Differential Expression of G1 Cyclins and Cyclin-Dependent Kinase Inhibitors in Normal and Transformed Melanocytes

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PURPOSE. To investigate the levels of the different regulatory proteins involved in the G1 progression and G1/S transition in normal and transformed human choroidal melanocytes (CM).

METHODS. Three choroidal melanoma cell lines and three CM cultures were used. The purity of the CM cultures was assessed by different approaches, including morphologic study, specific immunostaining, cell proliferation behavior, and transforming growth factor-β, responsiveness. The cell cycle protein levels were evaluated by specific immunoblotting of total extracts obtained from the different cell lines.

RESULTS. Alterations were observed in the expression of cyclins D1 and E in the transformed cells, whereas the amounts of the cyclin-dependent kinases (CDKs) CDK2 and CDK4 were almost identical in both cell types. Although the expression of cyclin H was slightly increased in transformed cells, neither the CDK7 level nor the CDK7 and cyclin H localizations were altered when compared with those in normal CM. The results suggest the absence of the CDK inhibitor (CKI) p21 in two of the three melanoma cell lines and, as a main feature, a striking underexpression of p27 in the three transformed cell lines. Finally, although the p16 level was almost the same in normal and transformed cells, a loss of p16-CDK4 interaction was observed in two of the three melanoma cell lines.

CONCLUSIONS. Deregulated expression of G1 cyclins and CKIs and alteration in the interaction of CKIs with CDKs may be implicated in the neoplastic transformation of human ocular melanocytes to malignant melanoma cells. (Invest Ophthalmol Vis Sci. 1998;39:876–884)

Choroidal melanomas are the most common intraocular neoplasms in adults.1 These tumors pose a serious threat to life: Despite recent advances in treatment, the prognosis for patients with such malignant disease remains questionable. The cellular events leading to neoplastic transformation of normal choroidal melanocytes (CM) are still virtually unknown. In vitro studies on growth regulation of skin formation of normal choroidal melanocytes (CM) are still questionable. The cellular events leading to neoplastic transformation of CMs are now possible. Recent technical advances have circumvented this problem, and in vitro investigations into the mechanisms that cause malignant transformation of CMs are now possible.

In several studies concerning a great variety of other cell types, investigators have demonstrated a close link between oncogenesis and the cell cycle machinery.4 Progression through the cell cycle is orchestrated by sequential activation of a series of serine–threonine kinases called cyclin-dependent kinases (CDKs). The CDKs' activity is primarily dependent on the association with their activating cyclin subunits. Key regulators of G1 progression and G1/S transition include cyclin Ds, which assemble with CDK4 and CDK6 in mid-G1, and cyclin E, which combines later with CDK2. Deregulated expression of G1 cyclins and CDKs may cause loss of cell cycle control and thus promote oncogenesis. For example, rearrangement, amplification, and overexpression of the cyclin D1 gene have been found in several types of human cancers.5 Although cyclin E is overexpressed in many human tumors,6,7 sometimes as a result of cyclin E gene amplification,8 there is no direct evidence at present that cyclin E acts as a protooncogene. However, the programmed overexpression of cyclin E induces the same cellular effects as does overexpression of cyclin D1—namely, G1 shortening, cell size reduction, and less dependence of the cells on mitogens.9,10 CDK4 and CDK6 are also overexpressed in several tumor cell lines,11 and the CDK4 gene is amplified in some tumors.8 Cyclin-dependent kinase activity is counterbalanced by a variety of low molecular weight CDK-inhibitory proteins (CKIs).12 Two gene families of mammalian CKIs have been identified. One family includes p21Cip1, p27Kip1, and p57Kip2. All have broad specificity, acting on the different G1 CDK
cyclins and on the S phase CDK2 cyclin A complexes. Among these CKIs, p21 may be involved in tumorogenesis, although there is no evidence that the p21 gene itself is mutated in human tumors. Similarly, the recently suggested link between p27 and oncogenesis remains to be clarified. The second and unrelated family of CKIs includes p16<sup>INK4A</sup>, p15<sup>INK4B</sup>, and p18, which specifically inhibit CDK4 and CDK6. The p15 and p16 genes lie in tandem on human chromosome 9p21, the site of the multiple tumor suppressor locus, which is rearranged, deleted, or mutated in many tumor-derived cell lines and in some primary tumors. As a main feature, p16 is often mutated in members of skin melanoma-prone families.

Finally, CDK activity is regulated by phosphorylation and dephosphorylation. In particular, full activation of CDKs requires phosphorylation on a conserved threonine residue (Thr160 in CDK2 and Thr172 in CDK4). A kinase responsible for phosphorylation of this residue has been identified in various organisms and has been designated CAF for CDK-activating kinase. CAF is constituted of a catalytic subunit CDK7, a regulatory subunit cyclin H, and an assembly factor. CDK7 level nor the CDK7 and cyclin H localizations were altered when compared with those in normal CM. Our results demonstrated alterations in the expression of cyclins D1 and E in the transformed cells, whereas CDK2 and CDK4 amounts were almost identical in both cell types. Although expression of cyclin H slightly increased in transformed cells, neither the CDK7 level nor the CDK7 and cyclin H localizations were altered when compared with those in normal CM. Our results also suggest the absence of p21 in two of the three melanoma cell lines under study and, as a main feature, a striking underexpression of p27 in the three melanoma cell lines. Finally, although the p16 level was almost the same in normal and transformed cells, a loss of p16-CDK4 interaction was observed in two of the three melanoma cell lines.

**MATERIALS AND METHODS**

**Cell Cultures**

Human CMs were isolated according to the method of Hu et al. from choroids of eyes obtained after death from the Toule Hospital. All cell cultures were performed in Gibco (Cergy Pontoise, France). Choroidal melanocytes were cultured in Ham's F12 medium (pH 7.3), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), 10 ng/ml cholera toxin, 5 ng/ml basic fibroblast growth factor-2, and 10% fetal calf serum. Choroid fibroblasts and RPE cells were grown, respectively, in Ham's F12 and Ham's F10 media (pH 7.3), supplemented with antibiotics and 10% fetal calf serum. Choroidal fibroblasts and RPE cells, respectively. For proliferation studies, cells were counted in triplicate (Coulter Counter; Coultronics, Margency, France).

**Immunocytochemical Studies**

Cells were plated and cultured in four-well chamber slides (Lab-Tek, Naperville, IL). After washing with phosphate-buffered saline (PBS), the cells were fixed in 70% ethanol for 10 minutes and were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Non-specific binding sites were saturated for 45 minutes in PBS containing 2.5% nonfat dry milk and 1% bovine serum albumin (BSA). Cells were washed in PBS mixed with 1% BSA and were incubated for 1 hour with 1:300 monoclonal anti-HMB-45, anticytokeratin F12-19, or polyclonal anti-S-100 antibodies. After washing in PBS mixed with BSA, cells were incubated with biotinylated secondary antibodies (1:200 antirabbit and antimouse immunoglobulins) for 45 minutes in PBS containing human serum type AB (1:50 dilution). After washing, cells were treated with 1:200 streptavidin-peroxidase for 45 minutes. Cells were then washed in PBS-BSA and stained for 20 minutes with 3,3'-diaminobenzidine-4HCl (DAB) in PBS that contained 0.01% H<sub>2</sub>O<sub>2</sub>. All reagents were obtained from Dakopatts.

For immunofluorescence staining, H<sub>2</sub>O<sub>2</sub> treatment was omitted. After incubation with 1:300 polyclonal anti-CDK7 (C18, UBI, Lake Placid, NY) and anticytin C (18, Santa Cruz Biotechnology, Tebu, France) antibodies, 1:100 dilution of biotinylated antirabbit immunoglobulins was applied, and cells were treated with 1:400 streptavidin-fluorescein isothiocyanate. Immunofluorescence was recorded with a fluorescence microscope (Axioskop; Zeiss, Oberkochen, Germany) using a ×40 oil planapo lens.

**Immunoblotting and Immunoprecipitation**

Total cell extracts were prepared by direct lysis of subconfluent cells in Laemmli sample buffer. To avoid possible interference of cyclic adenosine monophosphatase with the expression of proteins under study, cholera toxin and IBMX were omitted from the CM culture medium 48 to 72 hours before the cell extract was obtained. Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5%, 10%, or 12% polyacrylamide gels. Gels were stained with Coomassie blue to control for balanced loading or were electroblotted to nitrocellulose membranes for 1 hour at 20 V, using a semidy transfer apparatus (Pharmacia Biotech, Uppsala, Sweden). Membranes were submitted to immunoblotting using an enhanced chemiluminescence detection system (ECL,
To compare the levels of cell cycle regulators in normal and transformed choroidal melanocytes, we established primary cultures of choroidal melanocytes (CM). The characteristic morphology of these CM cultures, with typical bipolar or tripolar cells, is illustrated in Figure 1A. As a reference, we also show the respective morphologies of independent cultures of choroidal fibroblasts (Fig. 1B) and retinal pigment epithelial (RPE) cells (Fig. 1C), which were the two putative contaminating cell types. To discriminate between CM and choroidal fibroblasts, we investigated the cell proliferation behavior of CM in response to transforming growth factor β1 (TGF-β1). The cytokine inhibits growth of skin melanocytes while enhancing the proliferation of fibroblastic cells. Transforming growth factor β1 potently inhibited CM proliferation, whereas fibroblast growth was slightly increased (Fig. 2A). The three melanoma cell lines under study were more resistant than CM to the growth factor’s inhibitory action, confirming the data reported in normal and transformed melanocytes from skin.

Choroidal melanocytes grew more slowly than the melanoma cells (Fig. 2B); doubling times were 50 to 72 hours and 20 to 24 hours, respectively. Moreover, in contrast to the transformed cells, CM proliferation was dependent on the presence of cholera toxin, IBMX, and basic fibroblast growth factor-2 in the culture medium (Fig. 2B), although basic fibroblast growth factor-2 alone partially sustained CM growth (not shown).

To demonstrate definitively the purity of our CM cultures, immunocytochemical studies were carried out by using an antibody directed against HMB-45, a protein that is a specific melanocyte marker. All of the cells of CM cultures stained positively with HMB-45, whereas control cultures of RPE and fibroblasts did not (Fig. 3). As expected, CM stained positively with S-100 but not with anticytokeratin, whereas RPE cells stained with the two antibodies and the fibroblasts with none (not shown).

Because deregulation of some components of the cell cycle machinery could be responsible for the malignant transformation of the cells, we anticipated that normal and transformed human choroidal melanocytes might display differential expression of the proteins involved in the control of G1 progression and G1/S transition. For this study, we used three CM cultures (TCM-5, TCM-6, and TCM-8) that we had established from three different donors, and we compared them with three melanoma cell lines (SP-6.5, OCM-1, and MKT-BR).

The contents of CDK2 and CDK4 were approximately identical in normal and transformed melanocytes (Fig. 4). Note that CDK2 expression was markedly higher in CM and melanoma cells than in RPE and choroidal fibroblasts; note also that a weak extra band with a lower electrophoretic mobility was observed on CDK4 immunoblotting among the three melanoma cell lines. By contrast, cyclin E was overexpressed in melanoma cells when compared with that in CM, RPE cells, and fibroblasts. Cyclin D1 amounts were surprisingly low in normal (except TCM-8) and transformed melanocytes (in comparison with levels in RPE cells and fibroblasts), and there was a further underexpression in two of the three melanoma cell lines studied (OCM-1 and MKT-BR). Knowing that cyclin D1 is decreased in pRb-deficient cells, we investigated pRb in our cells, but levels were similar. Whether the lower electrophoretic mobility observed in TCM-8 CM compared with that in RPE cells and fibroblasts reflects a higher degree of pRb phosphorylation remains to be elucidated. However, it is interesting to note that this characteristic shift correlates with higher amounts of cyclin D1.

The CDK-activating kinase CAK has been proposed as a key regulator of CDK phosphorylation and activation. We therefore investigated the expression levels of its catalytic and
regulatory subunits. Melanoma cells and CM displayed identical amounts of CDK7 catalytic subunit, whereas the expression of the cyclin H regulatory subunit was slightly higher in the transformed cells (Fig. 5). Because CAK activity may depend on the subcellular localization of CDK7 and cyclin H, we performed immunocytochemical studies to determine eventual changes in normal and transformed cells. In the two cell types, CDK7 and cyclin H were mostly located in the cell nucleus (Fig. 6).

Accumulated evidence suggests that the levels of CKIs control the activity of CDKs. We investigated the expression of different members of the two CKI families. In two of the three melanoma cell lines (OCM-1 and MKT-BR), p21 was apparently absent (Figure 7). However, this result must be clarified, in that an extra band with a faster electrophoretic mobility was detected in these cells. More significant is our observation that p27 levels were strikingly lower in melanoma cells than in melanocytes or in the two other normal cell types, RPE cells and fibroblasts.

Finally, p16 was expressed almost identically in all cell types tested. This latter finding could be intriguing in that p16 is altered in several transformed cell lines and primary tumors.17,18 We anticipated that the p16-CDK4 interaction, rather than the CKI level could be affected in melanoma cells. To assess this hypothesis, we performed an immunoprecipitation experiment using the CDK4 antibody, which subsequently provided immunodetection of p16 in the immune complexes.

The results showed clearly that the p16-CDK4 interaction was abolished in two of the three melanoma cell lines (SP-6.1 and MKT-BR; Fig. 8).

DISCUSSION

Obtaining pure human CM cultures was a prerequisite for investigating the molecular mechanisms involved in their transformation to neoplastic melanoma cells. By using various complementary approaches (that is, performing morphologic study, immunostaining with a specific marker, and investigating cell proliferation behavior and TGF-β1 responsiveness), we demonstrated the purity of these cultures. Because a line of evidence suggests a close link between oncogenesis and the cell cycle machinery, we analyzed by immunoblotting the protein levels of G1 cyclins, their catalytic partners, and the CDK inhibitors in normal and transformed human choroidal melanocytes.

Deregulated expression of D-type and E-type cyclins has often been observed in neoplastic proliferation. We report significantly higher levels of cyclin E in choroidal melanoma cells than in normal melanocytes. This finding agrees well with results in several studies that show cyclin E overexpression in many human tumors.6-8 By contrast, our results indicating that cyclin D1 is not overexpressed in melanoma cells and is underexpressed in two of the three transformed cell lines may be...
FIGURE 3. Immunochemical staining of choroidal melanocytes, retinal pigment epithelial cells, and choroidal fibroblasts. Choroidal melanocytes (A), retinal pigment epithelial cells (B), and choroidal fibroblasts (C) were labeled with anti-HMB-45 (A, B, C). Immunostaining obtained in the absence of primary antibody was also shown (D, E, F).

Figure 3 illustrates the immunostaining of choroidal melanocytes, retinal pigment epithelial cells, and choroidal fibroblasts. The figure shows labeled samples (A, B, C) and controls in the absence of primary antibody (D, E, F). The staining patterns are characteristic of the respective cell types, with melanocytes showing a strong reaction with anti-HMB-45, while controls display no specific staining.

Surprisingly, although cyclin D1 gene amplification and high levels of specific mRNA have been repeatedly reported in human transformed cell lines and tumors, such effects have not always been correlated to an equivalent overexpression of the protein; in fact, cyclin D1 was often poorly expressed. Because overexpression of cyclin D above a certain threshold is toxic for cells, it has been postulated that only a moderate expression of cyclin D1 may confer a growth advantage to the tumor. Moreover, disruption of pRb function in pRb-negative tumor cells or in those expressing oncoproteins (SV40 T antigen, adenovirus E1a, and papilloma virus E7) causes a decrease of cyclin D1 and a loss of its binding to CDK4. Indeed, cyclin D1 expression is induced by functional pRb, and oncoproteins bind and inactivate pRb. We noted similar amounts of pRb in normal and transformed melanocytes, but whether pRb function was altered in melanoma cells and what causes such eventual damage remain questionable. The presence of oncoproteins in these transformed cells is at present only speculative. It is interesting that such oncoproteins have also been responsible for the resistance of infected cells to the antiproliferative action of TGF-β, a feature that we showed to be characteristic of choroidal melanoma cells.

Overexpression of CDKs has been tentatively correlated with oncogenesis. In some sarcoma cell lines, the CDK4 gene is amplified, and CDK4 protein is overexpressed in several tumor cell lines. Constitutive expression of CDK4 reduces the serum requirement in mink epithelial cell and may confer resistance to TGF-β. It is notable that CDK4 is also overexpressed in nearly 60% of skin melanomas. Our results were not in agreement with such observations in neither CDK2 nor CDK4 expression is altered in melanoma cells when compared with that in melanocytes. However, a second weak band was observed on immunoblotting for CDK4 among the three melanoma cell lines. Whether this extra band represented a phosphorylated or mutated form of CDK4 remains to be clarified.

Bartkova et al. suggest that the catalytic subunit of CAK (CDK7, previously named MO15) is moderately elevated in different transformed cells than in their normal counterparts. The authors did not investigate the fate of cyclin H, the regulatory partner of CAK. We demonstrated in this study that CDK7 content was identical in choroidal melanocytes and in melanoma cells, whereas cyclin H expression was slightly higher in the transformed cells. Cyclin H colocalized with CDK7 at the cell nucleus, a result that was expected but, to our knowledge, had not yet been proved. The nuclear localization of cyclin H and CDK7 was unchanged in melanocytes and in melanoma cells. Previous results indicate a constitutive expression of CDK7 and constant CAK activity in cycling mammalian cells. It has therefore been postulated that limited access to its substrates (CDKs, for example) or interference of CDKs by CKIs could be sufficient to assume cell cycle control. Also, it has been emphasized that CAK, which is a component of the transcription factor IIH (TFIIH), could exert its influence at the transcription level as a carboxy-terminal domain-kinase.

Our finding that p27 levels were markedly lower in melanoma cells than in normal melanocytes could be provocative, because a link between this CKI and oncogenesis is not completely accepted at present. Despite an extensive search for molecular aberrations modifying the p27 locus, no significant alterations of the p27 gene have been reported in human malignant disease. However, it is thought that p27 may play a central role in the negative regulation of cell growth in that it has been shown to mediate the G1 arrest induced by TGF-β, contact inhibition, and serum deprivation. Moreover, mice without p27 display multiple organ hyperplasia and,
CDK4 → 33
CDK2 → 33
Cyclin D1 → 33
Cyclin E → 49
pRb → 49
Actin → 49

**Figure 4.** Immunoblot analysis of cyclin-dependent kinase (CDK) and cyclin levels in melanocytes and melanoma cell lines. CDK4, CDK2, cyclin D1, cyclin E, and pRb contents were evaluated in total extracts from SP-6.5 (SP), OCM-1 (OCM), and MKT-BR (MKT) melanoma cell lines and from three choroidal melanocyte cultures (TCM-5, TCM-6, and TCM-8), as indicated in Materials and Methods. Retinal pigment epithelial cells and choroidal fibroblasts (FIBRO) were also used as controls. Insert on the right shows a portion of the anti-CDK2 blot with a longer time for enhanced chemiluminescence detection. Control for balanced loading was asserted by measuring the amount of actin in the different cell extracts.

Occasionally, pituitary tumors,\(^5^0\) which indicates a proliferative advantage in the absence of p27 function. Last but of importance, our finding fits well with those in recent reports that indicate that p27 protein levels are reduced in primary breast cancers and that this reduction is associated with tumor progression and is correlated inversely with survival.\(^1^5^,^1^6\)

Constitutive expression of the related CKI p21 suppresses growth of chicken embryo fibroblasts transformed by oncogenes\(^5^1\) and reduces tumorgenesis in vivo.\(^1^5\) However, p21-null mice undergo normal development,\(^5^2\) which is consistent with the low frequency of p21 mutations in human cancers.\(^1^4\) An interesting inverse correlation between p21 gene expression and evolution from normal skin melanocyte to metastatic melanoma has been described.\(^5^3\) However, conflicting data have been reported about p21 in skin melanoma cells indicating a reduction of p21 expression in 7 of 14 tumors.\(^5^4\) It is also overexpressed in certain human brain tumors\(^5^5\) and in cutaneous metastatic melanoma.\(^5^6\) Our results concerning the absence of p21 expression in two of the three choroidal melanoma cell lines remain to be clarified. In fact, an extra band appeared in these cells that had higher electrophoretic mobility. Whether it is a truncated or a phosphorylated form of p21 remains to be elucidated.

The potential involvement of p16\(^{INK4A}\) in the development of tumors was suggested initially by the finding that the p16 gene maps to a chromosomal region frequently mutated in a series of human cancers, including cutaneous melanoma, pancreatic and esophageal tumors.\(^1^7^–^1^9\) Spontaneous tumors develop in INK4-deficient mice at an early age, and the mice are highly sensitive to carcinogens.\(^5^7\) In contrast to a recent report showing that loss of p16 expression is a common event in primary cutaneous melanomas,\(^5^8\) we demonstrated in this study that p16 protein levels were identical in normal and transformed melanocytes. However, we showed that p16-CDK4 interaction was dramatically altered in two of the three melanoma cell lines. This finding is reminiscent of that in a previous report concerning p16 proteins from skin melanoma-prone families who are deficient in binding to CDK4.\(^5^9\)
FIGURE 5. Cyclin H and CDK7 expression in melanocytes and melanoma cells were evaluated in total extracts from SP-6.5 (SP), OCM-1 (OCM) and MKT-BR (MKT) melanoma cell lines and from three choroidal melanocyte cultures (TCM-5, TCM-6, and TCM-8), as indicated in the Materials and Methods section. Retinal pigment epithelial cells and choroidal fibroblasts (FIBRO) were also used as controls. Immunoblot analysis for CDK7 and cyclin H contents was performed by using the different cell extracts as indicated in the legend of Figure 4.

Although it is difficult to speculate on the basis of results of in vitro studies, our data suggest that deregulated expression of G1 cyclins and CKIs and alterations in the interaction of CKIs with CDKs may be implicated in the malignant transformation of choroidal melanocytes to melanoma. However, the
heterogeneity of the three melanoma cell lines studied should be noted in relation to cyclin D1 or p21 expression and p16-CDK4 interaction. This is indicative that various molecular events may be involved in the neoplastic transformation of choroidal melanocytes.

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References
40. Ewen ME, Sluss HK, Whitehouse LL, Livingston DM. TGF-β inhibi-
46.
45. Serizawa H, Miikela TP, Conaway JW, et al. Association of Cdk-
44.
41. Wang YL, Uhara H, Yamasaki Y, Nikaido T, Saida T. Immunohis-
38. Miiller H, Lukas J, Schneider A, et al. Cyclin D1 expression is
36. Quelle DE, Ashmun RA, Shurtleff SA, et al. Overexpression of
35. Lukas J, Miiller H, JB, et al. DNA tumor virus oncoproteins and
34. Bates S, Parry D, Bonetta L, et al. Absence of cyclin D/cdk com-
884 Mouriaux et al.
1995;374:283-287.
1993;7:1559-1571.
1994;14:7265-7275.
1995;91:2945-2949.
1996;380:262-265.
1994;374:283-287.
1994;262-265.
1993;74:262-265.
1994;91:2945-2949.
1994;14:7265-7275.
1994;8:9-22.
1996;380:262-265.
1995;374:283-287.
1994;8:9-22.
1994;262-265.