The Low Molecular Weight Cyclin E Isoforms Augment Angiogenesis and Metastasis of Human Melanoma Cells *In vivo*

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**Abstract**

Immunohistochemical analysis has consistently shown that cyclin E is up-regulated in human malignant melanomas *in vivo*. Here we analyzed such expression in more detail and show that cyclin E is overexpressed and present in low molecular weight (LMW) isoforms in metastatic melanoma and in a subset of primary invasive melanoma tumor tissues, but not in benign nevi. Human metastatic melanoma cell lines, but not normal melanocytes, also expressed the LMW cyclin E forms. The biological significance of these findings was established by showing that overexpression of two LMW cyclin E forms named cyclin E truncated 1 [cyclinE(T1)] and cyclin E truncated 2 [cyclinE(T2)] in a low tumorigenic and non-metastatic primary cutaneous melanoma cell line generated angiogenic tumors with prominent perineural invasion compared with full-length cyclin E. In addition, cyclin E(T1)– and cyclin E(T2)–expressing melanoma cells displayed a dramatic increase in the incidence and number of metastases in an experimental lung metastasis assay. Together, these results indicate that the LMW cyclin E forms are functional and likely act as regulators of human melanoma tumor progression and invasion. (Cancer Res 2005; 65(3): 692-7)

**Introduction**

Melanoma tumors display highly deregulated and poorly characterized cell cycles (reviewed in ref. 1). The cyclin-dependent kinases CDK2 and CDK6 and the cyclins D1, E, and D are consistently overexpressed in metastatic melanomas compared with nevus tissue (2–5). Overexpression of cyclin D1 is mostly prevalent in acral melanomas and seems to result from both amplification and non-amplification mechanisms (4). The mechanism by which cyclin E is overexpressed in melanoma tumors has yet to be determined. In breast tumor tissues, overexpression of cyclin E and its low molecular weight (LMW) forms is a prognostic factor for poor patient outcome (6, 7). The LMW cyclin E forms are NH2-terminus proteolytic products of the elastase class of serine proteases (8). Importantly, these forms are biologically hyperactive in breast cancer, induce genomic instability, and promote resistance to p21Waf1/Cip1, p27Kip1, and antiestrogens (9). Here we show that metastatic melanoma cells, tissues, and a subset of primary melanomas also express the LMW cyclin E forms. We show that the low tumorigenic and non-metastatic SB2 primary melanoma cells expressing cyclin E truncated 1 [cyclinE(T1)] or cyclin E truncated 2 [cyclinE(T2)] generated tumors in immunocompromised mice displaying increased microvessel density, low or negligible apoptosis, and increased metastases compared with full-length cyclin E or an empty vector. These findings suggest that the cyclin E forms are biologically active in melanoma and act as potential regulators of invasion and metastasis.

**Materials and Methods**

**Patient Material.** This study is based on benign nevi including dermal nevus, compound nevocellular nevus, congenital dermal nevocellular nevus, junctional dysplastic nevocellular nevus, superficial spreading malignant melanomas (SSMM), and melanoma metastases derived from the frozen melanoma bank at the University Hospitals Leuven, Katholieke Universiteit Leuven, Belgium.

**Cell Lines, Culture Conditions, and Transfections.** Normal melanocytes (10) and melanoma cell lines were cultured as described previously (11). The expression vectors for cyclin E and the LMW forms carried either a FLAG tag in the C terminus (9) or a Myc tag in the N terminus.

**Western Blots.** Levels of full-length cyclin E, its LMW isoforms, CDK2, proliferating cell nuclear antigen, and β-actin were analyzed by Western blot analysis of lysates prepared from specimens of frozen tumor tissue as previously described for breast tissues (12).

**Gel-Filtration Chromatography.** Nuclear extracts were isolated and fractionated by size-exclusion chromatography using SEC-450 columns [high-performance liquid chromatography (HPLC), Biorad HR, Bio-Rad, Hercules, CA] as described previously (13).

**Animals.** Male athymic BALB/c nude mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

**In vivo Tumor Growth and Metastasis.** To prepare tumor cells for inoculation, cells in exponential growth phase were harvested, washed, and resuspended in Ca2+/Mg2+-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion and only single-cell suspensions of >90% viability were used. S.c. tumors were produced by injection of 1 × 106 tumor cells/0.2 mL HBSS over the right scapular region. Growth of s.c. tumors was monitored by weekly examination of the mice and measurement of tumors with calipers. For experimental lung metastasis, 1 × 105 tumor cells/0.2 mL HBSS were injected into the lateral tail vein of nude mice. The mice were killed after 60 days, and the lungs were removed, washed in water, and fixed with Bouin’s solution for 24 hours to facilitate counting of tumor nodules under a dissecting microscope as described previously (14). Sections of the lungs were stained with H&E to confirm that the nodules were melanoma and to monitor the presence of micrometastases.
**In situ TUNEL Assay.** Tissues were fixed in 10% buffered formalin solution and then embedded in paraffin. Thin sections (4 μm) were prepared, and the terminal deoxynucleotidyl transferase–mediated dUTP end labeling (TUNEL) assay was done using a commercial kit (Promega, Madison, WI) according to the protocol of the manufacturer as described previously (15). Results are presented as the mean percentage ± SD of apoptotic cells from the total number of cells counted in 8 fields/slide.

**Statistical Analysis.** The in vitro data were analyzed for significance by using Student’s t test (two-tailed), and the in vivo data were analyzed by using the Mann-Whitney U test.

**Results and Discussion**

**Processing of Cyclin E into Low Molecular Weight Isoforms in Human Melanoma Tissues.** A Western blot analysis of cyclin E expression showed that three of five melanoma cell lines (Mel888, DM4, and IIB-Mel-J) expressed high levels of both full-length and LMW cyclin E isoforms (Fig. 1A, lanes 3–5), whereas the other two cell lines (A375 and UCD-Mel-N) expressed high levels of the full-length but low levels of the LMW forms (Fig. 1A1, lanes 1 and 6). In contrast, normal human melanocytes (Fig. 1A, lanes 7–9) and the primary melanoma cell line SB-2 (Fig. 1A, lane 2) displayed substantially lower levels of full-length cyclin E and negligible amounts of the LMW forms. To determine whether expression of the LMW cyclin E forms can also be observed in melanoma tumors in vivo, we analyzed by Western blotting the protein extracts from frozen metastatic melanoma specimens as well as primary invasive melanoma and nevus lesions. Of 13 metastatic melanomas, 10 displayed full-length (Mr 50,000) and LMW cyclin E isoforms (Mr 45,000, 44,000, and 35,000; Fig. 1B). Some of these tumors also displayed high levels of CDK2. Confirming previous results (2), 8 out of 13 tumors also displayed high levels of p27Kip1. Analysis of a small sample of eight invasive primary SSMM showed that two also expressed the LMW cyclin E forms (Fig. 1C, specimens 3 and 4), whereas four of eight displayed high levels of CDK2. Importantly, whereas one SSMM showed faint expression of cyclin E (Fig. 1C, specimen 5), a regional lymph node of the same patient showed high levels of the full-length cyclin E (Fig. 1C, lane 6). Metastatic melanomas from Fig. 1B5 (lanes 5 and 8) were used as positive controls (Fig. 1C, MM). CDK2 was expressed in five of seven primary melanomas (Fig. 1C, middle). In turn, levels of the proliferating cell nuclear antigen in three SSMM tumors correlated with cyclin E and CDK2 levels (Fig. 1C). It is important to mention that both β-actin and tubulin were below detection levels in primary melanoma and nevus specimens (Fig. 1C and D). These results are in agreement with previous findings showing that cytoskeletal elements are weakly or not expressed in primary melanoma tumor cells or benign nevi, whereas metastatic melanoma cells show substantial increase in such proteins (16).

Therefore, unspecific bands of which expression was recognized by the p27 antibody (asterisks) were used as surrogates for protein...
loading. Augmentation of cyclin E levels, or processing to the LMW forms, was not found in eight nevus lesions ranging from dermal nevus, compound nevocellular nevus, congenital nevus, and a junctional dysplastic nevocellular nevus (Fig. 1D). These results indicate that melanoma tumors also show the processing of cyclin E, which has been previously described in breast cancer cells and tissues (6).

The Low Molecular Weight Forms of Cyclin E Associate with CDK2 and Display Substantially Reduced p21Waf-1 Binding. To determine the biological significance of the LMW cyclin E forms in melanomas, we transfected the low tumorigenic and non-metastatic SB2 melanoma cells with constructs previously used to determine the function of the LMW cyclin E forms in breast cancer cell lines (6). We chose the SB2 cell line for transfection with full-length cyclin E and LMW cyclin E constructs, as these cells display low levels of full-length cyclin E and undetectable levels of the LMW forms (Fig. 1A, lane 2). The constructs used were cyclin E full-length Myc (EL) and the NH2-terminal truncated forms Trunk1-Myc (T1) and Trunk2-Myc (T2). The EL construct codes for the full-length Mr 50,000 form of cyclin E (termed EL1), the T1 construct for the Mr 45,000 and Mr 44,000 forms, and the T2 construct codes for the Mr 35,000 and Mr 33,000 forms. The cyclin E(T1) protein initiates at amino acid 40 and cyclin E(T2) at amino acid 65 (8, 17). The percentage of transfected cells ranged between 50% and 60% as estimated in parallel transfections with the green fluorescent protein (data not shown). The vectors carried the selection marker geneticin that allowed selection for pure populations of melanoma cells within 8 days. Several clones were selected and expanded for an additional 2 weeks, then analyzed for expression of the transgenes by Western blot (Fig. 2A). At least three independent clones/construct were immediately used for kinase assays, cell cycle distribution, and growth curves. Overexpression of cyclin E(T1) and cyclin E(T2) in epithelial breast cells increases their ability to enter S and G2-M phase by 2-fold over full-length or vector-only transfected cells (18). In contrast, overexpression of cyclin EL, cyclin E(T1), and cyclin E(T2) in SB2 melanoma cells did not result in appreciable changes in cell cycle distribution or growth potential (data not shown).

However, immunoprecipitation with a Myc antibody showed that the T1 and especially the T2 form displayed significantly reduced levels of p21Waf-1 compared with full-length cyclin E (Fig. 2A). CDK2 and p27Kip-1 levels were virtually identical in all clones. Decreased p21 association with CDK2/cyclin E complexes correlated with increased histone H1 kinase activity (Fig. 2B). To validate the results obtained with transfected constructs, we used HPLC fractionation of protein extracts derived from the UCD-Mel-N cell line. At least two fractions of > 120 to 150 kDa showed that the LMW forms of cyclin E did not contain the full-length cyclin E protein (Fig. 2C, fractions 27 and 29). CDK2 and p27kip-1 levels were virtually identical in all clones. Decreased p21 association with CDK2/cyclin E complexes correlated with increased histone H1 kinase activity (Fig. 2B). To validate the results obtained with transfected constructs, we used HPLC fractionation of protein extracts derived from the UCD-Mel-N cell line. At least two fractions of > 120 to 150 kDa showed that the LMW forms of cyclin E did not contain the full-length cyclin E protein (Fig. 2C, fractions 27 and 29). CDK2 and p27kip-1 levels were virtually identical in all clones. Decreased p21 association with CDK2/cyclin E complexes correlated with increased histone H1 kinase activity (Fig. 2B). To validate the results obtained with transfected constructs, we used HPLC fractionation of protein extracts derived from the UCD-Mel-N cell line. At least two fractions of > 120 to 150 kDa showed that the LMW forms of cyclin E did not contain the full-length cyclin E protein (Fig. 2C, fractions 27 and 29). CDK2 and p27kip-1 levels were virtually identical in all clones. Decreased p21 association with CDK2/cyclin E complexes correlated with increased histone H1 kinase activity (Fig. 2B). To validate the results obtained with transfected constructs, we used HPLC fractionation of protein extracts derived from the UCD-Mel-N cell line. At least two fractions of > 120 to 150 kDa showed that the LMW forms of cyclin E did not contain the full-length cyclin E protein (Fig. 2C, fractions 27 and 29). CDK2 and p27kip-1 levels were virtually identical in all clones. Decreased p21 association with CDK2/cyclin E complexes correlated with increased histone H1 kinase activity (Fig. 2B). To validate the results obtained with transfected constructs, we used HPLC fractionation of protein extracts derived from the UCD-Mel-N cell line. At least two fractions of > 120 to 150 kDa showed that the LMW forms of cyclin E did not contain the full-length cyclin E protein (Fig. 2C, fractions 27 and 29). CDK2 and p27kip-1 levels were virtually identical in all clones.
showed that fraction 27 did not exhibit kinase activity, whereas fraction 29, which contains both full-length cyclin E and its LMW forms, displayed substantial histone H1 kinase activity. Thus, the LMW forms of cyclin E seem to form different protein complexes in melanoma compared with breast cancer cells.

The Low Molecular Weight Cyclin E Forms Generated Vascularized Tumors in Mouse Xenografts. Because the in vitro analysis did not take into account the interaction of the melanoma cells with the tumor microenvironment, we injected cyclin EL-, cyclin E(T1)-, and cyclin E(T2)-expressing clones and empty vector control cells into nude mice, either orthotopically (s.c.) or through the tail vain (experimental lung metastasis assay). Growth of s.c. tumors was monitored by daily examination of the mice and weekly measurements with calipers. Figure 3 shows one set of data obtained with individual clones using six animals per group. Identical results were obtained in three independent experiments using additional clones expressing T1, T2, E1, and pcDNA (data not shown).

**Table 1.** Tumor microvessel density and apoptosis (TUNEL) in s.c. melanoma tumors

<table>
<thead>
<tr>
<th>SB2 clones</th>
<th>CD31</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA</td>
<td>20 ± 1.3</td>
<td>32 ± 3.6</td>
</tr>
<tr>
<td>Cyclin EL</td>
<td>30 ± 2.7</td>
<td>34 ± 8.2*</td>
</tr>
<tr>
<td>Cyclin E(T1)</td>
<td>41 ± 2.7</td>
<td>24 ± 2.7*</td>
</tr>
<tr>
<td>Cyclin E(T2)</td>
<td>38 ± 1.4</td>
<td>6 ± 6*</td>
</tr>
</tbody>
</table>

**NOTE:** The number of independent clones was *n* = 3 for vector, *n* = 2 for cyclin E(EL), *n* = 3 for cyclin E(T1), and *n* = 3 for cyclin E(T2). Results are presented as mean percentage ± SD from three independent experiments.

*A* *P* < 0.05, compared with empty vector cells.
experiments using two to three clones from each construct (data not shown). By 24 days, control and cyclin EL-expressing cells produced tumors of 20 and 28 mm³ in mean volume, respectively (Fig. 3A). In contrast, cyclin E(T1) and cyclin E(T2) produced tumors with a mean volume of 150 and 300 mm³, respectively. This indicates that transfection of the individual LMW forms enhanced tumor growth in vivo. More interestingly, the pathologic assessment of cyclin EL, cyclin E(T1), cyclin E(T2), and pcDNA tumors showed striking differences in cell morphology, vascularity, apoptosis, and perineural invasion (Fig. 3B). Cyclin E(T1) tumors consisted of tightly packed, small epithelioid tumor cells with scant cytoplasm. These tumors displayed high mitotic activity (mean, 54 mitoses/mm²), prominent vascularity, scattered apoptotic cells, and no apparent necrosis. Cyclin E(T2) tumors displayed larger epithelioid tumor cells with more abundant cytoplasm and less frequent mitoses than cyclin E(T1) tumors (22 mitoses/mm²); and in addition contained abundant inflammatory cells, scattered apoptotic cells, and large areas of necrosis and prominent vascularity (T2a). Importantly, T2 tumors displayed prominent perineural invasion (Fig 3B), which is a feature of aggressive human melanoma tumors. In turn, cyclin EL tumors displayed similar morphology and mitotic rate (28 mitoses/mm²) as cyclin E(T2) tumors, but reduced vascularity and perineural invasion as well as more abundant apoptotic cells and larger zones of necrosis. Finally, control (pcDNA) tumors displayed morphology, vascularity, apoptotic rate, and zone of necrosis similar to EL tumors. However, control tumors had lower mitotic rates (14 mitoses/mm²) than cyclin E(T2) and cyclin E(EL) tumors, and no observable perineural invasion. To confirm observations made on H&E slides, we determined tumor microvessel density and apoptosis when tumors reached ~2 mm in diameter. Cyclin E(T1) and cyclin E(T2) tumors were highly vascularized, whereas tumors formed by EL and neo-control cells had a significantly decreased vascular density (P < 0.05; Fig. 3C and Table 1). Importantly, pcDNA and EL tumors had large areas of cells undergoing apoptosis (Fig. 3C, arrows), compared with T1, whereas T2 had almost no evidence of apoptosis (P < 0.05). Intriguingly, although cyclin E(T1) and cyclin E(T2) tumors displayed similar levels of vascularity as detected by the CD31 antibody, T2 tumors displayed significantly less apoptosis than T1 (Table 1).

Increased Metastatic Potential by SB2 Cells Expressing Cyclin E(T1) and Cyclin E(T2). We also analyzed the metastatic potential of cyclin E transfectants (EL, T1, and T2) using an experimental lung metastasis assay (19, 20). This assay is the only reliable method to assess the metastatic potential of human melanomas, as spontaneous metastases of these cells are exceedingly rare in BALB/c nude mice (20, 21). SB2 cells are non-metastatic in nude mice and cells transfected with a pcDNA control vector also did not produce metastases. Cyclin EL-expressing cells showed relatively low incidence and number of metastases. In contrast, the LMW forms T1 and especially T2 promoted a striking metastatic growth (Table 2).

In summary, our studies found unexpected activities of the proteolytic fragments of cyclin E in vivo. Increased mitosis and angiogenesis of cyclin E(T1) and cyclin E(T2) tumors may result from direct and indirect effects, including increasing the cyclin E protein dosage of tumor cells and differentially affecting the cellular metabolism. The latter could result as a consequence of targeting different substrates or by inappropriate expression in the cell cycle. It might also arise as a consequence of the abrogation of amino acid sequences which serve as the substrate for specific kinases. For example, amino acids 25 to 28 contain the casein kinase 1 consensus sequence SARS, whereas amino acids 15 to 18 contain the casein kinase 2 consensus sequence TMKE, and amino acids 36 to 39 the forkhead-associated domain protein. The latter being directly involved in signal transduction, cell cycle control, DNA repair, and protein–protein interactions. Whether these or other sequences are important for the activity of the LMW forms of cyclin E remains to be determined. Finally, recent data obtained in cyclin E1 and cyclin E2 knockout mice (22) indicate that cyclin E is dispensable for proliferation of normal cells. However, cyclin E seems to be critical for oncogenesis and tumor progression of breast and other human cancers (reviewed in refs. 17, 23). Our data suggest that the LMW cyclin E isoforms may be important in these processes.

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Table 2. Metastatic potential of SB2 human melanoma cells transfected with cyclin EL, cyclin T1, cyclin T2, and control (pcDNA) vectors

<table>
<thead>
<tr>
<th>SB2 clones</th>
<th>Median (no. metastases)</th>
<th>Range (no. metastases)</th>
<th>Incidence (no. mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA</td>
<td>0</td>
<td>0</td>
<td>0/10</td>
</tr>
<tr>
<td>Cyclin EL</td>
<td>1</td>
<td>0-1</td>
<td>4/10</td>
</tr>
<tr>
<td>Cyclin E(T1)</td>
<td>5</td>
<td>3-9</td>
<td>10/10</td>
</tr>
<tr>
<td>Cyclin E(T2)</td>
<td>10</td>
<td>7-14</td>
<td>10/10</td>
</tr>
</tbody>
</table>

NOTE: Ten mice per group were injected i.v. with 1 × 10⁶ SB2 cells overexpressing cyclin EL, cyclin E(T1), cyclin E(T2), and an empty vector (pcDNA). Sixty days later, the animals were euthanized. The lungs were removed, washed, and fixed in Bouin’s solution to differentiate the neoplastic lesions from the organ parenchyma. The lung nodules were counted with the aid of a dissecting microscope. The significance of the in vivo metastasis results was determined by the Mann-Whitney U test.

*Represents number of lung colonies.

References

6. Harwell RM, Porter DC, Dames C, Keyomarsi K.

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