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SUPPRESSION OF CYCLIN D1 BUT NOT CDK4 OR CYCLIN A WITH INDUCTION OF MELANOMA TERMINAL DIFFERENTIATION

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To identify cyclins specifically associated with control of melanoma cell proliferation, we now compared expression of cyclin A, reported to be a marker for hematological malignancies, with that of cyclin D and its cdk4 kinase partner. All these proteins were expressed in proliferating B16 melanoma. However, L-tyrosine which induces melanoma terminal differentiation, selectively decreased cyclin D with no comparable effect on cdk4 or cyclin A. A 2-hour exposure of the cells to the tyrosine phosphatase inhibitor, sodium vanadate, further decreased cyclin D from differentiated cells, suggesting that tyrosine phosphorylation regulates cyclin D turnover. Addition of serum to starved cells also revealed that tyrosine did not block the early cyclin D increase associated with serum stimulation, but accelerated its subsequent loss. Our data suggest that cyclin D decrease with melanoma terminal differentiation could be an alternative mode of growth arrest even in cells harbouring a mutant or transcriptionally silent cdk4 inhibitor tumor suppressor p16^{Ink4} gene. These results also imply that cyclin D may be useful as a target and as a prognostic marker in melanoma therapy.

Sequential induction and down regulation of cyclins is known to occur during cell cycle progression, but this regulation may be lost with cancer (1,2). In normal cells, cyclin D1 interacts with cdk4, is up regulated early concurrent with growth factor stimulation and subsequently peaks by mid G1 and interacts with its cdk4 partner, usually decreasing as cells approach S phase (3). In contrast, cyclin A increases in late G1 and activates cdk2 from the onset of S phase (4). However, tumor cells exhibit an aberrant control of cyclins, in which their turnover, timing and degree of expression may be altered (5). This is exemplified by cyclin A which is down regulated by serum starvation only in normal cells (4). In contrast there is a loss in cyclin A down regulation

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under comparable conditions during tumor progression in Chinese hamster fibroblasts (6) and terminally differentiated melanoma (7). On the other hand, cyclin A has been postulated as a marker of cell proliferation in hematological malignancies (8). Since our laboratory developed a model system to induce non-toxic cell-specific terminal differentiation with L-tyrosine in B16 melanoma (7), we now used such system to investigate whether cyclin D or cdk4 are adequate markers for suppression of melanoma cell growth. Our data shows that in contrast to the behavior of cyclin A as a marker for unequal cell proliferation in hematological cancers (8), no such behavior is found for melanoma which show constitutive expression of both cyclin A and cdk4, the kinase usually activated by cyclin D (3). However, the turnover of the latter was found to be accelerated with induction of terminal melanogenesis (7).

MATERIALS AND METHODS

Tissue culture

a) Normal melanocytes, Melan A, from C57/BL mice, dependent on Phorbol esters and 10% serum to grow (9) were kindly provided by Dr. Dorothy C. Bennett, St. George 's Hospital Medical School, London, England.

b) B16 melanoma BL6 cells, also from C57/BL mice, originally obtained from Dr. I.R. Hart (10), were further selected in our laboratory for optimal induction of terminal differentiation with L-tyrosine (7,9). These cells were exposed for 1-2 days to 2 mM L-tyrosine either in complete serum-supplemented medium or in serum-free medium, whenever indicated.

Immune blots

Cells were lysed on ice in buffer L (50 mM Tris-HCl pH 7.4; 150 mM NaCl, 1% Nonidet P40, 0.5% Na deoxycholate; 0.1% SDS, 5 mM EDTA, 50 mM NaF; 0.1 mM Na vanadate, 1 mM PMSF, 10 micrograms per ml each of aprotinin, leupeptin and soybean trypsin inhibitor).

Extracts were spun at 13,000 X g for 15 min, and protein supernatants (150 micrograms as estimated by the Pierce micro BCA reagent) were used for 10 to 12% SDS-PAGE electrophoresis, followed by bi-directional transfer to nitrocellulose (7) and reaction with antibody to cyclin A (4), cdk4 (11) and cyclin D (3). These antibodies were kindly provided by Dr. M. Pagano, Mitotix, Boston. Subsequently, blots were reacted with protein A-peroxidase and reactions evidenced by ECL (Amersham).

RESULTS

Induction of melanoma cell differentiation preferentially accelerates loss of Cyclin D

G1-enriched populations of B16 melanoma were obtained as previously described (7) by a 48 hour exposure of sparse cultures to serum-free medium.

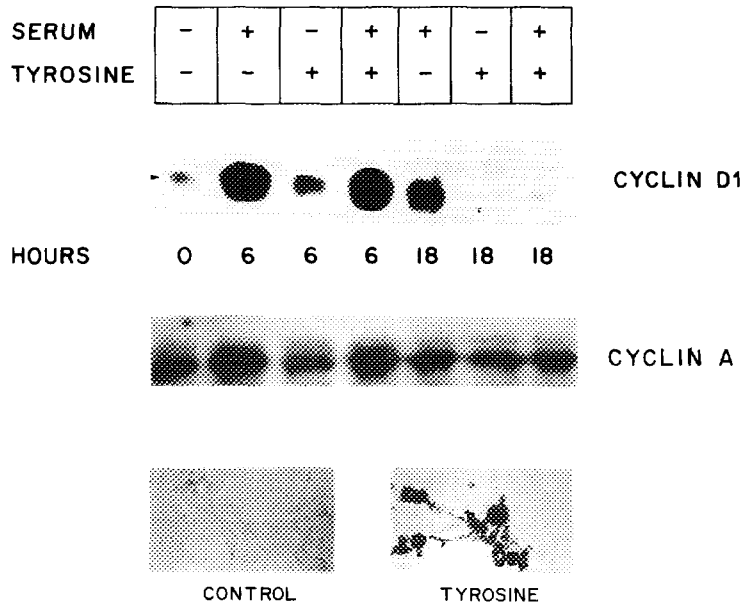

Figure 1. Tyrosine induces melanoma cell differentiation and increases loss of cyclin D in late G1/S.

Upper. Sparse cultures were serum-starved for 48 hours followed by stimulation with 10% serum and/or 2 mM L-tyrosine for 6 or 18 hours. Extracts were harvested at the indicated time intervals for detection of cyclin D or cyclin A by immune blotting.
Lower. Light microscopy of control cells exposed for 18 hours to 10% serum and tyrosine, cells treated in parallel with 10% serum and tyrosine.

Cell extracts were prepared from serum-starved cells and from those exposed to 10% serum and/or 2 mM L-tyrosine whenever indicated, for immune blotting with antibody to cyclin D or to cyclin A. This revealed lower levels of cyclin D than those of cyclin A in serum-starved cells (Fig. 1). Within 6 hours of serum stimulation, cyclin D and cyclin A were increased even in cells jointly exposed to 10% serum and L-tyrosine, although this was less evident in cells exposed only to L-tyrosine. Parallel experiments showed that these serum-starved cells increase DNA synthesis by 9-12 hours reaching a 7-10 fold maximal activation of S phase by 18-24 hours after addition of 10% serum (7)(not shown). In agreement with others who demonstrated that cyclin D peaks and then decreases after mid G1 (3,11), we observed a decrease in cyclin D protein by 18 hours after stimulation with 10% serum coinciding with maximal DNA synthesis (4,7). However, this decrease which was estimated to be about 50% (4,7) to that seen by 6 hours of

serum stimulation, was further lowered to less than 10% in the same cells treated for 18 hours with 10% serum plus L-tyrosine. No comparable change was observed in the same experiment with cyclin A. These changes in cyclin D by 18 hours of tyrosine treatment, were paralleled by increased pigmentation (Fig. 1, lower). Induction of differentiation did not affect total thymidine uptake but decreased *by 6 fold its incorporation into DNA (not shown).*

Cyclin D but not cdk4 is decreased with induction of melanoma terminal differentiation

We also used asynchronous cell cultures to investigate whether a 36 hour exposure to L-tyrosine affected the cyclin D-cdk4 complex, known to drive the early G1 transition of the cell cycle (11). In parallel with increased pigmentation (7), cyclin D levels were decreased about 5 fold in cells treated with L-tyrosine (Fig. 2, left). However, this decrease was even more significant (about 10 fold), when both control and cells treated with tyrosine for 36 hours were exposed for 2 hours prior to harvesting (Fig. 2, right) to 0.1 mM sodium vanadate, a tyrosine phosphatase inhibitor (12). This suggests that the known modification of cyclin D in tyrosine residues (13), favours its turnover only in differentiating cells (Fig. 2, right). In contrast, no comparable differentiation-associated effect became evident in the replica blot obtained by bi-directional parallel transfer (7), when reacted with antibody to cdk4.

Cyclin D1 is decreased in asynchronous melanocytes compared to their melanoma counterparts

Comparative cyclin D expression in normal Melan A melanocytes (9) and their B16 melanoma counterparts (10) was carried out with asynchronous cultures harvested at an intermediate cell density. This revealed clearly higher levels of cyclin D in B16 melanoma compared to their normal melanocyte counterparts, in contrast to comparable levels of upper migrating tyrosinase in both cell types (Fig 3).

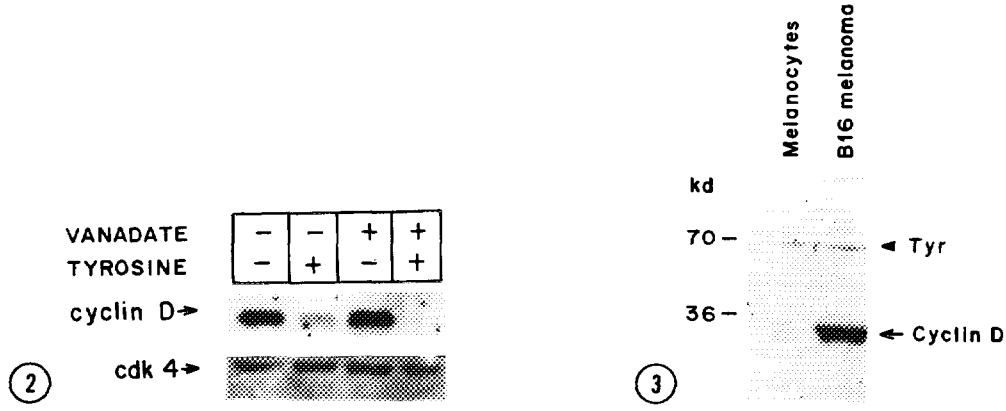

Figure 2. Cyclin D decrease in asynchronous differentiating B16 melanoma is favoured by prior treatment with vanadate.

Cyclin D and cdk4 were detected by immune blotting in sparse cultures exposed to 10% serum including 2 mM L-tyrosine for 36 hours and to 0.1 mM sodium vanadate for 2 hours prior harvesting, whenever indicated.

Figure 3. Cyclin D is decreased in melanocytes compared to melanoma.

100 µg protein from normal Melan A melanocytes and B16 melanoma were analyzed by immune blotting for cyclin D levels.

Note decrease in cyclin D in melanocytes subsequently assayed for tyrosinase protein, shown at similar levels in both cell types.

DISCUSSION

Cyclin D is known to function in the G1 phase of the cell cycle by regulating the kinase activity of cdk4, the enzyme believed to phosphorylate the Retinoblastoma protein, crucial for cell cycle progression and control (3,14). As a follow-up of our studies in B16 melanoma in which we showed that terminal differentiation decreases cdk2 which acts upstream of cdk4 (7,11), we now demonstrate that the cyclin D activator rather than the cdk4 catalyst is a target for terminal melanogenesis at an earlier stage of the cell cycle. This is of interest for various reasons: 1) syngeneic melanocytes express low levels of cyclin D protein compared to their melanoma counterparts suggesting that cyclin D over expression favours melanomas (Fig 3); 2) even in melanomas in which the p16 cyclin D/cdk4 inhibitor is mutated/deleted (15,16) or becomes transcriptionally silent (17), L-tyrosine induction of terminal differentiation may stop growth by decreasing cyclin D expression thereby preventing the activation of cdk4 (18). Although other mechanisms are not excluded, we believe that induction of differentiation partly accelerates the turnover of cyclin D and thereby slows cell cycle progression, to allow cell type specific gene expression

rather than that of genes involved with continuous cell proliferation. Another important facet of this work is that it implies that cyclin D rather than cyclin A expression correlates with control of melanoma cell proliferation since the latter is not comparably down-regulated with terminal differentiation, in contrast to cyclin D. These results differ from other reports indicating a general cyclin over-expression in breast cancer cells (5), and cyclin A as a marker for cell proliferation in hematological cancers (8), implying that different tumors types may preferentially overexpress different cyclin types. Our data suggests that cyclin D down regulation is an important target for melanoma therapy and prognosis follow-up, even in melanomas with genetically inactivated (15,16) or epigenetically silent (17) potential tumor suppressor $p16^{Ink4}$ *genes.*

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