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Abnormal Cell Cycle Regulation in Primary Human Uveal Melanoma Cultures

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Abstract Uveal malignant melanoma is the most frequent primary intraocular tumor in adult humans. The cellular events leading to neoplasic transformation of normal uveal melanocytes are not well known when compared to other cancers. In this study, we investigated the role of G1 and G1/S regulatory proteins of the cell cycle in human uveal melanoma (UM) primary cell cultures, since these proteins are common targets in tumor development. Further, freshly established and characterized tumor cells are a better model for in vitro studies when compared to cell lines established long ago. Human primary cell cultures from eight different UM were established, as well as one primary culture from rhesus uveal normal melanocytes (UNM). Primary human UM cultures were characterized by a low establishment and growing rate. From four successful cultures, three showed a high expression of cyclin D1, cyclin E, $p16^{INK4A}$, and $p27^{KIP1}$ with no variations in cyclin A, cyclin-dependent kinase 2 (CDK2), and CDK4. Interestingly, in one of the cultured tumors, tumor suppressor protein retinoblastoma (Rb) did not bind E2F despite the fact that Rb was found in its hypophosphorylated form. No mutations in either RB1 or the Rb-binding pocket of E2F-1 were detected. Furthermore, we identified seven proteins co-immunoprecipitating with Rb in this tumor, including Lamin A/C and six proteins not previously reported to bind Rb: Hsc70, high mobility group protein 1 (HMG-1), hnRPN, glyceraldehyde 3 phosphate dehydrogenase (G3PDH), EF-1, and EF-2. Our results indicate that the overexpression of cyclins D1/E and CDKIs p16 and p27, together with a deregulation of the Rb/E2F pathway, may be implicated in the development of human UM. J. Cell. Biochem. 93: 708–720, 2004.

Key words: uveal melanoma; cell cycle; Rb

Uveal melanoma (UM) is the most common primary intraocular tumor in adults, with a 5-year survival rate of up to 77–84% [Singh and Topham, 2003]. The incidence of this neoplasm is approximately six per million per year in whites, which is relatively low compared to that of cutaneous melanoma [Egan et al., 1988]. Unfortunately, the advances in eye cancer treatment have not paralleled those made in the treatment of other sites of cancer [Jemal et al., 2002], and the prognosis of patients with UM remains poor. Many investigations have demonstrated a close link between oncogenesis and the cell cycle machinery. While the molecular events leading to the transformation of cells are well characterized in many cancers, very little is known about those events in malignant UM [Loercher and Harbour, 2003].

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Progression through the cell cycle requires the action of cyclin-dependent kinases (CDKs). As cells enter the cycle, newly synthesized D-type cyclins associate with and activate their CDK4/6 partners in mid- to late G1 phase, whereas cyclin E appears later in G1 and activates its CDK2 kinase subunit near the G1/S boundary. Once in S phase, cyclin A emerges and also associates with CDK2.

Opposing the formation of cyclin-CDKs complexes are CDK inhibitors (CDKIs) of two classes: INK4 proteins, such $p16^{INK4A}$, $p15^{INK4B}$, and p18 that specifically inhibit cyclin-D kinases; and CIP/KIP proteins, such as $p21^{CIP1}$ and $p27^{KIP1}$ that also inhibit CDK2. These cell cycle inhibitors are frequently altered in various malignancies such as skin melanoma, where $p16^{INK4A}$ expression is often absent [Hussussian et al., 1994].

Other cell cycle regulators include the CDK substrates known as the retinoblastoma (Rb) family of nuclear pocket proteins, which include Rb itself, p130 and p107. These proteins form repressive complexes with E2F transcription factors that control genes needed for cell cycle progression. Phosphorylation of pocket proteins by CDKs frees these transcription factors and activates E2F target genes. G1/S phase transition is thought to be controlled by Rb since its functional inactivation becomes a significant event in tumorogenesis.

For many cancers, it has been shown that alterations in the expression of G1 cyclins, CDKs, CDK-activating enzymes, CDKIs, CDK substrates, and check point proteins can lead to loss of cell cycle control [Sherr, 1996]. In this study, we show a comparative analysis of the expression of different regulatory proteins involved in G1 and G1/S transition in primary cell cultures of rhesus normal uveal melanocytes (UNM) and human UM. Primary cultures were chosen as a model since it is well known that in vitro studies using freshly established and characterized cells are preferable to those using cell lines established long ago which may have poor biological relevance to their tumors of origin. We found an enhanced expression of cyclin D1 and E, and no variation in cyclin A, CDK2, or CDK4 expression in three of four successful UM cultures. Surprisingly, we also found overexpression of the CDKIs $p16^{INK4A}$ and $p27^{KIP1}$ in these cultures. We further describe the altered status of Rb protein in one of the UM cultures, where Rb occurred predominantly in a hypophosphorylated form, but nevertheless could not bind E2F-1. No mutations were found in RB1 or the E2F-1 Rb-binding pocket in this tumor. However, several Rb-binding proteins were detected that may explain this behavior, which may be implicated in the genesis of certain human UMs.

MATERIALS AND METHODS

Cell Cultures

Tumor samples were obtained after informed consent from 10 consecutive patients diagnosed with choroidal melanoma, followed by enucleation of the affected eye at the Ocular Oncology Unit (Complexo Hospitalario Universitario de Santiago de Compostela, Spain). Small tumor fragments were cultured on the same day that the surgery took place. Under sterile conditions, samples were cut into little pieces using a surgical blade and enzymatically dispersed using a solution of 0.4% (w/v) type IA collagenase, 0.2% (w/v) dispase, 0.1% (w/v) hyaluronidase, 0.01% (w/v) DNase I (all from Sigma-Aldrich, St Louis, MO), and 10% FBS in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA). Cell pellets were finally resuspended in DMEM containing 10% inactivated FBS and 20 μg/ml EGF, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin-B (all from Invitrogen). Cells were plated and incubated at 37°C in a humidified atmosphere of 95% air-5% CO_2.

Rhesus (*Macaca mulatta*) choroidal melanocytes (UNM) were cultured, as previously described by Goodall et al. [1994], from an enucleated eye kindly donated by Prof. Acuña (Laboratorios de Neurociencia y Computación Neuronal, University of Santiago de Compostela, Spain). The cell pellet was resuspended in DMEM plus 10% FBS, human transferrin 10 μg/ml, hydrocortisone 2.8 μg/ml, 2 mM glutamine, 10 mM PMA, 10 ng/ml cholera toxin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin-B [Goodall et al., 1994]. Cholera toxin was used during the establishment of this culture to avoid contamination by retinal pigment epithelial cells and choroidal fibroblasts.

The human retinal pigment epithelial cell line (hRPE), previously established by our group [Capeans et al., 1998], was grown in DMEM media supplemented with antibiotics and 10% FBS.

Immunocytochemical Studies

The histopathology study was performed by examination of the cells as they grew in vitro by HMB-45 immunostaining (DAKO, Glostrup, Denmark) [Burnier et al., 1991].

Proliferating Assays

For cell growth measurement, UM and UNM cells were seeded in sextuplicate, at a density of 4×10^3 cells/well, in 96-well microtiter plates (Nunc, Roskilde, Denmark). Cells were cultured in 100 μl of complete medium and incubated with bromodeoxyuridine (BrdU) from 1 to 15 days. BrdU uptake was assayed according to the method of the manufacturer (Roche, Basel, Switzerland) and measured in a Microplate Reader (Bio-Rad, Hercules, CA) at 405 nm with a reference wavelength at 490 nm.

Immunoblotting and Immunoprecipitation

Whole cell lysates were prepared by direct lysis of subconfluent cells in cold RIPA buffer containing 200 mM Tris (pH 7.4), 130 mM NaCl, 10% (v/v) glycerol, 1.9 mg/ml aprotinin, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 1% (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT, 1 mM sodium orthovanadate, and 1 M NaF. To avoid possible interferences, EGF was omitted 72 h before the start of the experiments. Equal amounts of protein (50 μg/lane) were run on sodium–dodecyl sulfate-polyacrylamide gels (SDS–PAGE), and electroblotted onto nitrocellulose membranes. The membranes were probed successively with primary antibodies and alkaline phosphatase-labeled secondary antibodies (Tropix-Perkin Elmer, Wellesley, MA). Specific antigen-antibody binding was visualized using a chemiluminescence method according to the manufacturer (Tropix-Perkin Elmer). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) [cyclin E (HE-12), cyclin A (BF683), CDK4 (C22), CDK2 (M2), p16 (N-20), p27 (N-20), Rb (IF8), E2F-1 (C20), actin (C11)], except anti-cyclin D1, which was from Novocastra (Newcastle upon Tyne, UK).

For immunoprecipitation experiments cells were lysed in RIPA buffer and passaged through a 19G needle. The lysate was cleared by centrifugation and an equal amount of protein (800 μg) was separated. Cyclins and Rb were immunoprecipitated with 2 μg of the corresponding antibody and Protein G Sepharose (Amersham Biosciences, Uppsala, Sweden). Samples were centrifuged and washed three times in ice-cold RIPA buffer. Immunocomplexes were boiled in Laemmli sample buffer [Laemmli, 1970] and separated and electroblotted as described earlier.

RT-PCR and mRNA Sequencing

mRNA was extracted from 1×10^6 UM-A and hRPE cells by using the RNA purification kit Quick Prep Micro (Amersham Biosciences). Reverse transcription was performed with 1 μg of extracted RNA. For mutation analysis of the *Rb* gene, three overlapping pairs of primers were designed based on the mRNA sequence obtained from the genebank (NCBI: M15400): Fw1Rb, 5′-ATT TTT GTA ACG GGA GTC GG GA-3′; Rv1Rb, 5′-GTC TGA AGA GTT TTA TCA TGA TCC-3′; Fw2Rb, 5′-CTA ATG GAC TTC CAG AGG TT-3′; Rv2Rb, 5′-TGA GGT TGC TTG TGT CTC TG-3′; Fw3Rb, 5′-CTC CTG TAA GAT CTC CAA AG-3′ and Rv3Rb, 5′-TTT TCT GGA ACT TCT CAG AAG T-3′. The amplification of these primers rendered 1,142, 979, and 996 bp fragments, respectively. To screen mutations on the *E2F-1* Rb-binding pocket sequence, the following pairs of primers were designed (NCBI: NM005225): FwE2F, 5′-CAG CCA GTC TCT ACT CAG CC-3′ and RvE2F, 5′-TCA GAA ATC CAG GGG GGT GAG-3′. The product of this amplification was a 53 bp fragment. Fragment 1 of Rb was amplified using FailSafe™ PreMix Selection Kit (Epicentre®, Madison, WI). Cycling parameters for this fragment were: 95°C, 1 min; 55°C, 1 min; and 72°C, 2 min for forty cycles. PCR reactions for Fragments 2 and 3 of RB and the E2F Rb-binding pocket were carried out in a total volume of 25 μl, containing 2 μl of cDNA, in reaction buffer (50 mM KCL + 10 mM Tris), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM of both forward and reverse primers, and 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCRs were performed during 35 cycles as follows: 1 min at 95°C, 1 min at 58 (for Rb2) or 55°C (for Rb3 and E2F); and 2 min at 72°C. The PCR products were purified through a Sequencing Reaction Cleanup Kit (Montage™ SEQ96, Millipore, Billerica, MA) and resuspended in 20 μl of injection solution. The purified PCR products were then sequenced by the dideoxy method Thermo Sequenase* Cy5 Dye Terminator Sequencing Kit in a MegaBACE™ 1000 sequencer (Amersham Biosciences).

Analysis of Rb-Binding Proteins: Protein Digestion and Mass Spectrometric Analysis

Rb immunoprecipitates were carried out as described above and analyzed by NuPAGE™ 4–12% (w/v) gradient SDS–PAGE gels (Invitrogen, Carlsbad, CA). Unspecific binding was detected by incubating protein lysates with normal mouse IgG (Santa Cruz, CA). Following electrophoresis, gels were fixed in 40% (v/v) ethanol: 10% (v/v) acetic acid and stained with the fluorescent dye OGT 1238 (propreitory to Oxford GlycoSciences, Abingdon, UK) on the basis of Hassner et al. [1984]. Monochrome images (8 bit) were obtained by scanning gels with a Fuji CCD Camera LAS-1000 plus (Tokyo, Japan).

Protein features assigned to mass spectrometric analysis were manually excised from the gel and dried in a SpeedVac. In-gel digestion was performed with trypsin (Roche, Basel, Switzerland) according to the protocol of Shevchenko et al. [1996]. The tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [García et al., 2004].

Statistical Analysis

All experiments were repeated at least three times. For Western blots, relative protein levels per band were estimated by densitometry. Statistical analysis was carried out with the non-parametric Mann–Whitney test comparing UM and RPE cells to UNM. Differences were considered significant when $P < 0.05$.

RESULTS

UM Primary Cell Cultures

Eight different primary cell cultures were established from 10 human UM tumors (UM-A–J). Cultured UM cells were characterized by a low establishment rate and did not cope with passaging in the same way (Table I). The cells that did not survive after the second passage were characterized by large extended cell bodies, with numerous vacuoles that may indicate cell senescence (Fig. 1A,B). One of these cultures (UM-H) reached the third passage and could be harvested for protein analysis. Three cultured tumors (UM-A, -F, -G) reached later passages (up to six) and were maintained in continuous culture or stored in liquid nitrogen (Fig. 1C–G). Cells from these three cultures were analyzed further. After passage 7, cells from the UM-A culture formed colonies of more refractile cells, characterized by a clear spindle shape and an accelerated rate of growth. Indeed, these cells quickly replaced the original cells, developing into a different phenotypic cell line (Fig. 1E). This in vitro re-transformed cell line was not further analyzed.

For our study, we had the opportunity to obtain an enucleated eye from a rhesus monkey and establish a primary culture of uveal normal melanocytes (UNM) (Fig. 1H). These cells were chosen as controls due to the difficulties in acquiring human uveal melanocytes from healthy donors. The physiological, genetic, and pharmacological similarities between the rhesus monkey and human eye [Bernstein and Wong, 1998; Bito, 2001] make these cells a close and reliable model for the human counterparts. UNM, initially cultured in presence of cholera toxin to avoid contamination with retinal pigment epithelium cells [Goodall et al., 1994], showed a bipolar or tripolar shape and abundant pigment. Cells from passages 3 to 6 were used in this study.

Human retinal pigmentary epithelial (RPE) cells were also used as controls, as previously described [Mouriaux et al., 1998].

TABLE I. Uveal Melanoma Primary Cell Cultures

Patient	Age	Status	C. type	Localiz.	Pigm.	Dia. (mm)	Ht. (mm)
UM-A	22	In culture	Spindle	Equat.	High	19.1	12.41
UM-B	83	Failed	Spindle	C. body	High	13.8	9.17
UM-C	78	1p	Spindle	Equat.	Medium	13.2	6.79
UM-D	80	2p	Mixed	Macular	High	13.5	10.17
UM-E	58	2p	Mixed	C. body	High	10.7	6.7
UM-F	54	In culture	Spindle	Equat.	Low	13.99	13.25
UM-G	75	In culture	Epithel	Macular	No	9.94	4.19
UM-H	64	3p	Spindle	Equat.	Medium	16.97	4.35
UM-I	70	2p	Spindle	Equat.	Low	14.2	9.15
UM-J	73	Failed	Spindle	Equat.	High	16.8	11.22

Age, in years at enucleation; C. body, ciliary body; C. type, cell type-Callender classification; Dia, tumor diameter; Equat., equatorial; Ht, tumor height; Localiz, tumor localization; p, passage; Pigm, tumor pigment grade.

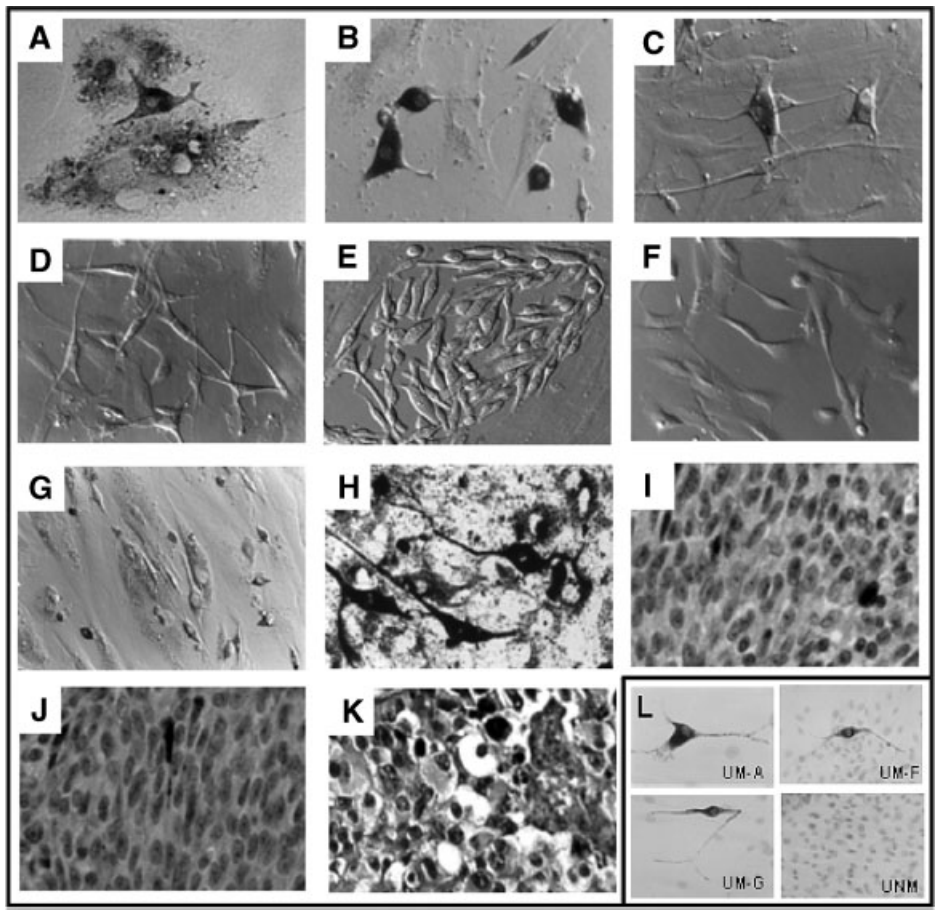

Fig. 1. Established primary cell cultures. Phase-contrast microscopy of (**A**, **B**) two slow growing UM cultures (UM-H and -E, respectively) at passage 2 [original magnification 60×], (**C–E**) UM-A culture at passages 1, 3, and 7 [original magnification 60×, 40×, 40×, respectively], (**F**) UM-F culture at passage 3 [original magnification 40×], (**G**) UM-G culture at passage 3 [original magnification 40×], (**H**) rhesus uveal normal melanocytes (UNM) at passage 1 [original magnification 40×]. **I–K**: Representative histology sections from UM-A, -F, and -G tumors, respectively [original magnification 40×]. **L**: UM and UNM cell cultures labeled with anti-HMB-45.

To further demonstrate the purity of the successful UM cultures, immunocytochemical studies were performed using an antibody directed against the melanosoma antigen gp100 (HMB-45) [Gown et al., 1986]. As expected, UM primary cultures were positive for HMB-45, whereas control cell cultures were negative (Fig. 1L).

Proliferation in Primary UM Cell Cultures

All UM cells were characterized by a slow growing rate as shown during cell culture and confirmed by BrdU uptake (Fig. 2). The determination of doubling times in these cells was an issue since proliferation rate decreased during passaging. At the fourth passage, UM-A, -F, and -G showed a doubling time of 6–10 days, compared to 24 h for the UNM primary culture (Fig. 2) and RPE cells [Capeans et al., 1998].

Expression of G1 and G1/S Regulating Proteins in UM

The expression of one or more G1 and G1/S transition regulating proteins was expected to be modified, as this is frequently the case in other cancers. Therefore, these proteins were analyzed in the successfully established primary cell cultures (UM-A, -F–H) (Fig. 3).

CDK2 and CDK4 protein levels did not differ amongst all the different cultured cells. By contrast, cyclins D1 and E were overexpressed in three of the UM primary cultures analyzed (UM-A, -F, and -H) as well as in the hRPE cell line. However, no differences in cyclin A levels were found when comparing UM to control cell cultures (Fig. 3).

It is well known that CDK inhibitors control the activity of cyclin/CDK function, therefore

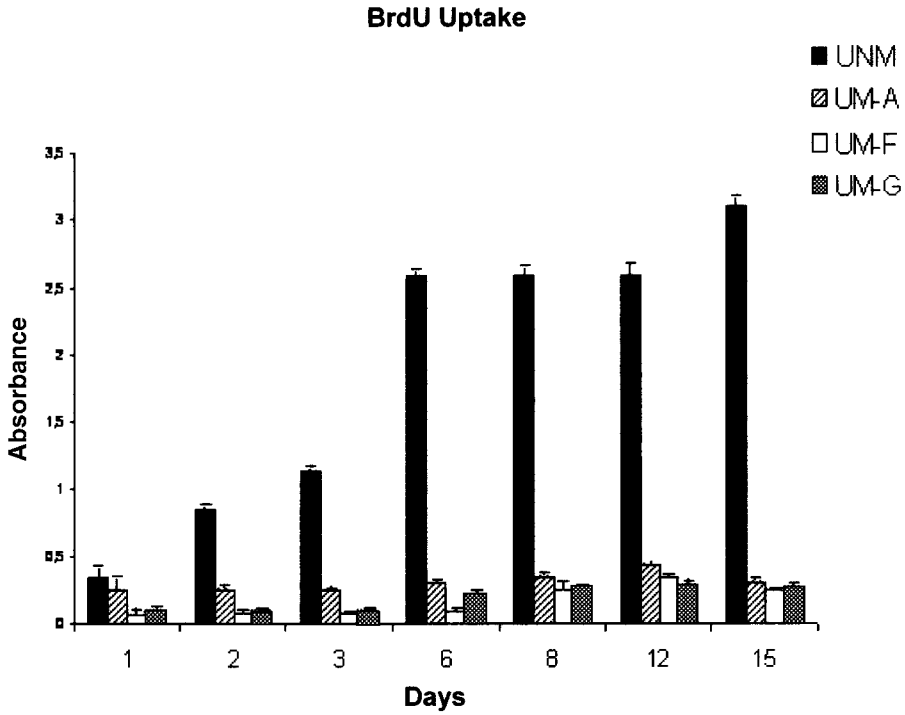

Fig. 2. Proliferation analysis of UNM and UM primary cell cultures. BrdU uptake from 1 to 15 days in normal (UNM) and uveal melanoma primary cultures (UM-A, -F, and -G) at passage 4. Mean of three independent experiments per sextuplicate ± SD are shown.

a low or lack of expression of these proteins in UM was expected. We initially focused on the expression of $p16^{INK4A}$, as this is frequently altered in cutaneous melanoma [Hussussian et al., 1994]. Surprisingly, p16 was overexpressed in three of the four UM primary cell cultures analyzed (UM-A, -F, and -H), whereas much lower levels were found in UNM (Fig. 3). The CIP/KIP family inhibitor p27 was also studied, and was discovered to be overexpressed in the same tumors as p16. In this case, p27 overexpression was also observed in hRPE cells.

Inhibitors, p16 and p27, act by disrupting complexes formed between cyclin and their kinases. Since we found high levels of these CDKIs in our cultures, we decided to study whether the complexes were formed adequately by studying Rb as the main substrate for these complexes. In an initial approach, the Rb phosphorylation state was studied. Using a specific antibody able to recognize the various forms of Rb phosphorylation, we could determine the Rb phosphorylation state in UM primary cell cultures (Fig. 3). UM primary cultures exhibited slightly lower Rb levels than normal melanocytes. However, the Rb phosphorylation pattern, which changes with the functional activity of Rb, was not the same for all cultures. In fact, RPE, UNM, UM-F, and -G presented a predominant band corresponding to the phosphorylated form of Rb, which is commonly found in proliferating cells. However, two UM tumors (UM-A and -H) showed an unexpected pattern with the majority of Rb in its hypophosphorylated or active state (Fig. 3). Rb in these tumors should be repressing E2F transcription factor, thereby preventing the cells from dividing. As this clearly was not the case, we studied in detail the molecular mechanisms responsible for Rb phosphorylation in UM-A primary cell culture.

Rb/E2F Pathway in UM

It is generally accepted that CDK2 and CDK4 are the main kinases involved in Rb phosphorylation [Hinds et al., 1992; Kato et al., 1993; Harbour et al., 1999]. CDK4 bound to cyclin D initiates Rb phosphorylation and Rb is then further phosphorylated by the complex cyclin E/CDK2. Therefore, we analyzed cyclin/CDK complexes by co-immunoprecipitation assays (Fig. 4). By immunoprecipitation of cyclin D1 followed by CDK4 immunodetection, we could prove that the assembly of both subunits into the cyclin/CDK complexes was significantly

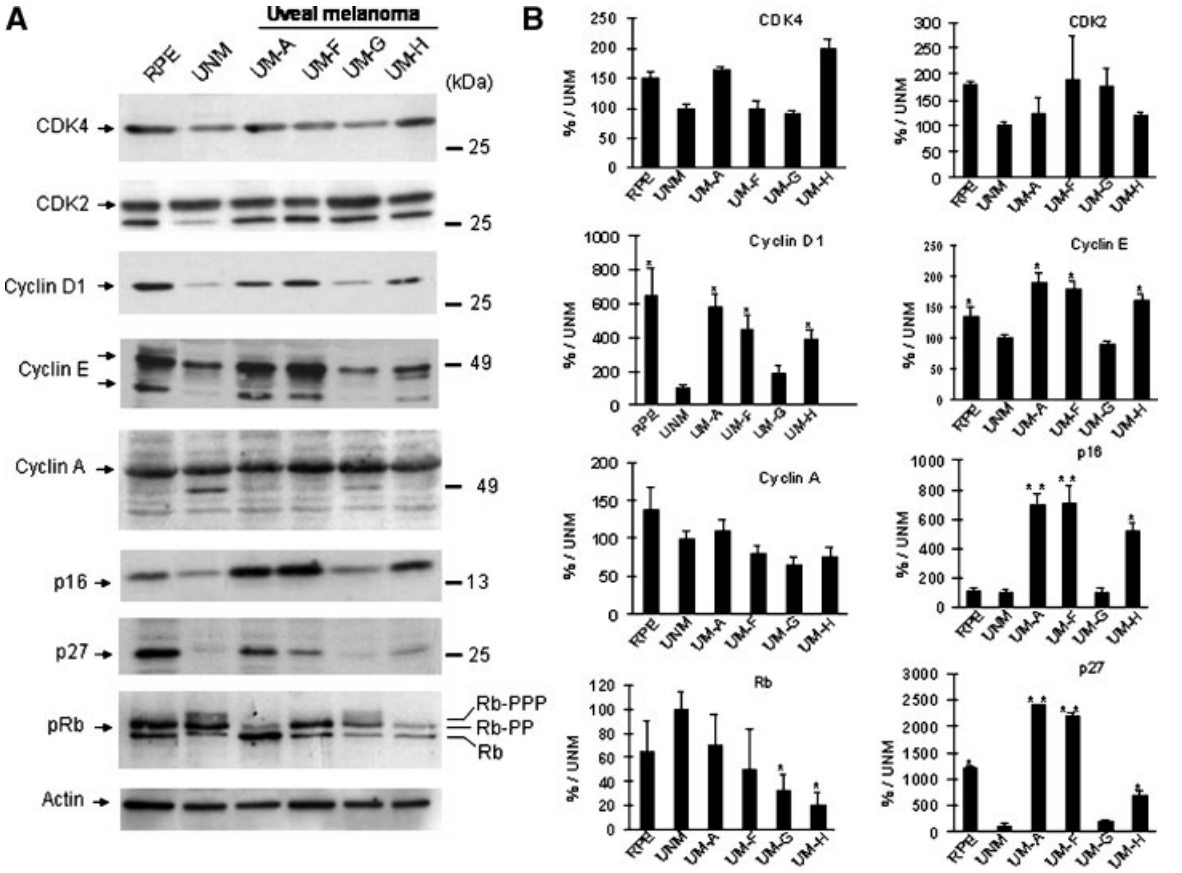

Fig. 3. Analysis of cell cycle regulatory proteins in UM primary cell cultures. **A**: Immunoblot analysis of G1 and G1/S transition regulatory proteins in control and uveal melanoma cell cultures. CDK4, CDK2, cyclin D1, cyclin E, cyclin A, p16, p27, and Rb expression were evaluated in whole extracts from the RPE cell line, UNM primary cell culture, and from four successful primary UM cultures (UM-A, -F, -G, -H). Equal loading was confirmed by measuring the amount of actin in the different cell extracts. Images are representative of at least three independent experiments. **B**: Linear exposed autoradiographs of at least three different replicate immunoblot experiments were quantifed by densitometry and mean ± SD are shown in the bar graphs for the indicated proteins, $*P < 0.05$, $**P < 0.01$.

reduced in UM-A compared to control cells (Fig. 4A). On the other hand, cyclin E and A could bind to CDK2 in UM-A and in control cells (Fig. 4B,C).

In order to investigate why the hypophosphorylated form of Rb does not stop the cell cycle in UM-A, we studied by co-immunoprecipitation if Rb and E2F-1 could still bind each other. We found that Rb does not bind the E2F-1 transcription factor in these cells (Fig. 4D). E2F-1 occurs in its free form and can therefore play its role in the transcription of genes required for cell cycle progression such as cyclin E or A that are expressed normally as previously shown (Fig. 3).

All these results suggested a possible mutation in the RB1 gene that gave rise to a non-functional Rb unable to bind E2F-1. As somatic mutations in RB1 are known to contribute to the development of several human tumors [Nevins, 2001], we sequenced RB of UM-A cells, by analyzing the complete sequence of Rb mRNA by RT-PCR followed by automated sequencing. Special attention was paid to the A/B pocket of this protein as this pocket is necessary to bind E2F-1 and is frequently mutated in many neoplasias [Nevins, 2001]. Detailed analysis of the resulting mRNA sequence showed no alterations or mutations which could explain the lack of Rb function in this UM (data not shown).

As Rb was free of alterations, we proceeded to sequence the E2F-1 transcription factor. The mRNA sequence that codes for the Rb-binding site of E2F-1 was analyzed and no mutations were found in this pocket either (data not shown).

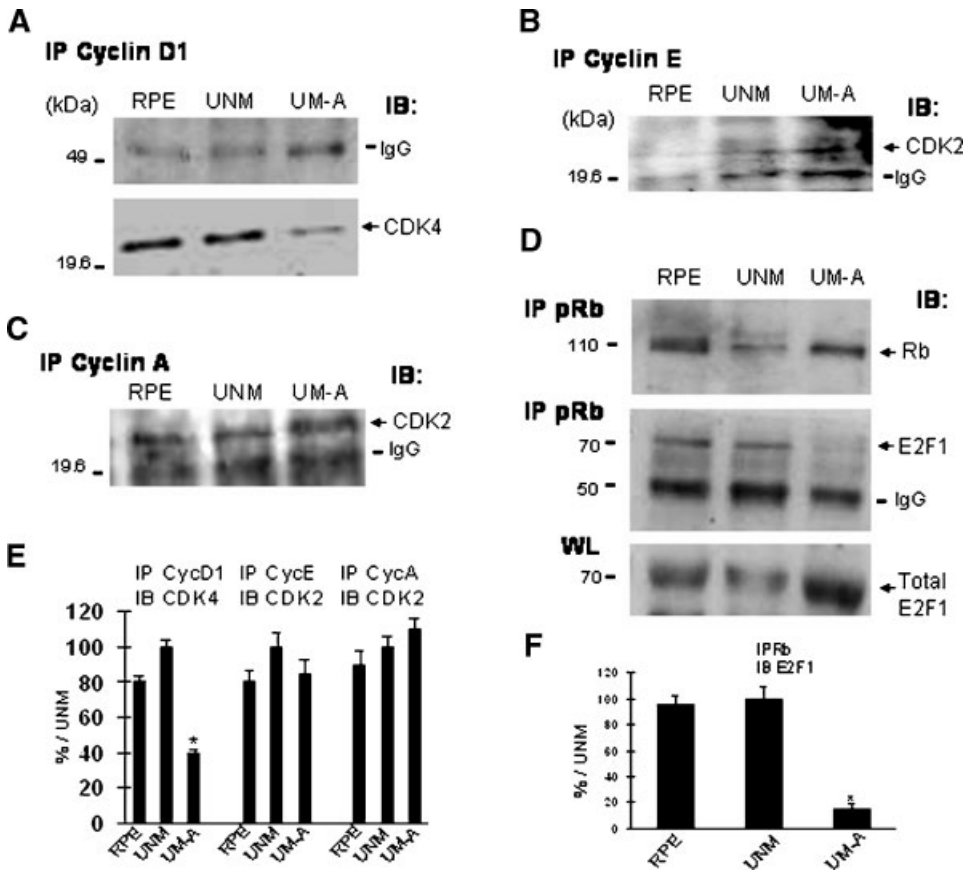

Fig. 4. Co-immunoprecipitation assays in control and UM-A cells. G1/S cyclin/CDK complex interactions were analyzed by (**A**) cyclin D1 IP followed by IB using an anti-CDK4 antibody, (**B**) cyclin E IP followed by IB using an anti-CDK2 antibody, (**C**) cyclin A IP followed by IB using an anti-CDK2 antibody. **D**: Rb was immunoprecipated followed by IB using anti-pRb and anti-E2F-1 antibodies. Total E2F-1 was assayed by IB using anti-E2F-1 in whole lysates. **E**: Linear exposed autoradiographs of at least three experiments related to panels (A–C) were quantified by densitometry and mean ± SD are shown in the bar graphs, $^*P < 0.05$. **F**: Quantification of Rb IPs followed by E2F-1 IB experiments done as in panel (E), $^*P < 0.05$. Images represent at least three independent experiments. IB, immunoblot; IP, immunoprecipitation; WL, whole lysates.

Rb-Binding Proteins in UM

Having established that Rb and E2F-1 DNA sequences were normal in UM-A cells, we considered the possibility that other proteins could interact with Rb affecting its binding to E2F-1. It has been shown that several Rb binding proteins, including several enzymes (kinases, phosphatases) and transcriptional regulators, affect Rb function [Woitach et al., 1998; Morris and Dyson, 2001]. In order to identify proteins bound to Rb in UM-A, but not in cultures derived from normal cells, Rb immunoprecipitations were performed and proteins resolved by SDS–PAGE (Fig. 5). Seven Rb binding proteins were found by mass spectrometric analysis in the UM-A tumor and not in homologous gel bands excised from normal uveal melanocytes immunoprecipitations: Lamin A/C, stress 70 protein (Hsc70), heterogeneous nuclear ribonucleoprotein (hnRNP), high mobility group protein 1 (HMG-1), glyceraldehyde 3 phosphate dehydrogenase (G3PDH), elongation factor 1α (EF-1), and elongation factor 2 (EF-2) (Fig. 5). Further experiments will contribute to a better understanding of the roles of these proteins in the functional inactivation of Rb in UM.

DISCUSSION

UM Primary Cell Cultures and Cell Cycle

We have studied the regulation of G1 cell cycle proteins in primary cell cultures of human UM. To our knowledge, this is the first study of the cell cycle proteins in UM primary cell cultures (passages 1–6). We propose that primary cell cultures are a physiologically more

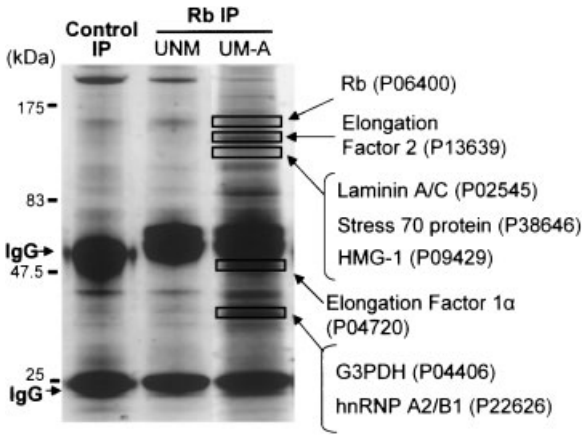

Fig. 5. Rb-binding proteins in UM-A cells. Rb immunoprecipitates from UNM and UM-A cells lysates were separated by 4–12% SDS–PAGE in order to do differential image analysis on the stained gel. UM-A protein lysates were also incubated with normal mouse IgG followed by protein G Sepharose (control IP) to detect unspecific binding and analyzed in the same gel. Black boxes represent the location of successfully identified protein bands in the stained gel. Protein names are shown together with their Swiss-Prot accession number. IP, immunoprecipitation.

relevant model than established cell lines to study UM as they more closely resemble the cells that form the tumor in the eye. Previous studies by Western blot in UM have shown discrepancies with regard to expression levels of cell cycle proteins between established cell lines and frozen tumor pieces [Mouriaux et al., 1998, 2000]. Indeed, we observed the re-transformation of one of the cultured UMs at the seventh passage, when the cells changed into a different phenotypical culture very similar to established UM cell lines described earlier [Diebold et al., 1997]. A detailed characterization of this cell line would need to be performed to show how these cells differ from primary cell cultures.

Despite the difficulties in culturing human UM cells, we achieved a success rate similar to that described by other authors [Aubert et al., 1993]. We found that primary cell cultures derived from UM have a slow growing rate and need between 6 and 10 days to double their number. This observation is consistent with previous results where the doubling time was found to be between 3 and 8 days [Wilson, 2000]. It is well known that UM is characterized by a low number of mitotic figures [Wilson, 2000] and that only 1–8% of tumor cells are BrdU positive [Char et al., 1989]. It is necessary to bear in mind that this tumor is slow growing compared to other neoplasias.

Many studies have demonstrated a close link between oncogenesis and the cell cycle machinery. Therefore, we analyzed G1/S phase regulating proteins in the successfully established primary UM cell cultures. We found that most of the tumors analyzed, presented a high expression of cyclin D1, E, and CDKIs p16, and p27, whereas no variations were found in cyclin A and CDK2/4 levels. Cyclin D1, E, and p27 overexpression was also found in RPE cells possibly due to an in vitro transformation after prolonged culture.

The presence of high expression levels of cyclin D1 and E in most of the UM cells studied herein suggests its implication in UM. This is consistent with the fact that cyclin D1 and E are overexpressed in many cancers [Motokura and Arnold, 1993; Yasmeen et al., 2003] and also that immunohistochemical analysis carried out in UM patients have revealed a high percentage of positives for cyclin D1 [Coupland et al., 1998; Errico et al., 2003]. However, no variations were found in cyclin A levels in the UM primary cell cultures studied. Interestingly, the UM-G culture showed a protein expression pattern that differed from the other cultured tumors studied, an observation we planned to analyze further in the future.

Unexpectedly, the analysis of the UM cultures showed a strikingly high expression of cell cycle CDKIs, $p16^{INK4A}$ and $p27^{KIP1}$. In contrast to skin melanoma [Hussussian et al., 1994; Reed et al., 1995], most of the UM primary cell cultures were overexpressing p16. Mouriaux et al. [2000] and Coupland et al. [1998] have shown previously that 34–45% of tumor-derived cells from UM patients were p16 positive. As with p16, the reduced expression of $p27^{KIP1}$ is considered to be a fundamental step for the development of human malignancies; however, we found enhanced expression of this protein in most of the UM primary cell cultures. Our results are consistent with findings by Mouriaux et al. [2000], who described an elevated expression of p27 and p16 CDKIs in extracts from frozen tumor pieces. We hypothesize that the high expression of CDK inhibitors may be a cell response to cell cycle alterations in an attempt to stop cell division. This may be the reason why the UM primary cultures showed a slow growing rate. Further, our results parallel those stating that high levels of cyclin D1 and E lead to increased p27 levels in some cell types and are associated with malignancies

such as invasive primary malignant melanomas [Bales et al., 1999] and aggressive B-cell lymphomas [Sánchez-Beato et al., 1999].

We also analyzed the checkpoint protein Rb in UM. So far, only a few investigations were aimed at the Rb pathway in UM even though altered Rb expression has been associated with a number of cancers [Lai et al., 2003]. Indeed, our results showed that in two of the four UM cell cultures analyzed, Rb was present primarily in its hypophosphorylated form. We performed a detailed study of Rb in one of these cultures (UM-A), and showed that it was active but unable to bind E2F-1, which explained why these UM cells continued to grow. We explained the presence of the hypophosphorylated form of Rb by the absence of cyclin D1/CDK4 complexes, which was likely to be due to the inhibitory action of p16. Moreover, the high levels of p27 could explain why other CDKs were unable to phosphorylate Rb even when cyclin E and A as well as CDK2 were expressed.

We proceeded to exclude the possibility that Rb was mutated causing its function to be altered, as was the case in several tumors including retinoblastomas, osteosarcomas, SCLC, renal cell carcinomas, and bladder carcinomas [Friend et al., 1986; Harbour et al., 1988; Cordon-Cardo et al., 1997]. However, we could not detect any mutation in the coding sequence for RB1, which could explain the lack of binding to E2F-1. Previous histopathological studies have shown that Rb was infrequently mutated in UM, but suggest a functional inactivation somewhere along its pathway [Brantley and Harbour, 2000a,b]. Brantley and Harbour [2000b] have shown that Rb is frequently inactivated in UM by phosphorylation of sites in the C-terminal region of the protein by cyclin/CDK complexes. This kind of inactivation can be ruled out in UM-A primary culture since Rb was present predominantly in its hypophosphorylated form, but it could explain the tumorogenesis of other cultures shown in our study.

Recently, Svensson et al. [2003] described an invasive phenotype in basal cell carcinoma, which is characterized by decreased phosphorylation of Rb and high expression of $p16^{INK4A}$. Further analysis is required to study a possible role of Rb and p16 in the dissemination of UM.

Another possible explanation for the lack of binding of E2F-1 to Rb could be that E2F-1 was affected by some mutation. However, alterations in genes coding for the E2F family of transcription factors is infrequent [Mundle and Saberwal, 2003] and indeed, no mutations were found in the Rb binding site of E2F-1 in UM-A.

Rb Binding Proteins and their Potential Role in UM

We have used proteomics technology [Gronborg et al., 2002] to characterize Rb binding proteins that could possibly explain the lack of function of Rb in UM-A, such as Mdm2, which is able to inhibit the growth suppression at G1 imposed by p53 and Rb by binding to them [Xiao et al., 1995; Chen et al., 1996a]. To date, several proteins have been reported to associate with Rb, suggesting its involvement in a plethora of functions including cell proliferation, inhibition of apoptosis, and promotion of cell differentiation [Morris and Dyson, 2001]. Mass spectrometry-based analysis of bands from SDS–PAGE gels that were specifically observed in Rb immunoprecipitated samples resulted in the identification of several proteins binding to Rb in UM-A (Fig. 5), some of which had been reported previously to bind to the hypophosphorylated form of Rb. This is consistent with our results obtained by Western blot analysis, which show that the main form of Rb in UM-A is the hypophosphorylated form.

One of the Rb binding proteins identified in UM-A was lamin A/C from the nuclear lamina. This protein has been shown to bind hypophosphorylated Rb in different cell types. The significance of this interaction is still unclear, but it has been suggested to be implicated in cell cycle regulation [Mancini et al., 1994; Ozaki et al., 1994].

On the other hand, it was interesting to find Hsc70 binding to Rb in UM, since proteins from the same family (Hsc73 and Hsp75) have been found to bind Rb preferentially in its hypophosphorylated form [Nihei et al., 1993; Chen et al., 1996b]. It has been suggested that these proteins could fold Rb into an active conformation. As the expression of heat shock proteins is of known prognostic significance in several tumor types, and as these proteins were reported to be also present in UM [Missotten et al., 2003], we are now considering their role in this neoplasia.

Another protein we found to bind Rb in UM-A was HMG-1, a protein that has been related to oncogenesis in many human cancers [Breznniceanu et al., 2003; Choi et al., 2003]. A protein belonging to the same family, HBP1, has also

been shown to bind Rb, but its role is still unknown [Dintilhac and Bernues, 2002].

We also identified proteins that are reported here for the first time to co-immunoprecipitate with Rb, and their roles when associated with this oncoprotein are not known. These proteins are hnRNP, G3PDH, and EF-1/2. They have been implicated in various other oncogenic processes [Rondinelli et al., 1997; Alaiya et al., 2000; Anand et al., 2002; Tominaga et al., 2003] but their interactions with Rb and potential implications in UM have to be further analyzed.

In conclusion, we described a deregulation of cyclin D1, cyclin E, $p16^{INK4A}$, $p27^{KIP1}$, and the Rb/E2F pathway in human UM primary cell cultures. We hypothesized that the overexpression of cyclin D1, E, p16, and p27 together with the inactivation of Rb by other proteins might participate in the oncogenesis and dissemination of UM tumors. Our results and further investigations in Rb and Rb binding proteins should provide new insights into the mechanisms underlying the molecular changes leading to the transformation of normal uveal melanocytes.

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