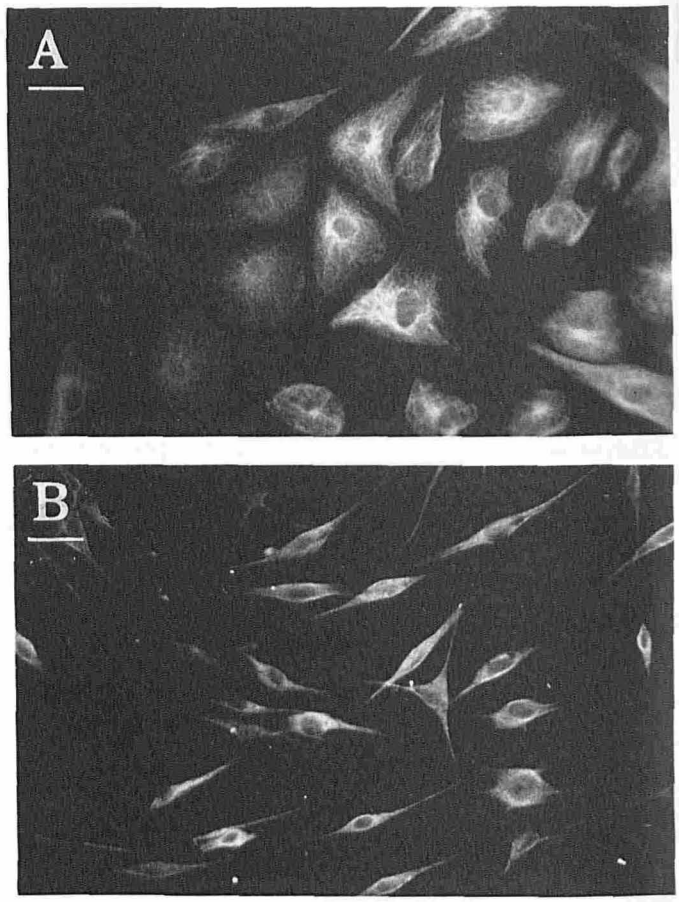

Figure 1. *A)* Immunofluorescent study of human melanocytes grown in MCDB 153 without TPA demonstrate a stellate and polygonal morphology with positive vimentin staining. *B)* When treated with TPA 50 ng/ml, a neuronal morphology is noted; however, the cells retain the positive vimentin staining. *Bar,* 10 μm.

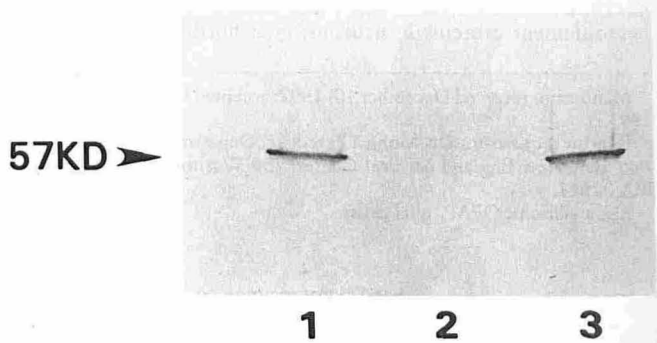

Figure 2. Immunoblot of fibroblasts *(lane 1)*, PC-12 cells *(lane 2)*, and melanocytes *(lane 3)*.

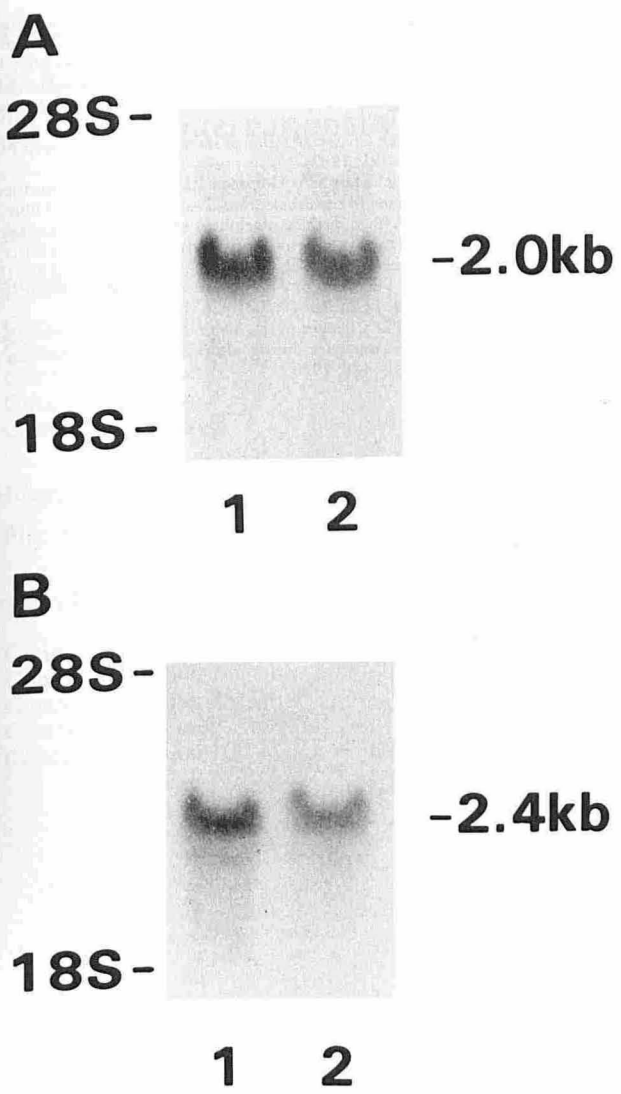

Figure 3. *A)* Northern blot analysis of unstimulated and TPA-stimulated melanocytes, probed with the cDNA for vimentin. *B)* Northern blot analysis of unstimulated and TPA-stimulated melanocytes, probed with the cDNA for tyrosinase.

tin autoradiograph (3 h). These observations demonstrate the presence of the mRNA for tyrosinase and suggest the cells were melanocytes. Whether or not the fivefold decrease in tyrosinase mRNA seen in the TPA-treated cells reflects a transcriptional downregulation in the expression of this mRNA could not be determined from these studies.

DISCUSSION

Human melanocytes are neural crest-derived cells that synthesize and distribute the pigment melanin to neighboring keratinocytes through a series of dendrites. In culture, melanocytes can assume intriguing morphologies depending on the characteristics of the culture medium. Phorbol esters induce a neuronal configuration in these cells that is associated with expression of the low-affinity receptor for nerve growth factor [7]. Because melanocytes are so polymorphic *in vitro*, we wondered what proteins could play a role in maintaining these different shapes. Using immunocytochemistry and immunoblotting, as well as Northern analysis, we demonstrate here that cultured neonatal human melanocytes express the intermediate filament vimentin.

Vimentin is an intriguing intermediate filament in that, of all intermediate filaments yet described, it is the most promiscuous. Classically described in cells of mesenchymal origin, it has been demonstrated in both immature and mature cells of varied lineage, as well as in transformed cells and many cells in culture [8–10]. This pattern of vimentin expression contrasts markedly with the other intermediate filament proteins, like keratins and neurofilaments, which are quite restricted in tissue-specific expression, and supports the contention that vimentin may have a much broader spectrum of biologic activity.

What does vimentin do in the cytoplasm of cells in general, and specifically, what role does it play in the cytoskeleton of the melanocyte? Like all intermediate filaments, vimentin is a constituent of the cytoplasm. Recent studies have demonstrated binding sites for vimentin on both the nuclear and plasma membranes, providing evidence that vimentin could function in attachment of the plasma membrane to the nuclear membrane, and, potentially, in signal transduction [22]. Shifts in the intracytoplasmic location of vimentin during the spreading of cultured BHK-21 fibroblasts, followed by the migration of various organelles into these areas, suggested that vimentin might function in intracellular organization and transportation of organelles in the cytoplasm [23]. Indeed, close association of melanosomes and 100-Å or intermediate filaments *in vivo* has been demonstrated previously [24]. However, what specific function vimentin performs in the cytoplasm of the human melanocyte could not be determined from our studies. In summary, we have demonstrated that neural crest-derived melanocytes express the intermediate filament vimentin in a variety of tissue culture situations. These data agree with previous reports demonstrating the expression of vimentin by melanocytes *in vivo*. The association of intermediate filaments with melanosomes *in vivo* raises the possibility that vimentin is this intermediate filament, and that this protein plays a role in the normal structure and function of the human melanocyte.

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