Molecular Biology of Human Melanoma Development and Progression

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In the United States, Australia, Northern Europe, and Canada, malignant melanoma is increasing at a faster rate than any other cancer, with the exception of lung cancer in women. Major advances have been made in the molecular biology and immunology of melanoma. These advances in basic science have led to more rational approaches to specifically targeting melanoma cells, with promising results in the clinic. An increased understanding of how melanoma spreads has led to more selective, less invasive surgical procedures that do not compromise patient health. Combinations of chemotherapy and immunotherapy are now available for patients with advanced melanoma that affect both the length and quality of the patients’ lives. This review of the molecular biology of melanoma development and progression discusses the disease’s etiology, molecular genetics, cell-surface antigens, experimental models, biological markers, and new forms of treatment. As we continue to learn more about malignant melanoma, we will be able to devise more specific and effective treatments that will give patients with this potentially deadly disease longer and more productive lives. Mol. Carcinog. 23:132–143, 1998. © 1998 Wiley-Liss, Inc.

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ETIOLOGY

The incidence of melanoma is increasing dramatically in the developed world [1], having nearly doubled between 1980 and 1990. While the etiologic factors leading to melanoma are unknown, three factors should be considered. First, Caucasians have a tenfold higher risk of melanoma on sun-exposed areas than blacks do [2]. Second, epidemiologic studies point to a role for sunlight in the development of melanoma [1]. Third, hereditary melanoma exists and accounts for 5–10% of all melanoma cases [3]. Many patients with hereditary melanoma have multiple nevi, and patients with many nevi have a greater risk of melanoma than those with few nevi [4].

Ultraviolet Light

The ultraviolet light (UV) present in sunlight, especially UV within the B spectrum (290–320 nm wavelength), has been implicated as a possible cause of melanoma development. UV has been found to increase DNA instability [5], inhibit antioxidants [6], and suppress the immune system [7]. Intermittent intense exposure to sunlight during childhood leads to a significantly higher incidence of melanoma in adults [8].

UV induces the expression of transforming growth factor (TGF)-α [9] and nerve growth factor [10] on melanocytes. N-ras mutations are frequently observed opposite pyrimidine dimers [11], a known consequence of UV damage. We recently identified a human melanoma in a neonatal foreskin xenograft grafted to an immunodeficient mouse whose skin was treated with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) followed by chronic UV exposure.

In three experimental animal systems, UV either causes or contributes to melanoma development. The South American opossum Monodelphis domestica, which lacks a DNA repair mechanism, develops melanomas at a high rate after approximately 1 yr of UV exposure [12]. Administration of DMBA followed by croton oil with UV leads to melanoma in mouse skin [13]. Crosses between platyfish and swordtail fish produce offspring that after chronic UVB exposure develop melanomas [14].

Molecular Genetics

Karyotype analysis of dysplastic nevi and melanomas demonstrates nonrandom changes in chromosomes 1, 6, 7, 9, and 10. Nonrandom karyotypic
changes in chromosome 1 were observed in 53 of 58 advanced melanomas [15]. In most cases, the abnormality was a deletion or translocation of 1p12-22. Bale et al. [16] identified 1p36 as a frequently altered region. Trent et al. [17] identified nonrandom deletions in chromosome 6 in melanoma cell lines, and loss of heterozygosity was found on 6q in melanoma metastases [18]. Melanoma in the platyfish and swordfish suggests that the tumor suppressor gene Tu, which maps to 7p11-13 in humans, is responsible for the tumor phenotype [19]. Alterations on chromosome 9 include a 2–3 Mb deletion proximal to the interferon (IFN)-α gene cluster in 85% of melanoma tumor and cell line DNAs [20]. This region contains the tumor suppressor gene p16. Multiple alterations on chromosome 10q have been associated with the early stages of melanocytic neoplasia [21].

Oncogenes and Suppressor Genes

ras

Expression of all three ras genes (N-ras, Ha-ras, and Ki-ras) has been detected in human melanomas. Of the three genes, N-ras appears to be the most commonly mutated, with up to 60% of primary melanomas containing mutated N-ras [22]. N-ras mutations are most often found in melanoma samples from sun-exposed areas of the body, suggesting an association between UV and these alterations.

c-myc

Melanoma cell lines frequently demonstrate overexpression of c-myc [23], but in vitro effects cannot be excluded. Increased c-myc expression is generally associated with tumorigenicity parameters such as anchorage-independent growth in soft agar and transformation of NIH/3T3 cells.

c-ret

Mice containing the ret transgene demonstrate hyperpigmentation, aberrant melanogenesis, and melanoma development [24].

nm23

Expression of nm23 inversely correlates with tumor progression. Melanoma patients developing metastases during the first 2 yr after diagnosis have lower nm23 levels than patients with less aggressive disease [25]. Transfection of the metastatic melanoma cell line K-1733 with nm23 led to reduced metastatic potential [26].

Tu

Malignant melanomas form in the progeny of crossed platyfish and swordtail fish if the oncogene Tu in the platyfish is crossed with a swordtail with an absent repressor gene R. Tu encodes a membrane receptor similar but not identical to epidermal growth factor receptor (EGFR) [19].

Both mutation and overexpression of this tumor-suppressor gene have been reported [27,28], although the frequency from one report to the next is quite variable [29].

c-kit

It appears that embryological expression of c-kit is important in early melanocytic migration to the skin from the neural crest. Expression of c-kit is downregulated in 60% of melanomas but not in melanocytes [30].

p16

The product of the cyclin-dependent kinase (CDK) N2 gene, p16, is a tumor suppressor protein involved in the G1/S checkpoint of the cell cycle. Mutations, loss of heterozygosity, and deletions of the CDKN2 locus have been reported in sporadic and familial cutaneous malignant melanomas [31]. These alterations lead to deficient binding to cdk4 [32] and as a result ineffective inhibition of cell proliferation. Deficient binding of p16 and cdk4 has also been reported to be a result of mutations in the p16-binding domain of cdk4 [33]. Whereas all benign melanocytic nevi were found to express p16 protein, 42% of primary malignant melanomas lacked p16 expression [31]. Within the latter group, expression was inversely correlated with tumor thickness. Other reports demonstrated that p16 expression is lost in 59% of melanoma metastases [34] and 77% of melanoma cell lines [35]. Reintroduction of p16 into melanoma cells deficient in p16 results in a dramatic change in cell morphology toward a dendritic phenotype resembling that of adult melanocytes [36] and increases the radiation sensitivity of melanoma cells [37].

It has been postulated that p16 is the melanoma susceptibility gene. Reports differ regarding the frequency of mutations in melanoma-prone families, so that conclusions cannot yet be drawn. One study supporting the concept involved the screening of 15 Dutch pedigrees with familial atypical multiple-mole melanoma syndrome. A 19-bp germline deletion was found in 13 families, all of which originated from an endogamous population [38]. A second supporting report came from Hussussian et al. [39], who identified eight p16 germline substitutions in 13 of 18 familial melanoma kindreds. A study with an opposite conclusion was reported by Kamb et al. [40], who evaluated 38 melanoma-prone families for p16 mutations and found potential predisposing mutations in only two.

Some have postulated that UV induces p16 mutations. This is based on the finding of a high frequency of C:G → T:A transitions as well as tandem CC → TT mutations, with over 90% occurring at dipyrimidine sites [40].

The TP-ras transgenic mouse expresses an activated
Ha-ras gene (with a mutation in codon 12) regulated by a tyrosinase promoter. The transgene is expressed in melanocytes of the skin, eyes, and brain. The mice develop cutaneous melanoma when treated with DMBA. Five of nine cell lines created from the tumors had decreased or no p16 DNA, suggesting that the TP-ras transgenic mouse may be a valuable model for studying melanoma prevention and treatment [41].

**p15 and p19**

The gene that codes for the p15 protein, like p16, is located on chromosome 9p21. Homozygous deletions of p15 have been found in cell lines generated from a human melanoma [42]. The function of p15 appears to be very similar to that of p16. p19 arises from transcription of an alternative reading frame of the p16 gene, and ectopic expression of p19 in murine cells induces G1 and G2 arrest [43]. It is possible that some melanoma-prone families with linkage to 9p but no evidence of p16 mutations have mutations in p15, p19, or both.

**Summary**

While alterations in many genes and in gene expression can be found in both primary melanomas and melanoma cell lines, only one gene, p16, appears to be altered consistently in most reports. The location of p16 on chromosome 9p in an area of frequent karyotypic alterations in melanomas, as well as its mutation frequency in familial melanoma, makes it the leading candidate for the long-sought-after gene for increased melanoma susceptibility.

**CLINICAL AND PATHOLOGIC EVIDENCE OF TUMOR PROGRESSION**

The features of clinical and pathologic tumor progression have been well described [44]. Tumor thickness is the best prognostic indicator for primary melanoma. Patients with tumors less than 0.75 mm thick have a 98% 5-yr disease-free survival rate compared with a 36% 5-yr survival rate for patients with tumors at least 3.5 mm thick [1]. Other clinical variables include sex (women tend to survive longer than men) and location (patients with tumors on the extremities tend to survive longer than patients with tumors on the upper back and neck). Ulceration is a poor prognostic indicator, whereas lymphocytic infiltration around the tumor has a small positive effect on outcome [29]. Nevi are capable of evolving into melanomas, with giant congenital nevi having the highest risk of transformation [45]. It is estimated that over 85% of familial melanomas arose from precursor lesions [46].

Melanocytic nevi are benign tumors that can either regress or progress; regression is far more common. Dysplastic nevi (also called atypical moles and melanocytic dysplasia) are intermediate lesions thought to have a greater propensity for progression. Radial growth-phase (RGP) melanomas are similar to in situ melanomas, except that the former have invaded the dermis. Neither lesion is thought to metastasize [47].

Vertical growth-phase (VGP) melanomas have the ability to metastasize. The prognosis of VGP melanomas is related to a variety of factors, including mitotic rate, tumor thickness, metastasis (lymph node or distant), presence of infiltrating lymphocytes, anatomic site, sex, and regression. Other factors include satellitosis, ulceration, vascular invasion, and nodular growth [48].

**IN VITRO GROWTH REQUIREMENTS**

Many factors have been found to stimulate the growth of normal melanocytes [49]. These factors include activators of protein kinase C such as 12-O-tetradecanoylphorbol-13-acetate (TPA), growth factors such as basic fibroblast growth factor (bFGF), calcium, cholera toxin, and inflammatory mediators such as interleukin (IL)-1 and tumor necrosis factor-α.

Nevus cells are similar in morphology to melanocytes but are more heterogeneous, having a range of locations in the skin (epidermis, dermis, or junction between epidermis and dermis). Their growth requirements are similar to those of melanocytes. In contrast to normal melanocytes, nevus cells are less dependent on bFGF [50], due to the ability of the nevus cell to synthesize the protein.

Nevus cells can grow in soft agar with low efficiency, but spontaneous transformation in culture and tumor development after injection into immunodeficient mice have not been observed [51]. While common acquired and congenital nevi have a normal karyotype, approximately 25% of compound and dysplastic nevi have abnormal karyotypes [52].

Relatively little is known about dysplastic nevi and RGP melanomas in vitro because of the scarcity of clinical material available for study. When available, primary cultures from dysplastic nevus lesions may be overgrown by either common acquired nevi or melanoma cells. RGP cultures, on the other hand, become overgrown by populations of rapidly growing cells that have lost their pigmentation markers but maintain their expression of melanoma-associated antigens [50]. RGP cells have an extended life span, may or may not grow in soft agar, and form tumors in immunodeficient mice only after many months.

VGP melanomas can be divided into early (no tumor recurrence), intermediate (recurrence after 9–84 mo), and late (metastases present at the time of primary surgery) types. Doubling times are longer and colony-forming efficiency lower in early melanomas [51]. All VGP melanomas are tumorigenic, although at different rates. Metastatic lines can grow in media without growth factors [53]. TPA inhibits VGP cells [51].
EXPERIMENTAL TUMOR DEVELOPMENT
AND PROGRESSION

Both normal melanocytes and common acquired nevus cells can be transformed with simian virus 40 (SV40) T antigen [54]. These SV40-transformed cells have several characteristics of spontaneously transformed melanocytes, including growth in soft agar, growth independent of exogenous growth factors, inhibition by TPA, and infinite life span. The cells are not, however, tumorigenic. N-ras transfection of the SV40-transformed cells makes the cells tumorigenic [51].

Non-metastatic cell lines can become metastatic through natural selection. For example, after successive subcutaneous and intravenous injections of an early melanoma, it became metastatic in immunodeficient mice [55]. These selected cells grow better in serum-free medium, have a higher colony-forming efficiency, and have greater invasiveness because of their higher activities of tissue-type plasminogen activator and collagenase than their unselected counterparts.

Murine melanocytes can be transformed in a variety of ways. Murine melanocytes transfected with either c-myc or N-ras are both immortal and tumorigenic, whereas those transfected with bFGF are immortal but not tumorigenic [56]. Mice transgenic for SV40 large T antigen under the control of the tyrosinase promoter, as well as transgenic mice that overexpress re, develop melanomas, as do newborn mice treated with carcinogens plus TPA or UVB.

MELANOMA-ASSOCIATED ANTIGENS

Integrins

Integrins are heterodimers with an α and a β subunit and are divided into subfamilies defined by the β subunit. Unlike in most other tumors, in which integrins are downregulated with disease progression, integrin expression is upregulated in metastatic compared with primary melanoma cells [57]. α3β1 integrin expression is a marker of melanoma metastasis, as it occurs in metastatic but not primary melanomas [58]. The β1 (vitronectin) subunit is upregulated in melanoma progression and absent in melanocytes, nevi, and RGP melanomas but present in 80% of VGP and 100% of metastatic melanomas [59]. The α3β1 integrin binds to a large number of extracellular matrix proteins, including vitronectin, fibrinogen, von Willebrand’s factor, thrombospondin, fibronectin, osteopontin, and collagen. α3β1 plays an important role in invasion and metastasis. Treatment of A375 melanoma cells with antibodies to α3β1 stimulates invasion of melanoma cells through a reconstructed basement membrane [60].

Extracellular Matrix Proteins

The basement membrane zone separates the dermis from the epidermis in normal skin. The base-

ment membrane zone contains the hemidesmosome, a region of plaque and keratin filaments; the lower portion of the basal epithelial cells; the lamina lucida and lamina densa (basal lamina); and sub–basement membrane elements including anchoring filaments, type VII collagen, and the upper portion of the dermis. Melanocytes are generally within the basal epithelial layer in contact with the basement membrane, although suprabasal melanocytes can also be identified [61].

Passage of melanocytes through the basement membrane to the papillary dermis is associated with nevus formation. Nonetheless, individual cells or nevus cell clusters are surrounded by a basement membrane. This is not true in melanoma, in which the basement membrane is discontinuous.

Fibronectin and tenascin are two extracellular matrix proteins secreted by melanomas. Fibronectin contains the RGD tripeptide involved in platelet adhesion with extracellular matrix proteins. RGD peptides promote cell attachment. Tenascin is generally absent from normal tissues but is found during wound healing and in the lamina lucida [51]. Melanomas produce tenascin constitutively [51]. Tenascin secretion is associated with tumor progression both in vitro and in vivo. Melanocytes produce little tenascin, whereas metastatic melanomas produce it constitutively. While tenascin contains the RGD sequence of fibronectin, it is hidden in the folded protein.

YIGSR peptides derived from the laminin β1 chain can inhibit migration of cells to laminin, bind to the non-integrin laminin-binding receptor, and decrease metastasis of murine melanoma cells [62]. The synthetic 19–amino acid peptide SIKVAV derived from the laminin α chain can increase the number of lung metastases when injected with B16F10 murine melanoma cells [63].

Cell-Cell Adhesion Molecules

Cell adhesion molecules (CAMs) allow cells to sort into groups and form complex structures and are therefore important in organogenesis and tissue reconstruction. Most CAMs that are melanoma-associated antigens are members of the immunoglobulin supergene family. Intracellular CAM-1 (ICAM-1) and MUC18 are the most highly expressed CAMs in melanoma [64]. Both are markers of poor prognosis. There appears to be an interaction between cells involving growth factors and homeobox-containing genes (HOXs), which code for transcription factors and in turn influence the expression of adhesion molecules such as integrins, ICAM-1, and neural CAM (N-CAM) [65]. HOX genes help regulate when and how other genes are expressed in a cell.

The percentage of melanoma cells expressing ICAM-1 increases directly with tumor thickness [64] and is higher in metastatic than in primary lesions. Soluble ICAM-1 levels in serum also correlate with
clinical progression of melanoma [66]. Soluble ICAM-1 can inhibit lysis of melanoma cells by natural killer cells [67], suggesting that the soluble receptor acts as an antagonist for tumor-host adhesions.

MUC18/melanoma-associated CAM is remarkably specific, being present on most melanomas but not on other tumors. Expression of MUC18 is directly related to tumor thickness [68], is first identified when a tumor reaches 1 mm in diameter, and is upregulated as melanomas progress into VGP. Recent evidence suggests that MUC18 may be an isoform of a molecule expressed by endothelial cells recognized by the antibody S-Endo-1 [69]. Expression of MUC18/melanoma-associated CAM on melanocytes and nevi is downregulated by undifferentiated keratinocytes; differentiated keratinocytes or fibroblasts have no effect [48].

N-CAM is broadly expressed during embryonic development. It is expressed in a minority of melanomas both in vitro and in vivo [70]. The L1 adhesion molecule is neuron specific, being expressed on a variety of neuroectodermal tumors including melanoma [71]. Vascular CAM-1 (VCAM-1), the ligand for VCAM-1, is expressed on nevus cells and a minority of primary melanomas, and expression is decreased in metastases [72].

Gangliosides

There are five major gangliosides expressed by melanocytic cells: GM₃, GM₁₄, GD₂, GD₃, and 9-O-acetyl GD₃ [48]. GD₂ is the most strongly melanoma-associated ganglioside, being expressed only on VGP primary and metastatic melanomas [73]. With progression, melanoma cells shift in expression from GM₁₄ toward GD₂, GD₃, and GD₃ are found in adhesion plaques on melanoma cells, concentrated at the site of cell-matrix interactions and on cell surfaces [74]. In addition to their role in adhesion, gangliosides can stimulate lymphocytes [75] and astroglial cell proliferation, suggesting that they may have a direct effect on cell proliferation. Gangliosides appear to be immunogenic, and vaccines containing these antigens are currently in clinical trials in the United States and in Europe [76].

Major Histocompatibility Antigens

The cellular immune response to tumors requires presentation of tumor antigens to T lymphocytes. HLA molecules are required for antigen presentation, so tumors may escape immune surveillance by regulating HLA antigen expression. Compared with melanocytes, melanoma cells have a reduction or loss of HLA class I antigens but express HLA class II antigens [68]. Addition of v-ras to cultured human melanocytes leads to induction of HLA class II antigens [77].

Loss of class I antigens occurs in primary tumors, and there is no difference in expression between primary and metastatic tumors [48]. Class I antigens are composed of a membrane glycoprotein bound to β₂-microglobulin. Loss of β₂-microglobulin is usually associated with loss of recognition of the antigen by T cells, which allows melanomas to escape immune recognition [29]. Class II antigens are expressed more strongly in metastatic than in primary melanomas, and class II antigen expression increases directly with tumor thickness. Thus, class II antigen expression may provide tumors with a selective advantage.

Cytokines can regulate HLA antigen expression. IFN-γ and tumor necrosis factor-α induce HLA expression. T lymphocytes are a known source of IFN-γ. Thus it appears that HLA class II antigens may be regulated by T-lymphocyte infiltration.

Growth Factors, Cytokines, and Their Receptors

bFGF

Normal melanocytes require bFGF, nevus cells have a reduced requirement, and primary and metastatic melanomas are bFGF independent. Melanomas express bFGF mRNA, whereas melanocytes do not [78]. Inhibition of bFGF by antisense oligos suppresses melanoma growth [79], as does the introduction of antibodies to bFGF into the cell cytoplasm [80]. bFGF functions not only to stimulate melanocyte and melanoma growth but also to stimulate angiogenesis, stroma formation, and invasion [48]. bFGF is mitogenic for fibroblasts and keratinocytes [81]. bFGF activates the proteolytic enzymes tissue-type plasminogen activator and urokinase-type plasminogen activator [82] and collagenases [83] and thus may allow invasion of cells through surrounding tissues.

Three other members of the fibroblast growth factor family, hst-1, int-2, and keratinocyte growth factor, are expressed in human melanomas [84]. hst-1 and int-2 are amplified in a subset of melanomas [84], although the significance of this amplification is unclear.

TGF-α

TGF-α belongs to the epidermal growth factor family and has a similar tertiary structure [85]. Melanoma cells secrete high levels of TGF-α-like factors [86]. Whether they also express cell surface-bound TGF-α is not known. TGF-α mRNA can be identified in primary melanomas and melanoma cell lines but not in normal melanocytes [87]. The epidermal growth factor/TGF-α receptor is not detected on normal melanocytes or common acquired nevi, is expressed in 20% of dysplastic nevi and RGP melanomas, and is expressed in more than 80% of VGP primary and metastatic melanomas [73]. Exogenous epidermal growth factor stimulates the growth of normal melanocytes and melanoma cells in early passages [88].

TGF-β

Active TGF-β appears to have a negative growth regulatory role in most normal and malignant epi-
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The motility factor for melanoma cells [101]. Because IGF-1 is a growth factor for melanoma cells [78]. IGF-I is a primary melanoma but not metastases [100]. IL-6 inhibits the growth of RGP and VGP melanoma cell lines but may be stimulatory in other cells [98]. IL-8 is secreted by most if not all melanoma cells in culture. IL-8 is chemotactic for neutrophils, T cells, and basophils [96]. IL-8 also induces migration of melanoma cells and keratinocyte proliferation. IL-8 may act as an autocrine factor for melanoma cells [48].

**EGFR**

EGFR is increasingly expressed with melanocyte transformation and is therefore a potential marker of melanomagenesis [29].

**Pleiotrophin**

Also known as heparin-binding growth-associated molecule, pleiotrophin is a neurotrophic factor secreted by melanoma cells. It is also secreted by breast cancer cells [93] and can be inhibited by heparin analogs [93]. Pleiotrophin is highly angiogenic. Inhibitors of pleiotrophin inhibit metastasis formation. Because melanoma cells do not appear to express the pleiotrophin receptor, pleiotrophin may play a paracrine role in melanoma progression.

**Melanocyte growth stimulatory activity and IL-8**

Melanocyte growth stimulatory activity is identical to the gro gene product [94], which belongs to the family of monocyte inflammatory proteins [95]. In addition to its mitogenic activity, melanocyte growth stimulatory activity appears to be a mediator of chemotaxis and inflammation.

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**IL-1 and IL-6**

IL-1 is produced in a variety of cell types [97] and exists in two isoforms, α and β. Both isoforms are strong inhibitors of cell proliferation [98] in some melanoma cell lines but may be stimulatory in others [99]. IL-6 inhibits the growth of RGP and VGP primary melanomas but not metastases [100].

**Insulin-like growth factor-1 and scatter factor**

Insulin-like growth factor-1 (IGF-I) and insulin are important melanoma growth factors [78]. IGF-I is a motility factor for melanoma cells [101]. Because IGF-I is produced by fibroblasts, it may be an important paracrine stimulator of melanoma cells.

**Scatter factor (hepatocyte growth factor)**

Scatter factor (hepatocyte growth factor) is a fibroblast-derived growth factor that stimulates cell motility [102]. The scatter factor receptor met is found in many different malignant tumors, including melanomas [103]. On normal melanocytes, scatter factor acts synergistically with bFGF.

**Other factors**

Melanocyte-stimulating hormone (MSH) has widely diverging effects on the growth of non-human melanomas [104]. Human melanoma cell lines and tumors express MSH [105]. MSH is involved in melanocytic pigmentation [106]. MSH receptors are overexpressed in melanoma cells [107]. Whether this hormone has additional growth-regulatory functions is yet to be determined.

Vascular endothelial growth factor (VEGF) or vascular permeability factor can be induced by hypoxia [108]. Transfection of VEGF cDNA into SK-MEL-2 cells leads to the development of well-vascularized tumors with minimal necrosis. Transfection of antisense VEGF into the cells leads to small, minimally vascularized tumors with extensive necrosis [108]. Melanoma cell lines that develop tumors with a low metastatic potential in nude mice have low in vitro VEGF levels, whereas highly tumorigenic melanoma lines have high VEGF expression [109]. The in vivo expression of VEGF is not related to metastasis, for all tumors examined had high levels of VEGF, probably due to hypoxia, because in cell lines low levels of VEGF in vitro could be upregulated by low oxygen tension. The receptor for VEGF is expressed by melanoma cells but not melanocytes [110].

**Protein Tyrosine Kinases and Protein Kinases**

Protein tyrosine kinases are either transmembrane receptors or cytoplasmic kinases, are often implicated in signal transduction, and include many oncogene products [111]. Protein tyrosine kinases expressed by melanoma cells include EPH, FGF-R, INS-R, TYRO-3, NF-R, CSK, and FES.

The important role of protein kinases in melanoma is highlighted by the effect of the protein kinase C activator TPA on melanocyte growth stimulation and on melanoma growth inhibition. Protein kinase C β is expressed in melanocytes but not melanomas [112].

**Cation-Binding Proteins**

Melanoma cells overexpress a large number of calcium-binding proteins. S-100 is a highly acidic cytoplasmic protein that is part of the family of calcium-modulating proteins. It is present in neural crest tumors, including melanomas, as well as in benign tissue of neural crest origin [113]. To the pathologist S-100 remains the single most useful tool for differentiating melanoma from other neoplasms such as squamous cell carcinoma [29].
Calmodulin modulates the activity of several key enzymes and other factors in growth regulation. Qualitative and quantitative differences in expression exist between melanocytes and metastatic melanomas [114]. Calcinexin and calnexin are both overexpressed in metastatic melanoma [115].

p97 is highly expressed by some primary melanomas and by most melanoma cell lines [116]. The N terminus has homology with both transferrin and lactotransferrin [117]. The purified form of p97 can bind iron.

Proteolytic Enzymes and Their Inhibitors

Melanoma cells are active secretors of proteases thought to be involved in invasion and metastasis [118]. At least five groups of proteases can be found in the culture supernatants of melanoma cells, including tissue-type and urokinase-type plasminogen activators [119], collagenases type IV [120], stromelysin, heparanase, cathepsins, and tissue factor. The proteases are secreted as inactive precursors that require activation by other proteases.

Expression of proteolytic enzymes is also regulated by specific inhibitors. Melanoma cells express inhibitors for collagenase type IV and plasminogen activators. α2-Macroglobulin is a protease inhibitor on melanoma cells that binds several growth factors, including TGF-β [121]. The expression of serine and metalloproteinases appears to be a reliable marker for progression, but more detailed analyses are needed.

Cell-surface proteases form a separate class of proteases. They are less specific than other proteases, and their function is less well understood. Aminopeptidase N appears to be involved in invasion by digesting nidogen/entactin [122]. Neither the role of leucine aminopeptidase in melanoma cells [123] nor the function of neutral endopeptidase or cell-surface gelatinase is clear.

Melanocyte Lineage Markers

These structures can be detected by molecular techniques or by histochemical or immunohistological analyses. S-100 and HMB-45 are not melanocyte-lineage specific but can be used in combination with other markers for the identification of melanoma cells.

Melanin production increases with maturation of melanocytes. Melanoma cells present all grades of pigmentation from normal to amelanotic. One of the key enzymes in melanin synthesis is tyrosinase. One study found no correlation between tyrosinase levels and melanoma progression; even amelanotic melanomas had tyrosinase activity [124]. The function and significance of a number of other proteins involved in melanin production, including tyrosinase-related peptides 1 and 2, gp100, and lysosome-associated membrane protein-1, await further investigation.

Miscellaneous Melanoma-Associated Antigens

Chondroitin sulfate proteoglycan, also called high-molecular-weight melanoma-associated antigen (HMW-MAA), might be involved in cell-cell contact, cell-substrate adhesion, or motility. It is highly immunogenic in mice injected with melanoma cells or nevus cells [125]. Most melanoma cells express between 10⁵ and 6 × 10⁶ binding sites [126]. HMW-MAA is expressed on the melanoma surface as microspikes on the upper cell surface and at the cell periphery [127]. Peripheral HMW-MAA is involved in the initial interactions between adjacent cells, and they form complex footpads that make contact with the substratum. Monoclonal antibodies to HMW-MAA do not affect melanoma cell adhesion but block the chemotactic and chemokinetic motility of the cells [128] and reduce soft-agar growth [127]. HMW-MAA appears to be a cell-substrate adhesion receptor that cooperates with the α6β1 adhesion receptor in binding to a heparin-binding domain of fibronectin.

Melanomas express several distinct intracellular antigens that are recognized by autologous cytolytic T-lymphocytes [129]. These antigens include melanoma antigen genes (MAGEs). The MAGE family comprises 12 known genes, of which six are expressed in tumors but not normal tissues, except for testis and placenta [130]. All of the MAGE genes are located on Xq28 and are clustered in three main regions within 3.5 Mb. Human MAGE genes are conserved in primates but less well conserved in other vertebrate species. Sixteen of 100 primary tumors (16%) versus 69 of 145 metastases (48%) expressed MAGE-1 [131]. Benign and dysplastic nevi did not express any of four MAGE genes evaluated. The usefulness of MAGE antigens for immunotherapy is currently being evaluated [132]. The functions of these genes are unknown.

Antigens Associated with Cell Proliferation

Proliferating-cell nuclear antigen and Ki-67 are proliferation-related antigens that have been studied in melanoma [133]. While an increase in expression was noted in metastases compared with primary tumors, expression in the primary lesions did not correlate with metastasis risk or disease-free survival.

**bcl-2**

This oncogene inhibits programmed cell death (apoptosis). There is constitutive expression of bcl-2 by basal melanocytes and in nevi [134]. Mature highly differentiated melanocytes are less likely to express bcl-2 [134]. The expression of bcl-2 by melanocytes suggests that the cells are protected from cell death.

MARKERS DISTINGUISHING CELLS FROM DIFFERENT STAGES OF MELANOMA PROGRESSION

Of the many markers that have been evaluated, the most significant for diagnosis appear to be β3 of...
the vitronectin receptor, ICAM-1, and MUC18/melanoma-associated CAM. No marker that can predict therapy response has been found. Although a number of tumor-associated antigens are upregulated, some are downregulated, the most significant of which is dipeptidyl peptidase (CD26), which is lost in melanoma cells [57].

**MELANOMA BIOLOGY AND TREATMENT**

Therapy with monoclonal antibodies directed against melanoma-associated antigens has been attempted. For example, anti-ganglioside antibodies have been used to treat systemic disease [135]. When murine antibodies are used, as they have been in many cases of antibody-directed therapy for melanoma, problems of sensitization and elimination of the antibody by the reticuloendothelial system occur [136]. These problems can be circumvented by using human or humanized antibodies. Because of heterogeneous expression of antigens by tumors, however, the use of multiple antibodies may be required to improve the efficacy of this treatment modality.

Cytokines and expanded lymphocytes removed from patients have been used in an attempt to induce a lymphocyte-mediated tumor killing [137]. Circulating lymphocytes, as well as lymphocytes from around tumors (tumor-infiltrating lymphocytes), have been used. While this therapy thus far has been disappointing, it remains a field of intense interest.

Active specific immunotherapy, that is the induction of specific immunological responses to tumor cells by using anti-idiotypic antibodies, vaccines, and gene therapy, is ongoing [138,139]. Anti-idiotypic antibodies containing antigenic determinants of gangliosides and chondroitin sulphate proteoglycan have induced humoral immunity in patients and improved survival [140]. A polyvalent vaccine from pooled melanoma cell lines as well as the ganglioside vaccine GM2, are good examples of active specific immunotherapy [141,142].

Clinical trials using peptide vaccines, including those from the MAGE antigens, are ongoing [143]. Host variables govern responses to such vaccines. Each of the MAGE proteins requires presentation by a specific HLA antigen, and these antigens are present in only a subset of the population. Because each of the MAGE proteins is expressed on only a subset of melanomas, treatment schedules using vaccines of this type should include both HLA testing and assessment of the antigenic profile of the tumor before therapy.

The IFNs have been shown to have antitumor, antiproliferative, and immunomodulatory effects on melanoma cells in vitro [144]. Preliminary phase I and II clinical trial data suggested that IFNs had antitumor activity in vivo. Subsequent trials demonstrated that α-IFN, particularly IFN-α-2b, has activity against metastatic melanoma as single-agent therapy and in combination with other agents, including dacarbazine, IL-2, and other biologic therapies [145]. A phase III trial evaluated the efficacy of IFN-α-2b in high-risk melanoma patients with no evidence of disease after surgical therapy. Patients received 1 yr of IFN-α-2b or observation. Those receiving treatment had significantly increased disease-free survival (1.7 vs. 1.0 yr, \( P = 0.005 \)) and overall survival (3.8 vs. 2.8 yr, \( P = 0.047 \)). Treatment was associated with an increase in the 5-yr survival rate from 36% to 47% and an increase in the 5-yr disease-free survival rate from 26% to 37% [146]. Based upon these results, IFN-α-2b has become the standard treatment for individuals with melanoma at high risk for relapse after surgical therapy.

**MOLECULAR BIOLOGY AND DETECTION OF OCCULT DISEASE**

The presence of lymph-node metastases in patients with melanoma decreases their 5-yr survival rate approximately 40% compared with patients with no evidence of nodal metastases [146]. Routine histologic evaluations of lymph nodes for metastases assess less than 1% of the submitted material and often miss micrometastatic disease. The sensitivity of routine histologic evaluation is one abnormal cell in a background of 10⁵ lymphocytes. If serial sectioning and immunohistochemical staining are used, the sensitivity increases to one abnormal cell in 10⁴ normal lymphocytes [146]. The latter technique is not standard practice due to its greater expense and increased time requirement.

New technology allows the mapping of the cutaneous lymphatic flow from the primary tumor and identification of the sentinel lymph node in the regional lymphatic drainage basin. This procedure, as initially demonstrated by Morton and colleagues [147], reveals the first site of metastatic disease. If the sentinel lymph node is negative for disease, the rest of the lymph nodes in the basin are essentially always negative [148]. Sentinel lymph-node dissection allows detailed evaluation of one or two lymph nodes rather than the 10, 20, or more lymph nodes assessed in a standard dissection. Nonetheless, 25% of patients proven to be free of lymphatic metastasis by histologic evaluation have disease recurrence and die, suggesting that these patients have occult lymph-node metastases missed by histologic review or suffer from blood-borne metastases without lymphatic spread.

Two laboratory techniques have recently been used to evaluate histologically negative lymph nodes for the presence of micrometastatic disease. One involves bisecting a lymph node, submitting half to pathological evaluation, and placing the other half into tissue culture. Thirty-one percent of subjects who were histologically lymph-node negative were found to have malignant cells growing in culture. Patients
histologically node negative but with malignant cells identified in tissue culture had a higher recurrence rate than did individuals negative by both histological review and cell culture [149].

Another strategy for identifying occult micro-metastases looks for the presence of tyrosinase mRNA, a transcript present almost exclusively in melanocytic lineage cells. Reverse transcription–polymerase chain reaction (RT-PCR) and standard histological review were used to evaluate lymph nodes from 29 patients with primary melanoma. RT-PCR was positive in 19 samples, including all nine of the lymph nodes found to contain metastases by histological review [150]. RT-PCR has been shown to be able to identify one melanoma cell within 10⁶ lymphocytes and so is two orders of magnitude more sensitive than standard histological review. The 29 individuals were followed for 3 yr. Those positive for lymph-node metastases by both histological analysis and RT-PCR had the highest rate of disease recurrence, 42%; those RT-PCR positive but histologically negative had a recurrence rate of 22%; and those negative by both evaluations had a recurrence rate of 6.6%.

RT-PCR has also been used to detect occult circulating tumor cells in the blood of melanoma patients [151]. Four different markers (tyrosinase, MAGE3, MUC18, and p97) were evaluated. All four markers were identified in all 10 melanoma cell lines examined but in none of 14 healthy volunteers. Among 74 patients with melanomas of various clinical stages, the pattern of marker detection was tyrosinase, 59%; MAGE3, 9%; MUC18, 66%; and p97, 65%. The detection of circulating tumor markers was significantly greater in the 53 patients with disease than in the 21 disease-free patients (P = 0.025). There was also an increase in the ability to detect one or more of these markers with increasing disease stage. The detection of one or more markers in the circulation may provide a means of early detection of metastatic or recurrent disease or a means of evaluating the response to specific therapeutic modalities.

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