Histopathogenesis of malignant skin melanoma induced in genetically susceptible transgenic mice

(melanocytic hyperplasia/nev/ulceration/invasion/metastasis)

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ABSTRACT Animal models of human malignant skin melanoma were created in melanoma-susceptible inbred-strain transgenic mice by grafting skin from donors of high-susceptibility lines to hosts of a low-susceptibility line, thereby overcoming the problem of early death of the more susceptible animals from eye melanomas. As already described [Mintz, B. & Silvers, W. K. (1993) Proc. Natl. Acad. Sci. USA 90, 8817-8821], melanocytes within the grafts selectively proliferated in close proximity to areas of greatest wound healing, presumably in response to mitogenic factors from cells contributing to wound repair. An orderly sequence of externally visible events culminated in malignant melanoma. We examine here the histogenetic concomitants of these changes and find that they define a stepwise sequence strikingly comparable to that leading to human cutaneous melanoma. Moreover, the histological details suggest some of the underlying mechanisms.

While the early lesions are first seen in the superficial dermis in the mouse, and in the basal layer of the epidermis in the human, both progress by radial growth followed by vertical growth. Melanocytic hyperplasia resulted in nests of densely melanized fusiform cells which were losing their dendrites. Some discrete lesions in the deep dermis appeared as blue nevi. As radial proliferation advanced, cellular atypia increased, and the previously independent melanocytes cohered closely and formed a small solid tumor; the cells were usually then hypomelanotic or amelanotic. Ulceration of tumor through the epidermis occurred early. The tumor mass grew rapidly in the deep dermis and invaded and destroyed subcutaneous tissue and muscle. Primary tumors in the skin were often heterogeneous, with lobules or regions differing in pigmentation or atypia. However, the cells in circulating emboli, or in metastases in lymph nodes and lungs, appeared relatively homogeneous. These genetically uniform transgenic mouse models provide experimental access to the multistage genesis of melanoma.

Unexpectedly, melanocytes in the grafted skin proliferated excessively and selectively, close to areas of greatest wound healing, especially near the graft margins (3), thereby strongly suggesting a causal role of growth factors and cytokines associated with wound repair (4). The melanocytic lesions progressed to malignant melanoma in all skin grafts from the more susceptible of two transgenic donor lines tested, and in one-fourth of the grafts from another line; no melanomas of host origin developed. We have proposed that the induction of this malignancy in susceptible skin by wound healing is merely a caricature or exaggeration of the same basic biological and molecular events as ordinarily underlie the genesis of melanoma, without skin grafting, and that the exaggeration makes those events more accessible to analysis (3).

From the externally visible evidence of progressive changes in the lesions, we concluded that the development of the transgenic melanomas bears a striking resemblance to that of human skin melanomas and is an excellent in vivo model of the human malignancy (3). This model has the further advantages of genetic uniformity and of the absence of any other kinds of skin tumors in the animals. In the present report, we describe the histogenetic evidence in support of a multistep tumor progression paralleling the sequence in human cutaneous melanomas. In addition, details visible only at the microscopic level suggest mechanisms on which some of the changes leading to malignancy may depend.

MATERIALS AND METHODS

The same transgenic mouse lesions as in the companion report (3) were examined histologically here. These originated in donor body-skin grafts from line 8 (hemizygous for the transgene (Tag/-), and line 9 (Tag/Tag homozygotes), each developing in a line 12 (Tag/-) host. Wild-type body-skin grafts, experimentally wounded but ungrafted transgenic skin, and unmanipulated wild-type and transgenic skin were also included. Tissues were fixed in neutral formalin, embedded in paraffin, and stained with hematoxylin/eosin. Some sections were immunostained as described (1) to visualize the S-100 or HMB-45 proteins associated with malignant melanoma. Staining for 3,4-dihydroxyphenylalanine (dopa) was used in some cases to detect nonmelanized pigment cells (along with melanized ones).

RESULTS AND DISCUSSION

Melanocytes in C57BL/6 Wild-Type and Transgenic Body Skin. Pigmented melanocytes in the fur-bearing body skin of the wild-type C57BL/6 mouse occur chiefly within the hair follicles. They are virtually absent in the body skin outside the follicles, although they are present in the skin of other regions such as the ears and tail (5). Some dopa-positive unpigmented melanocytes (or melanoblasts) persist in the...
epidermis and superficial dermis of body skin and synthesize melanin when the mice are exposed to ultraviolet light (6). In contrast, pigmented melanocytes occur in the epidermis and dermis of human body skin, especially in the basal layer of the epidermis (7). The epidermis itself is much thinner in the haired areas of mouse skin than in human skin and has less-defined strata.

The body skin of Tyr-SV40E transgenic control mice was found to differ from wild-type controls of the same inbred strain in having occasional pigmented melanocytes in the superficial dermis and mid-dermis. In older transgenics, the cells were seen in the mid-dermis and deep dermis. Small flat black spots or macules also appeared spontaneously in the older transgenic mice. The macules had regular borders and consisted of groups of loosely associated pigment cells in the dermis. The accumulation of pigmented cells in unusual numbers or sites has also been described in various internal organs of the transgenic mice and was referred to as melanosis (2).

**Melanocytic Hyperplasia in Grafted Transgenic Skin.** After transplantation, the number of isolated melanocytes in the dermis increased, in comparison with ungrafted transgenic skin of similar age, so that scattered dendritic pigment cells were not difficult to find (Fig. 1a). A much more striking change appeared within a few weeks after grafting and was readily apparent externally as a blackened localized patch just inside the margins of the graft and occasionally as a more irregular black tracery in the interior of the graft (3). These areas consisted of nests of discrete melanocytes in variable numbers; they clearly represented melanocytic hyperplasia, similar to early lesions after application of carcinogens to mouse skin, or carcinogens followed by exposure to ultraviolet light (8, 9). The pigmented cells in our grafts were usually in the papillary and mid-dermis intermingled with dense collagen fibers (Fig. 1b).

**Changes in Melanocyte Morphology.** At this stage, the melanocytes gradually displayed several noteworthy changes. They were relatively elongated rather than epithelioid, their dendrites were greatly reduced in length and number, or even absent (Fig. 1b and f), and melanization of the cells was markedly increased. Normal melanocytes in the skin characteristically have long dendritic processes through which pigment granules are exported to keratinocytes (or to hairs, when the cells are in the base of the hair follicles). Decreased dendriticity of the hyperplastic transgenic melanocytes could thus lead indirectly to accumulation of pigment granules in the cells and to hyperpigmentation. The intensified melanization is usually transitory; as the transgenic cells continue to change in other morphological respects (described below), they often become hypomelanotic or amelanotic.

The loss of dendrites is of particular interest because their continued presence would be an impediment to intimate adhesion between cells, as will presently occur in the formation of a solid melanoma. Experiments with cultured human melanocytes have shown that decreased dendricity may result when keratinocyte-derived soluble factors in the medium are withdrawn, and the decrease is reversible (10). Certain mouse pigmented mutations are also known to cause reduction of dendrites (11). One of these encodes an unusual type of myosin heavy chain at the dilute locus (12) and may be involved in pigment granule transport along cytoskeletal elements.

**Nevi.** A few of the isolated flat black macules in the interior of some grafts became slightly wider and thicker, extending more deeply into the dermis, and could then be termed moles or nevi. In time, some nevi expanded further in width and depth, the cells became even more intensely melanized, and the lesions were externally deep blue in color, similar to human blue nevi. Such lesions contained more melanocytes than nonblue nevi and were usually localized in the deep dermis, often at the interface with the subcutaneous tissue. The cells were S-100 positive but HMB-45 negative. In the most densely pigmented areas of the grafts, discrete blue nevi were not apparent externally; however, similar lesions were found histologically. Among the several dozen melanocytes in such nevi, some were spindle shaped and others were epithelioid (Fig. 1 c and d). The admixture with dense dermal collagen fibers, other connective tissue elements, and melanin-containing macrophages (or melanophages) was similar to the description of human blue nevi (13).

**Melanomas.** In no case did a discrete and isolated blue nevus, situated outside the regions of wound-associated dense melanocytic hyperplasia, become a melanoma in the course of the graft experiment, spanning over 1 year. It is therefore likely that many of these are indolent lesions generally destined to remain benign, as is often the case in the human cellular blue nevus (13). Nevertheless, the very gradual nature of the series of changes seen in our skin grafts, and the physical contiguity of nevi, blue nevi, atypical nevi (14), and melanoma, support the view that certain nevi are in fact the precursors of the mouse melanomas, as is likely in human melanomas (15). In Fig. 1d, this contiguity is apparent in the deep dermal blue nevus (right arrowhead), the transitional area (immediately above) with decreasing cellular melanization, and the early melanoma (left arrowhead). It seems possible that parameters are shared by nevi to make this transition; such factors may be more readily available where injury and wound repair are in progress.

As documented in Fig. 1 c, d, and g, atypical nevi were increasingly comprised of rounded cells lacking dendrites and having an augmented nucleocytoplasmic ratio, prominent nucleoli, and scant pigmentation; such cells were at first intermingled with the elongated and heavily pigmented cells characteristic of earlier stages. These atypical cells tended to proliferate radially into the neighboring dermis. The irregular greyish areas previously observed spreading from more densely pigmented regions (see figure 4 b–d in ref. 3) seem to be cord-like extensions of nevi that represent incipient melanoma. The apparent succession of stages from melanocytic hyperplasia to nevus, to blue nevus, to atypical nevus, to outgrowth, to melanoma, was seen in several grafts where all these lesions were in close proximity. A case in point is the graft in Fig. 1e. At this low magnification, three of the relevant areas are indicated by arrowheads; the same right and center areas are shown at higher magnification, respectively, in Fig. 1f and g, and another section through the left area is enlarged in Fig. 1h. An additional view near the base of the same tumor appears in Fig. 1i. The earlier of the lesions shown (Fig. 1f, arrowhead) is part of a dermal nevus of hypermelanized elongated cells with few or no dendrites. The next region (Fig. 1g) contains dispersed hypermelanized cells at the leading margin (left arrowhead), contiguous with increasingly hypomelanotic cells (toward the right), followed by a solid mass (right arrowhead) of primarily amelanotic cells closely packed together, finally forming a solid tumor. Growth of such masses now accelerated and mitotic figures were more frequently seen.

The close adhesion of the cells at this stage is likely to require molecular changes in addition to the mere loss of dendrites. Some of the proteins linking components of the cytoskeleton and of the cell membrane are involved in control of cell shape and of adhesion between cells; some also regulate responses to growth factors (16). An attractive possibility is that the loss of dendrites is part of changes in such cytoskeleton–cell membrane linkages, in this case facilitating adhesion between the cell membranes of the melanocytes.

The melanomas induced in transgenic skin grafts by wound healing resembled a spontaneously occurring malignant melanoma found in the skin at the base of an ear in a 13-wk line 8 (Tag/--) mouse. This was a large amelanotic tumor of epithelioid type (Fig. 2a) and was unusual in having progressed so far by that age. Metastases of similar histological
FIG. 1. Progressive melanocytic changes, from hyperplasia to malignant melanoma, in Tyr-SV40E transgenic skin grafted from donors of high susceptibility to hosts of low susceptibility. Skin grafts were from homozygous (Tag/Tag) donors of line 9 (a, b, and d) or hemizygous (Tag/−) donors of line 8 (c, and e–i); all hosts were from line 12 (Tag/−). (a) Dendritic melanocyte in the dermis. (b) Nest of hyperplastic melanocytes. (c) Blue nevus in deep dermis. (d) Blue nevus (right arrowhead) and early melanoma (left arrowhead). (e) Low magnification of neighboring melanocytic lesions at different stages in one skin graft; two areas marked by arrowheads are shown in greater detail in f and g, and a section through the other area is shown in h. (i) Subcutaneous invasion of the same melanoma as shown in h into the muscle. See text for details. (a, ×140; b–d, ×70; e, ×20; f–h, ×35; i, ×55.)

type in the animal's lungs appeared to come from this skin tumor rather than from other melanomas (chiefly ocular) in the same animal, as the others were of slightly different prevailing histological type.

Human skin melanomas originate from lesions in the basal layer of the epidermis (17, 18), while the mouse lesions were first seen in the superficial dermis (Fig. 1 b–d). This difference may be related in part to the thinness of the mouse epidermis and the concentration of pigmented cells chiefly in the hair follicles of mouse skin. Nevertheless, in both species the lesions progress by radial growth, followed by vertical growth into the dermis and subcutaneous tissue.

Ulceration. A more minor difference between mouse and human melanomas seems to be a tendency for slightly earlier
FIG. 2.  (a) A spontaneous malignant skin melanoma at the base of the ear in a line 8 (Tag/−) transgenic mouse.  (b–h) Melanomas originating in grafted skin.  Grafts in b–f were from line 8 (Tag/−) transgenic mice, those in g and h were from line 9 (Tag/Tag), and all were growing in line 12 (Tag/−) hosts.  (b) Nodular melanoma.  (c) Multilobular melanoma with lobes differing in melanization.  (d) Amelanotic melanoma at edge of a graft.  (e) Detail of another amelanotic melanoma.  (f) Metastasis in a lymph node from the skin melanoma shown in e.  (g and h) Embolism (arrowhead) and metastases in lungs, from skin melanomas in two other transgenic skin grafts.  See text for details.  (a and e, ×70; b–d and f, ×20; g and h, ×35.)

ulceration of the mouse tumors (see especially figure 4f in ref. 3).  The association of ulceration with stages later than nevi in the mouse is shown by comparing the closely spaced lesions in Fig. 1e.  At higher magnification, the skin overlying the early nevus is clearly intact (Fig. 1f), but it is ulcerated over the nearby small melanoma (Fig. 1g), as well as over the more advanced tumor (Fig. 1h).  Thus, it is possible that cells in the radially expanding stage may themselves be already acquiring the ability to lyse or penetrate the extracellular matrix, basement membrane, and overlying epidermis.

Tumor Heterogeneity.  Phenotypic heterogeneity was common within and among tumors (Figs. 1h and 2 b–e), even in skin from genetically identical mice.  Many tumors in the grafts were multilobular, as in human melanomas.  In the mouse skin grafts, the tumor lobes differed substantially in pigmentation, eosinophilia, and degree of atypia (Figs. 1h and
The spontaneous transgenic skin melanoma partly shown in Fig. 2a was in fact also multilobular and heterogeneous. A pigmentated lobe of the experimental tumor in Fig. 2c, although macroscopically dense black, was microscopically a mixture of heavily pigmented and unpigmented cells; upon subcutaneous transplantation, this continued largely to be the case, but an occasional unpigmented segment arose. Some tumors did not appear multilobular but had regional differences within the tumor mass. Another variant was the occasional sharply discrete nodular tumor; the one in Fig. 2b is at an early stage and has not ulcerated. Some nodular tumors grew deeply into the subcutaneous tissue and could also protrude from the skin surface. Most tumors were hypomelanotic or amelanotic (Fig. 2d and e). (In Fig. 2d, the host tissue adjacent to the graft is distinguished by the presence of many hair follicles.)

Despite heterogeneity, the prevailing cell type within the tumors was epithelioid, with a lightly stained cytoplasm and hyperchromatic nuclei with prominent nucleoli; mitoses were numerous (Fig. 2e) and melanization was highly variable. All tumors were intensely positive for S-100 and most had some cells positive for HMB-45. The relative tumorigenic potential of the spindle cells, small hyperchromatic cells, or other atypical subpopulations of cells is unknown. Whether the phenotypic heterogeneity is due to polyclonal origin or to divergent cellular evolution within the tumor is also unknown. Heterogeneity among cells of long-transplanted mouse melanoma cell lines has been well documented (19). The transgenic mouse melanomas provide an opportunity to assess the origin and consequences of heterogeneity in newly developed primary skin melanomas.

**Invasion.** The rapid downgrowth of tumors proceeded from the dermis into the subcutaneous tissues, especially adipose and muscle tissue. In Fig. 1f, the same tumor as shown in Fig. 1h has penetrated into and destroyed parts of the muscle (arrowheads). All tumor-bearing grafts in the experiment had invasive tumor.

**Metastasis.** Metastases were seen in three graft hosts (see table 1 in ref. 3) in the lymph nodes and lungs. In contrast to the obvious phenotypic heterogeneity of the primary tumors from which these metastases arose, the metastases themselves (and emboli) appeared relatively homogeneous, thereby suggesting origin from a subpopulation in the primary tumor. In Fig. 2f, a conspicuous amelanotic metastasis (from the primary skin tumor shown in Fig. 2e) extends out from the lymphoid tissue. In the lung of another mouse, amelanotic tumor emboli are located in a blood vessel (Fig. 2g, arrowhead) while larger tumor masses are already established in the parenchyma. In Fig. 2h, from still another mouse, an amelanotic metastasis protrudes from the surface of the lung. Lymph nodes and lungs are the most common sites of distant metastases in human skin melanomas (20). As the primary tumors in our mice varied greatly in size and progression at the time of autopsy, this may have precluded obtaining metastases in a larger number of hosts.

**The Melanoma Model.** The present histopathological findings support and extend the proposition, based on external observations (3), that the skin graft-bearing Tyr-SV40E mice are uniquely favorable experimental models of human malignant skin melanoma. The series of changes and types of lesions in the mice parallel those in the human disease in virtually all respects. The mouse model offers the prospect of detailed molecular dissection of the in vivo steps toward malignancy and the mechanisms underlying melanoma metastasis. Unlike other candidate models of melanoma, these animals have no other skin tumors, are not immunologically compromised, and do not require repeated treatment. They are genetically uniform, with the same inbred-strain background as mouse strains bearing most of the genes known to affect pigment cells in particular and cell growth in general, so that such genes can be readily introduced into the transgenic system as single variables. And the use of transgenic lines with maximum vs. moderate melanoma incidence, as skin graft donors, enables suspected therapeutic vs. causal factors, respectively, to be tested in them.

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