Characteristics of Cultivated Adult Human Nevocellular Nevus Cells

Barbara A. Gilchrest, M.D., Valori Treloar, M.D., Anita M. Grassi, M.D., Mina Yaar, M.D., George Szabo, Ph.D., and Evelyn Flynn, B.A.

Cutaneous Gerontology Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University (BAG, VT, AMG, MY), and Laboratory of Electron Microscopy, Harvard School of Dental Medicine (GS, EF), Boston, Massachusetts, U.S.A.

Nevus cells are of biologic interest because of their uncertain relationship to epidermal melanocytes and of clinical interest because of their statistical association with melanoma. We report a technique that allows reliable cultivation of nevus cells from small acquired and congenital nevi and permits in vitro characterization of this cell type. Morphologically, cultured nevus cells were found to closely resemble epidermal melanocytes from the same or comparably aged donors, manifesting marked dendricity and specific ultrastructural features characteristic of melanocytes; but could be distinguished by the presence of occasional large binucleate or trinucleate cells and by the frequent finding of grouped melanosomes in nevus cell cytoplasm. Growth kinetics were also similar for nevus cells and epidermal melanocytes, with population doubling times of 1–2 weeks in hormone-supplemented serum-free medium, and substantial growth enhancement by fetal bovine serum. As previously noted for epidermal melanocytes, nevus cells in serum-free culture demonstrated striking substrate responsiveness, with far greater attachment rates and degree of cytoplasmic spreading on fibronectin or type I/III collagen than on laminin, type IV collagen, or uncoated plastic. These strong similarities in vitro suggest that morphologic and behavioral differences observed between epidermal melanocytes and nevus cells in the skin may result from local environmental influences rather than from intrinsic cellular differences. The availability of a satisfactory culture system for nevus cells may facilitate future investigations into their malignant potential and other biologic features. *J Invest Dermatol 87:102–107, 1986*

Nevocellular nevi (moles) are extremely common human tumors. Approximately 1% of newborns are reported to have congenital nevi [1,2] and the average young adult has 15–40 [3–5]. Most are completely benign and constitute at most a cosmetic concern. However, recent epidemiologic studies have emphasized a much greater than random statistical association with malignant melanoma [6–8], especially for congenital nevi [9–11], and a dominantly inherited syndrome of dysplastic nevi with a high rate of malignant conversion has now been identified in several kindreds [12,13].

At present, decisions to excise prophylactically or simply to observe nevi are based on the moderately controversial statistical associations mentioned above, reinforced by clinical and histologic features of individual lesions, although the latter are helpful primarily for "advanced" lesions and offer little prognostic guidance in routine cases. Efforts to identify cell surface markers, characteristic of benign vs malignant pigment cells [14–17] offer considerable promise, but have not yet obviated the clinical dilemmas so often presented by congenital or otherwise "suspicious" nevi.

Aside from the above practical concerns, nevi raise questions of biologic interest. Although it is widely assumed that nevus cells, like melanocytes [18], are of neural crest origin [19], it is not known whether they are identical (except for location) to epidermal melanocytes. Similarly, there is virtually no information regarding factors that might determine their different migration patterns in the skin, tendency to "nesting" in the dermis, individual cellular morphology, susceptibility to malignant transformation, or frequent disappearance in late middle age [5]. Investigation of such features has been hampered by lack of adequate in vitro systems for this cell type. We now report that a minor modification of our previously published technique for human epidermal melanocytes [20] allows reliable cultivation of nevus cells from adult donors through 2–3 passages, a period sufficient to allow detailed in vitro characterization.

MATERIALS AND METHODS

Cell Sources Fifteen compound nevi were obtained at the time of elective surgical removal from 12 healthy adult donors aged 19–66 years. All nevi were benign by clinical criteria, ranged in diameter from 3–15 mm, and were located on the trunk or arm. Using combined clinical, historical, and histologic criteria [21], 10 nevi were judged to be acquired and 5 congenital.

Tissue Culture Specimens were immediately placed in sterile tissue culture medium containing 10% bovine serum, 75 U/ml penicillin, and 50 μg/ml streptomycin, then stored until processing at 4°C for up to 24 h. Deep dermis and subcutaneous fat were removed with dissecting scissors, and the remaining tissue was cut into 3-mm^2 fragments, washed in calcium-free phosphate-buffered saline (PBS), and incubated overnight at 4°C in 0.25% trypsin. The epidermis was then removed mechanically from the dermis with forceps, and the resulting dermal fragments were incubated at 37°C for up to 1 h in a 50:50 solution of 0.25% trypsin and 0.1% collagenase. Serum-containing medium was then added to the enzyme solution, dermal fragments were removed, and the disaggregated cells were centrifuged and resus-

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Reprint requests to: Barbara A. Gilchrest, M.D., USDA Human Nutrition Research Center on Aging, 711 Washington Street, Boston, Massachusetts 02111.

Abbreviations:
PBS: phosphate-buffered saline
PD: population doublings
PDT: population doubling time

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pended in hormone-supplemented medium (vide infra), counted, and inoculated on uncoated tissue culture dishes (Falcon) at approximately 10^5 cells/cm^2. Cultures were maintained at 37°C in 8% CO_2 and provided with fresh serum-free medium twice weekly. Residual dermal fragments were attached to scored dishes and grown as explant cultures under identical conditions. When a margin of normal skin accompanied the nevus specimen, this epidermis was processed to yield epidermal melanocyte cultures [20].

Hormone-Supplemented Medium Medium 199 (Gibco) was supplemented with 10 ng/ml epidermal growth factor (Collaborative Research), 10 μg/ml insulin (Sigma), 10^{-9} M triiodothyronine (Sigma), 10 μg/ml transferrin (Sigma), 1.4×10^{-6} M hydrocortisone (Calbiochem), 1.2×10^{-9} M cholera toxin (Calbiochem), and 100 μg/ml of a dialyzed bovine hypothalamic extract known to contain a growth-promoting activity for melanocytes [22]. Fetal bovine serum 2% was added to the cultures for 24 h at the time of plating and at each cell passage; cultures were otherwise maintained in serum-free medium. All cultures were routinely scanned under phase microscopy throughout the in vitro lifespan to determine cellular morphology and to detect fibroblast or keratinocyte contamination. Representative fields were photographed, and any dishes suspected of containing mixed cell populations were discarded. At each passage, selected dishes were further studied by electron microscopy to determine cellular ultrastructure and to verify cell type.

Electron Microscopy Cultures to be examined ultrastructurally were fixed in situ with Ito-Karnovsky fixative (4% formaldehyde, 5% glutaraldehyde, 0.02% 2,4,6-trinitrophenol) for 2 h at 25°C, rinsed twice with cold 0.1 M cacodylate buffer, and postfixed in 1.0% osmium tetroxide for 1 h. Cells were dehydrated in a graded series of ethanols and embedded in Epon. Sections were cut parallel to the culture surface with a diamond knife using a Porter-Blum MT-2B ultramicrotome, stained with uranyl acetate and lead citrate, and photographed using an AEI Corinth 275 electron microscope.

Attachment Studies Ability of the nevus cells to attach and spread on uncoated plastic tissue culture dishes and on several matrix materials was compared in a protocol previously utilized for epidermal melanocytes [23]: 4–10 × 10^4 trypsinized cells from primary or first-passage cultures were added to paired 35-mm dishes and observed and photographed in a blinded fashion at 2, 6, and 24 h under phase microscopy. At 24 h each dish was gently rinsed with PBS, trypsinized, and the resulting cell suspension counted in a hemacytometer chamber. Treated dishes were coated immediately prior to each experiment with one of the following: 10 μg/cm^2 of mixed type I and III collagen (Sigma) prepared as previously described [23], or 50 μg/dish (approximately 6 μg/cm^2) laminin supplied by Drs. George R. Martin and Hynda Kleinman of the National Dental Institute, Bethesda, Maryland.

Proliferation Studies Cells from near-confluent primary, secondary, or tertiary cultures were trypsinized, counted, and repassaged into paired dishes at 2.5 × 10^4 to 3 × 10^5 cells/35-mm dish, allowed to proliferate 2 weeks under the conditions specified above; then rinsed, trypsinized, and recounted. Population doublings (PD) were calculated from the equation $PD = 1n\,(N/N_0)\,(1n\,2)^{-1}$ and population doubling time (PDT) in days from the equation $PDT = 14/PD$.

RESULTS

Of the 15 nevus specimens, 12 yielded apparently homogeneous nevus cell cultures of sufficient longevity to permit data collection. There were no detectable differences in any culture parameter between the 4 presumably congenital nevi and the 8 presumably acquired nevi or as a function of donor age. Consequently, all culture data were pooled in the following analysis.

Morphology By the end of the first day in culture and for at least 4 weeks, nevus cells were markedly dendritic (Fig 1), closely resembling cultured epidermal melanocytes derived from normal skin of comparably aged donors, in contrast to the epithelioid nevus cell nests observed in histologic sections of the parent lesions (Fig 2). At the ultrastructural level (Fig 3), cultured nevus cells were characterized by a large oval nucleus, prominent nucleoli, relatively sparse melanin granules, and varying numbers of mitochondria, ribosomes, and glycogen droplets. Nevus cell borders characteristically had numerous filopodia, in contrast to

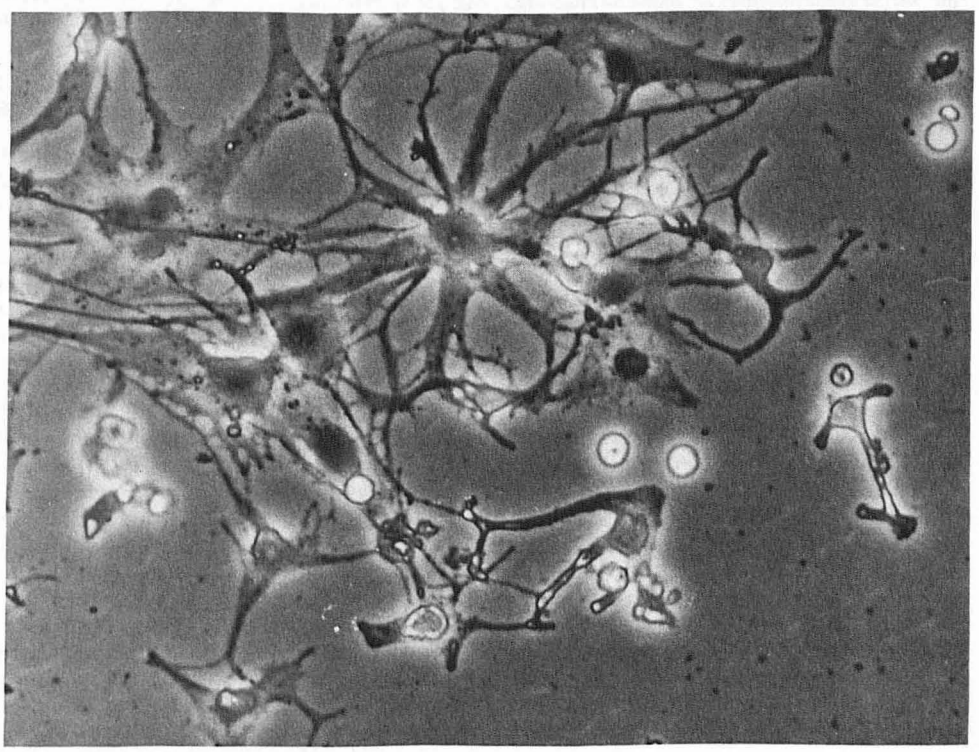

Figure 1. Nevus cells in vitro. First passage culture derived from a 42-year-old donor, showing marked dendricity. Phase contrast micrograph. Original magnification, × 650.

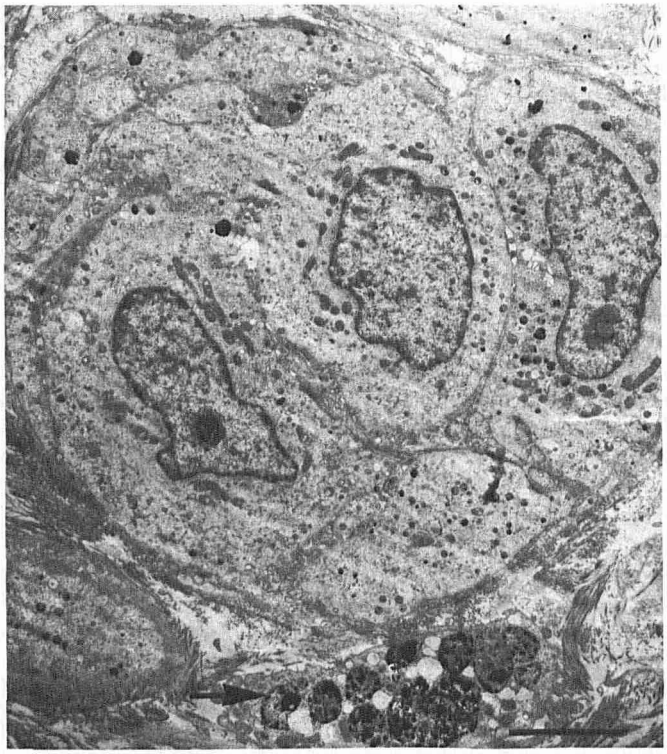

Figure 2. Dermal nevus cell nest in a cross-section of an acquired nevus. Note whorl-like array of 5 epithelioid nevus cells containing sparse pigment granules. An adjacent melanophage (*arrow*) is characterized by numerous phagocytic vacuoles, many containing abundant melanosomes. *Bar* = 5 μm.

the relatively smooth borders of epidermal melanoctyes maintained under identical culture conditions. Occasional large binucleate and trinucleate cells were noted in all nevus cell cultures examined, but were absent from melanocyte cultures. Contaminating cell types identified included rare fibroblasts, a single mast cell, and several phagocytic cells containing melanin granules (melanophages).

The most distinctive feature of the nevus cells both in tissue sections and culture dishes was the frequent presence in their cytoplasm of grouped melanosomes (Fig 4). These round to oval melanosomes were contained within single membrane-bound vesicles, reminiscent of melanosomes "packaged" within Caucasian keratinocytes. Melanosome grouping was seen at least occasionally both in vitro and in vivo in all the dermal nevi studied. In contrast, it was observed within the epidermis in only one biopsy section, where it was restricted to a small number of epidermal melanocyte-like cells overlying dermal nevus cell nests, and was never observed in cultures of epidermal melanocytes derived from biopsies of normal skin lacking nevi.

Attachment and Spreading Three separate experiments utilizing second- or third-passage cultures derived from acquired nevi yielded the same rank order of substrate preference as judged by cell counts and degree of cytoplasmic spreading [23]: fibronectin > type I/III collagen > laminin = type IV collagen > plastic alone. Nevus cells inoculated on fibronectin or type I/III collagen were strikingly larger in cytoplasmic area than were paired inocula on laminin, type IV collagen, or uncoated plastic. Within 2 h, a majority of the cells on fibronectin were stellate (polydendritic) and those on type I/III collagen were bipolar or tripolar, while at 24 h, many cells on the other substrates remained rounded to near-rounded with minimal cytoplasmic spreading and dendrite formation. Percent attachment at 4 h and 24 h varied from 40–85% for nevus cells inoculated on fibronectin-coated dishes and was consistently less for the same cells on the other substrates tested, but the differences in attachment rates were not statistically significant and the small number of cells counted per dish rendered suspect the absolute values.

Growth Rate Nevi from 5 donors provided sufficient nevus cells for 21 separate calculations of the population doubling time (PDT) in serum-free medium (Table I). PDT was long in all cultures and increased moderately between second and third passage, from 11.4–17.0 days on average. In these calculations, PDT was not corrected for attachment rate, and such a correction would be expected to decrease PDT by approximately 25–50%, based on determination with other nevus cultures (vide supra). Nevus cell growth beyond the third passage was very poor, and fibroblast contamination or cell detachment was observed.

Nevus cell growth was notably enhanced by addition of 2–10% fetal bovine serum to the medium, but frequent overgrowth of the cultures by dermal fibroblasts prohibited reliable calculation of growth rate.

DISCUSSION

Although there have been several recent reports regarding cultured human nevus cells [16,24–29], all are seriously flawed by their failure to unequivocally identify the cell intended for study. In some studies, in which "nevus cells" observed under phase microscopy are described as "fibroblast-like" and rapidly growing [26,27,29], it is difficult to exclude the possibility that the cultures contained predominantly fibroblasts. In others, derived from whole skin specimens [16,24,25,28,30,31], there is no apparent mechanism for distinguishing epidermal melanocytes from nevocellular nevus cells, as both populations were present in the starting material and no differentiating morphologic features were claimed. In contrast, in the present study, the identity of the cultured cells was repeatedly confirmed by electron microscopic criteria throughout the culture lifespan and the phase microscopic appearance of such confirmed cultures was correlated with that of paired cultures used in attachment or growth assays.

This close monitoring and familiarity with distinguishing morphologic features of the various cultured skin-derived cells virtually guaranteed freedom from fibroblast contamination. The other necessary distinction, that between epidermal melanocytes and nevocellular nevus cells, was accomplished in part by removing the epidermal portion of the skin specimens prior to processing the dermal nevus cells and in part by identifying a cellular marker (grouped melanosomes) for the nevus cells. This latter criterion was needed to eliminate, or at least greatly reduce, the possibility that the "nevus cell" cultures were in fact derived from hair bulb melanocytes rather than from the more numerous nevus cells. While this approach to identifying the cell population under investigation is clearly suboptimal, the inherent concerns appear valid. It is hoped that ongoing work, such as attempts to identify by antibody techniques surface markers unique to nevus cells [14–17,29,32], will provide more definitive and convenient means of determining culture origin and purity.

In addition to focusing attention on the critical issue of nevus cell identification, the present study provides to date the most comprehensive in vitro characterization of this important cell type. Nevus cells, whether derived from congenital or acquired lesions, were found to be highly dendritic, as observed in this study and previously [33] for adult-derived epidermal melanocytes, with morphologic evidence of active metabolism and melanin synthesis. Over a period of several weeks, the nevus cells on average became more epithelioid in morphology and contained more melanosomes. Proliferation, as judged by frequent observation under phase microscopy and by the ratio of cell yield to initial cell inoculum after various periods of growth, was slight even in serum-containing medium. Culture lifespan was limited to 2–3 postprimary passages and 2–3 months, with 6–8 estimated cumulative PD in more successful cultures. The nevus cells were clearly morphologically responsive to their substrate, demon-

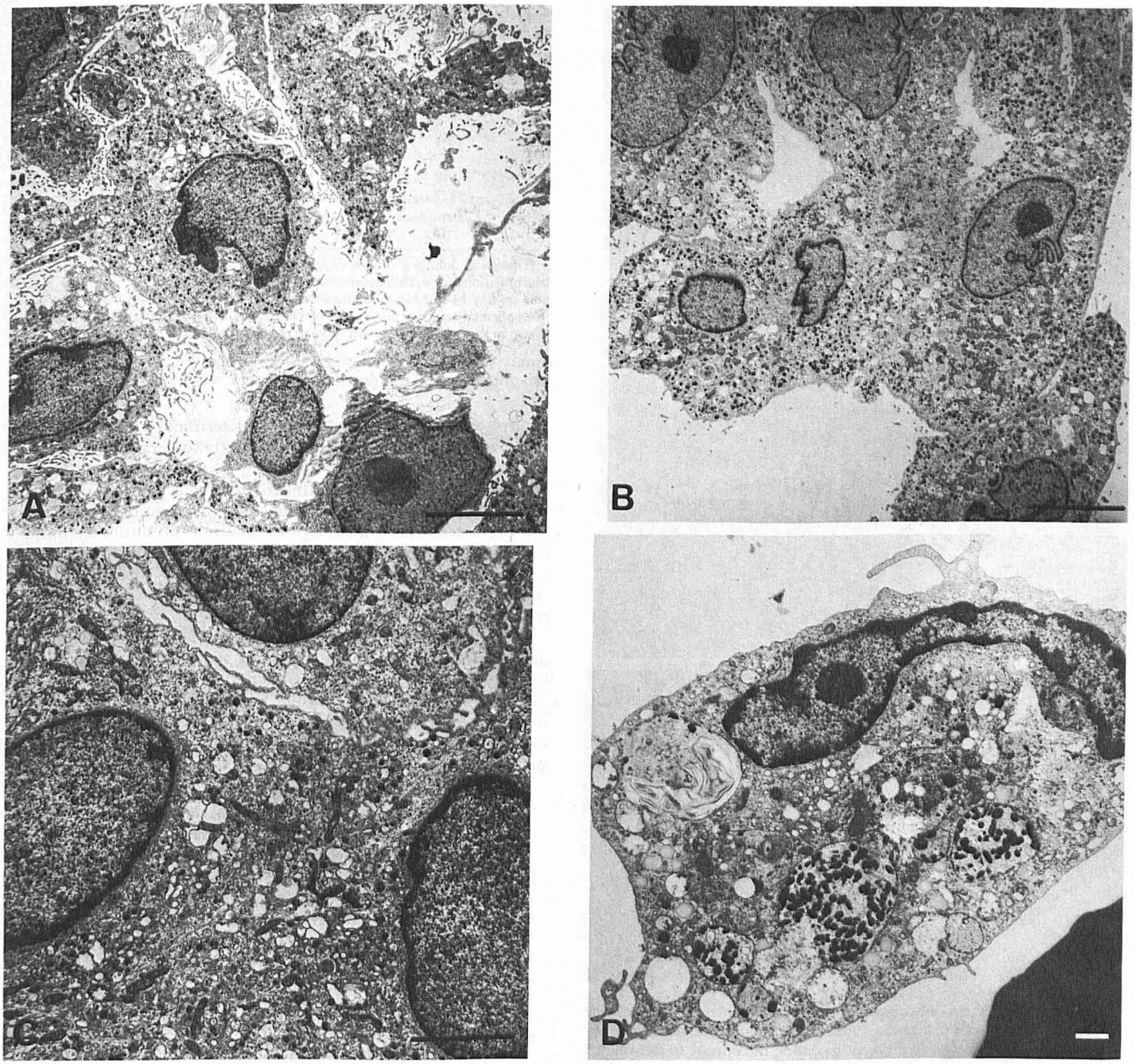

Figure 3. Pigment cell ultrastructure in vitro. *A*, Nevus cell culture day 22, derived from the acquired nevus of a 27-year-old donor. Note long filiform projections from the cell surfaces. *Bar* = 10 μm. *B*, Epidermal melanocyte culture from the same donor as (*A*). Nuclear and cytoplasmic features are very similar, but the cell borders lack prominent filopodia. *Bar* = 10 μm. *C*, Nevus cell at high magnification. Melanin granules, mitochondria, endoplasmic reticulum, and glycogen droplets are easily appreciated in the nevus cell cytoplasm. Lower cell is seen to be binucleate, with no cytoplasmic interruption whatever between 2 widely separated oval nuclei. *Bar* = 5 μm. *D*, Contaminating melanophage. This cell differed from the dermal nevus cells in the same culture dish by the presence of large membrane-bound phagosomes containing either numerous melanosomes or cell membranes and cytoplasmic debris (myelin bodies), as well as by its different nuclear morphology. *Bar* = 1 μm.

strating higher attachment rates and greater cytoplasmic spreading on dermal matrix materials than on basement membrane components or on plastic, although severely limited cell numbers prohibited meaningful quantitation.

Overall, cultured nevus cells strongly resembled cultured epidermal melanocytes obtained either from the uninvolved margins of excised nevi or from normal skin or other adult donors. Growth rate and substrate responsiveness were essentially identical to those previously observed for adult melanocytes under the same culture conditions [20,23]. Indeed, nevus cells could be differentiated from melanocytes, if at all, only by the presence of grouped melanosomes and the occasional finding of large binucleate cells, never observed in melanocyte cultures. The striking similarities of these cells in vitro raise the question of their respective origins, a point on which only speculation is presently available. Are melanocytes and nevus cells intrinsically different and therefore dissimilar in their appearance and location in the skin? Or, despite the existence of "dermal melanocytes" [34], are these cells identical except for local environmental influences that render: (1) the dermal nevus cell epithelioid and prone to form tight aggregates

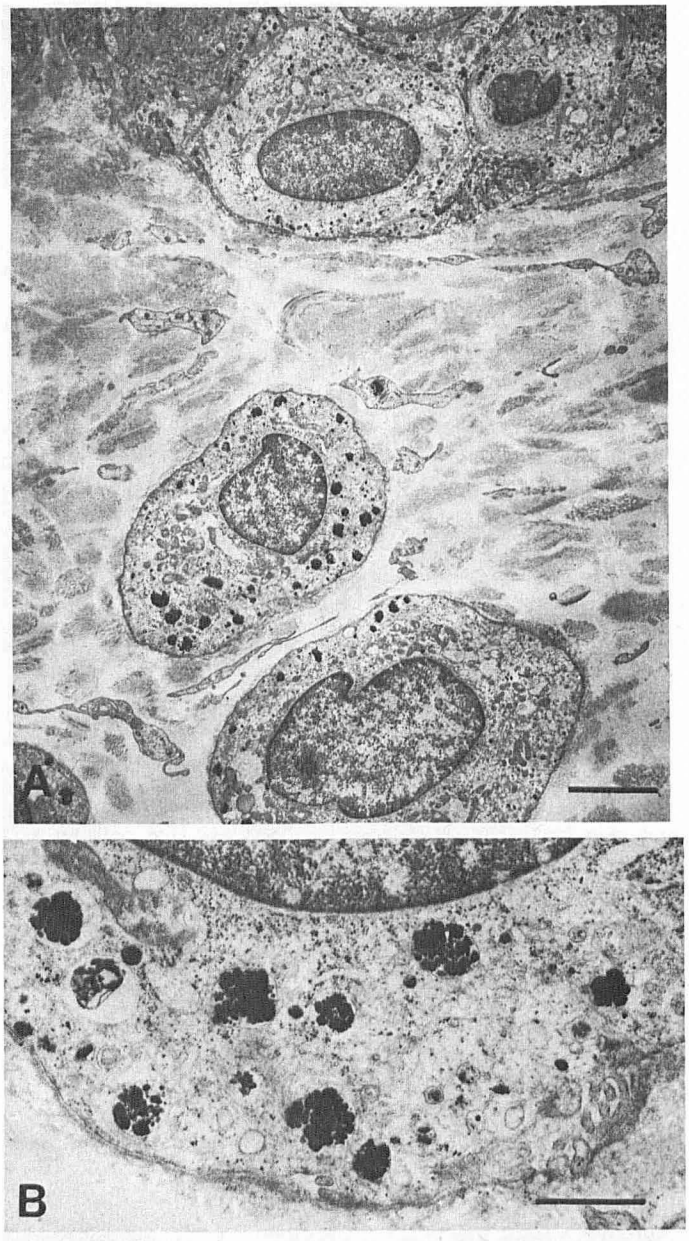

Figure 4. Grouped melanosomes. *A*, Cross-section through an acquired nevus of a 26-year-old Caucasian donor. Characteristic nevus cells in the papillary dermis show large pigment granules, in contrast to the smaller pigment granules visible in the overlying epidermal melanocytes along the basement membrane. *Bar* = 5 μm. *B*, Higher magnification of the nevus cell cytoplasm reveals grouped membrane-bound melanosomes, accounting for their apparent larger size at low magnification. *Bar* = 1 μm.

or nests; and (2) the epidermal melanocyte dendritic and prone to isolate itself from other melanocytes in a sparse array atop the basement membrane? The latter hypothesis is favored, although certainly not proved, by the present data and by the fact that both cell types give rise to malignant melanomas apparently indistinguishable from each other.

The phenomena of multinucleate nevus cells and grouped melanosomes within nevus cells, previously noted in histologic studies of nevi in vivo [4,35,36], are unexplained. The former may represent either cell fusion or incomplete mitosis, while the latter is most readily explained by pigment donation between nevus cells, in analogy to the process known to occur between epidermal melanocytes and keratinocytes. Unfortunately, no nevi from black donors were available for study to determine whether melanosomes of a size normally transferred singly to keratinocytes might nevertheless be "packaged" in nevi.

Table I. Growth of Nevus Cells in Serum-Free Medium

Tissue Donor	Donor Age (years)	Passage Level	No. Separate Experiments	Population Doubling Time (days)[a]
1	26	1	2	13.2 ± 1.9
		2	4	11.4 ± 4.7
2[b]	52	2	4	10.1 ± 2.7
		3	5	18.2 ± 4.5
3	42	2	1	11.0 ± 1.0
4	27	2	3	14.0 ± 2.6
		3	1	11.0 ± 3.6
5	37	2	1	8.6 ± 1.1

[a]Population doubling time is expressed as the average mean ± SD of duplicate determinations for each culture dish: $[1n\ (2)]\ [1n(N/N_0)]^{-1}$ [14], where N = cell yield on day 14 and N_0 = cell inoculum.

[b]Congenital nevus by history and by clinical and histologic criteria, approximately 12 cm^2 in surface area. All other nevi were judged acquired by the same criteria and were 3–5 mm in diameter.

Ethical considerations prohibited utilizing atypical or dysplastic nevi in these studies, as the amount of tissue required would have compromised routine histologic evaluation. As culture techniques improve, however, it will be of interest to determine whether such nevus cells differ in proliferative capacity or other culture characteristics from "routine" nevi with a lesser risk of malignant conversion.

The approach described in this paper permits cultivation of nevus cells from small congenital or acquired nevi in sufficient numbers and for an adequate time period to accomplish a partial in vitro characterization. Further work in this area should prove helpful in elucidating the biology of this intriguing cell type and ultimately in addressing the important clinical problem of its malignant transformation.

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