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Reduced cyclin D1 ubiquitination in UVB-induced murine squamous cell carcinomas

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Abstract

Ubiquitination of cyclin D1 signals for its proteosomal degradation. To assess the possibility that reduced cyclin D1 proteolysis is a putative mechanism for its accumulation during UVB-induced skin tumorigenesis, ubiquitination activity of cyclin D1 was assessed in UVB-induced murine SCCs. Cyclin D1 was rapidly ubiquitinated by control skin extract, whereas ubiquitination of cyclin D1 was significantly reduced in SCCs. Mutant cyclin D1, in which residues important for GSK3β-mediated degradation of cyclin D1 are altered to non-phosphorylatable alanine, was not ubiquitinated. We also observed phosphorylation-dependent inactivation of GSK3β in SCCs. Our results indicate reduced ubiquitination of cyclin D1 in UVB-induced murine SCCs and suggest that inactivation of GSK3β-dependent cyclin D1 degradation pathway contributes to the accumulation of cyclin D1 in UVB-induced murine SCCs.

Keywords: Ubiquitination; Cyclin D1; UVB; Skin; Squamous cell carcinoma; Proteolysis; Phosphorylation; GSK3β

Cyclin D1 overexpression is known to contribute to the malignant progression of various epithelial tumors [1–6] and this cyclin cooperates with ras and adenovirus E1A to transform cultured cells [7,8]. Overexpression of cyclin D1 in normal cells results in a malignant phenotype (e.g., overexpression of cyclin D1 overrides tumor growth factor β-1-mediated antimitotic signals), whereas suppression of cyclin D1 activity in malignant cells results in disappearance of the malignant phenotype [9,10]. Transgenic mice overexpressing cyclin D1 frequently develop breast cancers [11,12]. EBV ED-L2 promoter-directed cyclin D1 expression in esophagus results in alterations in cell cycle regulation and expression of epidermal growth factor receptor and p53, leading to esophageal squamous dysplasia, a precursor of carcinoma, which increases following *N*-nitrosomethylbenzylamine treatment [3,12]. Amplification at chromosomal location 11q13 containing cyclin D1 is found in esophageal cancers [3,13,14] and cyclin D1 overexpression occurs in more than 50% of primary breast cancers, which farther increases with the grade of malignancy [5].

Cyclin D1 overexpression occurs in dysplastic skin and has been shown to correlate with the degree of solar UVB exposure [15]. In addition, cyclin D1 overexpression occurs in more than 50% of non-melanoma skin cancers [16]. Antisense cyclin D1 induces apoptosis, leading to shrinkage of human SCCs [10]. In murine skin, bovine keratin 5 promoter-driven cyclin D1 overexpression results in hyperplasia but no spontaneous tumorigenesis [8,17,18]. However, cyclin D1 null mice show reduced susceptibility to skin tumorigenesis in a two-stage carcinogenesis protocol [19]. Furthermore, cyclin D1 has been shown to have a unique role in promoting ras-initiated tumor development in skin [20]. Recently, we observed progressive enhancement of cyclin D1 expression during UVB-induced SCCs development [21]. However, the mechanism of cyclin D1 overexpression is unknown. It is possible that cyclin D1 accumulation in UVB-induced SCCs is secondary to

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impaired ubiquitination and subsequent proteosomal degradation [22]. In this study, we provide evidence that cyclin D1 degradation is impaired in UVB-induced skin carcinogenesis. Our data indicate that ubiquitination of cyclin D1 is reduced in UVB-induced SCCs and that GSK3β-dependent phosphorylation of threonine 286 residue in cyclin D1 is crucial for its proteosomal degradation.

Materials and methods

Immunohistochemical analyses. The protocol for UVB-induced photocarcinogenesis in SKH-1 hairless mice and generation and sampling of SCCs were performed as described in [21]. Strips of skin (1×0.4 cm) from both irradiated and control animals were fixed in cold formalin solution overnight at 4 °C. The sections were washed for 30 min each sequentially in $1\times$ PBS and 0.9% NaCl/alcohol solutions containing 30%, 50%, and 70% ethanol. The sections were then embedded in paraffin wax and sectioned onto slides. The embedded tissue sections were deparaffinized in xylene, rehydrated, and boiled for 5 min in 0.01 M citrate buffer, pH 6.0, to retrieve antigens. Cyclin D1 was stained with anti-cyclin D1 mouse monoclonal antibody (1:100 dilution, Santa Cruz) using Vector M.O.M immunodetection kit following manufacturer's instructions. The sections were then washed and incubated with biotinylated secondary antibody. Color reaction was observed using the ABC peroxidase detection system (Vector Lab) using 3,3′-diaminobenzidine. The sections were counterstained with Harris hematoxylin (Sigma), dehydrated through alcohol and xylene, and mounted using permount (Sigma).

Preparation of tissue lysate and Western blotting. Tissue and tumors were washed with ice-cold 1×PBS and homogenized in ice-cold lysis buffer (10 mM Tris, pH 8). The tissue homogenate was then sonicated 10 s twice on ice. Clear lysate was prepared by centrifugation at 10,000*g* for 10 min. Extracts were aliquoted in small volumes and stored at −80 °C before use. One hundred μg total extract was separated by SDS–PAGE and proteins were electrophoretically transferred to nitrocellulose membranes. Nonspecific sites on the membrane were blocked with 5% (W/V) non-fat dried milk in TTBS (0.1% Tween 20, 20 mM Tris base, 137 mM NaCl, and 3.8 mM HCl, pH 7.6) for 1 h at ambient temperature followed by overnight incubation with the primary antibody at 4 °C. After washing the blot three times in TTBS for 10 min each, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000, Jackson Labs, Maine). The blot was washed three times in TTBS for 10 min each and developed with ECL according to manufacturer's instructions (Amersham, Arlington Heights, IL).

In vitro ubiquitination assay. HA-tagged wild-type cyclin D1 or HA-tagged mutant cyclin D1 (T286A/T288A) was in vitro translated from ^{HA}cyclin D1-pCDNA3 or ^{HA}cyclin D1 (T286A/T288A)-pCDNA3 (a gift from Dr. Doris Germain, the Peter MacCallum Cancer Institute, Australia), using T_NT coupled reticulocyte lysate system following manufacturer's protocol (Promega). [^{35}S]Methionine is used to generate ^{35}S-labeled cyclin D1. Two hundred μg tissue extract was incubated with 5 μl in vitro translated cyclin D1 in ubiquitination buffer (10 mM Tris, pH 7.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$) in a total volume of 125 μl. The reaction mixture was incubated for various time points and stopped by adding 4× SDS sample buffer. The reaction mixture was boiled for 3 min followed by SDS–PAGE. For [^{35}S]methionine-labeled cyclin D1, the gel was fixed in 20%/10% methanol/acetic acid for 30 min and incubated with Amplify (Amersham) for 20 min. The gel was then dried and exposed to Kodak X-ray film to detect ^{35}S-labeled proteins.

Immunodepletion and immunoprecipitation. Two hundred μg tissue extract was incubated with the rabbit polyclonal anti-cyclin D1 antibody (20 μg, Santa Cruz) conjugated with protein A/G–agarose beads for overnight at 4 °C with rotation to remove endogenous cyclin D1. The depletion of endogenous cyclin D1 was verified by SDS–PAGE followed by Western blotting with anti-cyclin D1 antibody. Immunoprecipitation was performed using standard protocol employing anti-HA antibody [23].

Results

Cyclin D1 is overexpressed in UVB-induced SCCs

We examined the expression of cyclin D1 in UVB-induced skin SCCs using immunohistochemical studies. As shown in Fig. 1, strong staining for cyclin D1 was observed in SCCs whereas no cyclin D1 expression was detected in non-irradiated, age-matched control skin. Western blot analysis showed substantial increase in cyclin D1 level in SCCs. Cyclin D1 was detected in 68% of papillomas (13/19) and 55% of SCCs (17/31) [21].

In vitro ubiquitination of cyclin D1 is reduced in UVB-induced SCCs

We recently showed that UVB induces proteolytic degradation of cyclin D1 in A431 cells, suggesting that UVB alters proteosomal degradation of cyclin D1 in skin cells [24]. To test whether altered cyclin D1 proteolysis plays a role in cyclin D1 accumulation in UVB-induced SCCs, we analyzed cyclin D1 ubiquitination in UVB-induced SCCs in vitro. This in vitro assay utilizes protein extracts from tissues capable of performing cyclin D1 proteolysis. Ubiquitination leads to the formation of high molecular weight forms of cyclin D1 [25]. Protein extracts from either mock-irradiated age-mat-

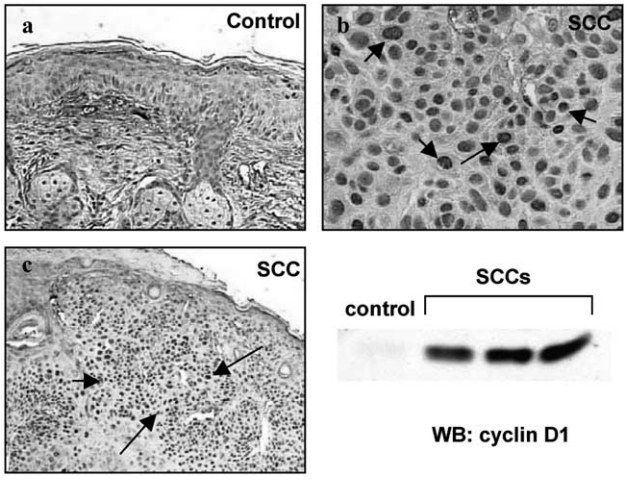

Fig. 1. Overexpression of cyclin D1 in UVB-induced SCCs. Immunohistochemical and Western blot analyses of cyclin D1 in UVB-induced SCCs. Cyclin D1 immunohistochemistry of murine skin sections from mock-irradiated control skin and SCCs are stained with anti-cyclin D1 antibody (Santa Cruz). (Magnification (a) 100×; (b) 400×; (c) 100×).

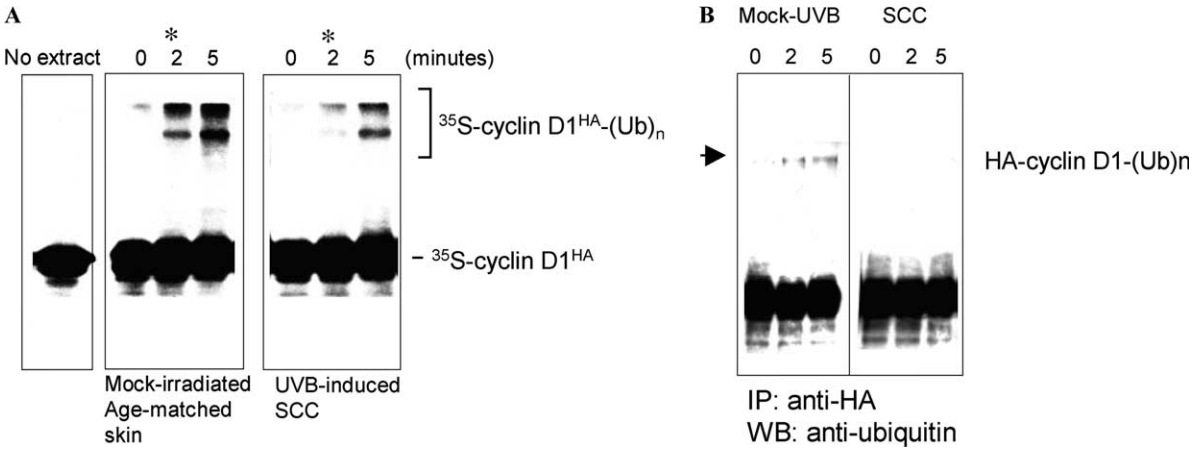

Fig. 2. Total cell lysates were prepared in the absence of both detergent and reducing agent to retain ubiquitination activity as described in [32] from UVB-induced SCCs and mock-irradiated skin. 200 mg protein of either mock-irradiated control skin or SCCs extract was incubated with 5 ml of in vitro translated 35S-cyclin D1 (A), or in vitro translated HR-cyclin D1 (B). In vitro ubiquitination assay was performed for various time points indicated. (A) Extracts were analyzed by SDS–PAGE and autoradiography. (B) Extracts were immunoprecipitated with anti-HA antibody followed by Western blotting with anti-ubiquitin antibody.

ched control skin or UVB-induced SCCs were prepared in the absence of both detergent and reducing agent to preserve ubiquitination activity. The extracts were incubated with in vitro translated [^{35}S]methionine-labeled cyclin D1 for indicated periods of time at 30 °C and the reaction was terminated by boiling the reaction mixture. The denatured proteins were separated by SDS–PAGE and exposed to an X-ray film to detect ^{35}S-labeled cyclin D1 (Fig. 2A). [^{35}S]Methionine-labeled cyclin D1 rapidly formed high molecular weight species within 2 min in the presence of the mock-irradiated skin extracts (Fig. 2A, left panel). In contrast, these high molecular weight species were significantly reduced in extracts prepared from UVB-induced SCCs (Fig. 2A, right panel). Next we assessed whether these high molecular weight species represent ubiquitinated cyclin D1. For this experiment, following termination of the ubiquitination reaction, cyclin D1^{HA} was immunoprecipitated using anti-HA antibody (Santa Cruz) and developed by Western blotting with anti-ubiquitin antibody (Sigma) (Fig. 2B). Cyclin D1^{HA} in mock-irradiated control skin extract formed high molecular weight species within 2 min, which was not detectable in UVB-induced SCCs extracts. This indicates that the high molecular weight band contains cyclin D1^{HA} and further confirms the fact that cyclin D1^{HA} is ubiquitinated only by mock-irradiated control skin extracts. These results suggest that the cyclin D1 ubiquitination activity is significantly diminished in UVB-induced SCCs.

Mutant cyclin D1 (T286A/T288A) is not ubiquitinated

Ubiquitin-mediated degradation of cyclin D1 is known to involve activation of the PI3K–Akt–GSK3β pathway. It has been suggested that phosphorylation of threonine 286 in murine cyclin D1 (or threonines 286 and 288 in human cyclin D1) by GSK3β is required for its ubiquitination and degradation [23]. It was shown that the half-life of mutant cyclin D1 (T286A/T288A) protein was higher than the wild-type cyclin D1 supporting the importance of these residues for its stability [23]. To determine whether phosphorylation of threonine 286 is essential for cyclin D1 degradation in skin, a plasmid encoding a non-phosphorylatable human cyclin D1 mutant in which two threonines at residues 286 and 288 are altered to non-phosphorylatable alanines, as well as wild-type cyclin D1, was used in our in vitro ubiquitination assay as shown in Fig. 3. To exclude any interference by endogenous cyclin D1 present in the extract, mock-irradiated normal skin extracts, which are ubiquitination-competent, were immunodepleted with

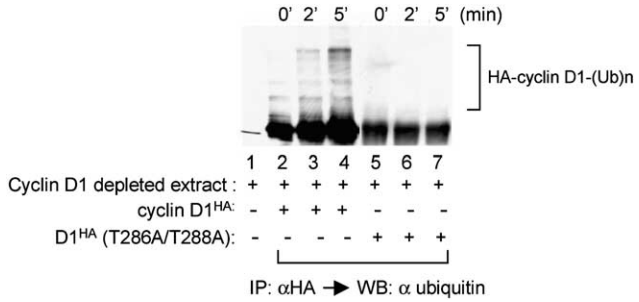

Fig. 3. Lack of in vitro ubiquitination of mutant cyclin D1 (T286A/T288A). Mock-irradiated normal skin extract was immunodepleted with anti-cyclin D1 antibody (Santa Cruz). Cyclin D1 depleted extract was incubated with in vitro translated cyclin D1^{HA} (lanes 2–4) or cyclin D1^{HA} (T286A/T288A) (lanes 5–7) for 2 min (lanes 3, 6) or 5 min (lanes 4, 7). Extracts were then immunoprecipitated with anti-HA antibody (Santa Cruz) followed by Western blot with anti-ubiquitin antibody (Santa Cruz). Lane 1 shows Western blotting of depleted extract with anti-cyclin D1 antibody.

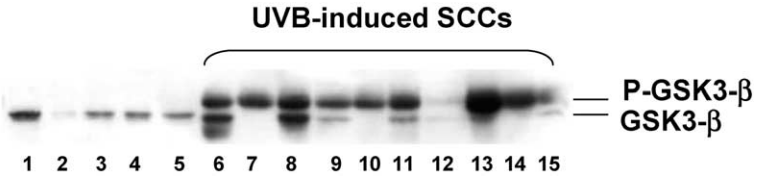

Fig. 4. GSK3β is phosphorylated in UVB-induced SCCs in SKH-1 hairless mice. The total cell lysate was prepared from epidermal and tumor extracts from UVB-irradiated mice. Lanes 2–5 represents a number of weeks with UVB irradiation (1, 5, 9, and 13 weeks, respectively), and lanes 6–15 indicate extracts prepared from UVB-induced SCC samples. Each lane contains 80 μg protein lysate as detected by Western blot analysis with anti-GSK3β antibody.

anti-cyclin D1 antibody (Santa Cruz). Cyclin D1-depleted extracts were incubated with either in vitro translated cyclin $D1^{HA}$ (lanes 2–4) or mutant cyclin $D1^{HA}$ (T286A/T288A) (lanes 5–7) for 2 min (lanes 3, 6) or 5 min (lanes 4, 7). Extracts were then immunoprecipitated with anti-HA antibody (Santa Cruz) followed by Western blotting with anti-ubiquitin antibody (Sigma). Lane 1 shows the level of endogenous cyclin D1 after immunodepletion. As shown in lanes 3–4, the high molecular weight species, indicative of ubiquitinated cyclin D1 intermediates, formed rapidly. In contrast, the mutant cyclin D1 (T286A/T288A) was not ubiquitinated. We also performed this ubiquitination assay in the presence of non-depleted extract and obtained similar results (data not shown). These data suggest that phosphorylation of these residues is important, at least in part, for cyclin D1 ubiquitination in murine skin.

GSK3β is present in an inactive phosphorylated form in SCCs

GSK3β-dependent phosphorylation of threonine 286 in murine cyclin D1 is required for its ubiquitination-dependent proteolysis. GSK3β is a ubiquitously expressed protein-serine/threonine kinase. Akt-dependent phosphorylation at its serine 9 residue inhibits its kinase activity. Using antibodies that detect both phosphorylated and unphosphorylated forms of GSK3β, we show that GSK3β is present mainly as phospho-GSK3β in SCCs whereas in non-irradiated age-matched control skin (lane 1) and in UVB-irradiated non-tumor skin (lanes 2–5) an active non-phospho form of GSK3β exists (Fig. 4).

Discussion

Cyclin D1 is a rate-limiting activator of cyclin-dependent kinases (cdk4/6) governing the G1/S transition, which is believed to be a critical cell cycle check point [9]. The G1/S transition requires activation of the cyclin D/cdk4/cdk6 [26] and cyclin E/cdk2 complexes [27,28], which in turn phosphorylates the retinoblastoma protein (Rb). Dissociation of E2Fs from Rb activates a series of target genes, the expression of which is required for cells to enter S phase [29]. In addition, cdk4 facilitates activation of cdk2/cyclin E complexes by sequestering CKIs of the p21/p27 family [30]. cdk2/cyclin E is independently required for initiation of DNA replication. During normal cell cycle progression, the balance between protein synthesis and protein degradation tightly regulates cyclin protein levels. Cyclin D1 abundance is controlled by proteolysis involving two distinct and independent mechanisms. During cell cycle progression cyclin D1 is phosphorylated on threonine 286 following activation of the PI3K–Akt–GSK3β pathway [23] thereby targeting it for degradation by the proteasome. The observed inactivation of GSK3β by its hyperphosphorylation in the present study may result in the accumulation of unphosphorylated cyclin D1. Alternatively, the anaphase promoting complex (APC) is responsible for the rapid degradation of cyclin D1 in cells irradiated with ionizing radiation [31]. APC-dependent degradation of cyclin D1 does not require threonine 286 phosphorylation, but the presence of a "cyclin degradation box" [31,32]. However, as observed in the present study, threonine 286 phosphorylation is essential for proteolytic degradation of cyclin D1 in skin. Inactivation of one or more components of the cyclin D1–Rb pathway is observed in most tumors, suggesting that deregulation of surveillance check point mechanism(s) in this pathway likely contributes to uncontrolled cell proliferation [26,31,33,34].

Cyclin D1 accumulation occurs in various human malignancies including those of breast, esophagus, liver, lung, and in carcinomas of head and neck. D-type cyclins [5,6,35–39] are overexpressed during the course of skin tumor development. Overexpression of cyclin D1 has also been observed in human skin tumors [16,35,36] and in carcinogen-induced skin cancers [8,20,40]. We also observed cyclin D1 overexpression in UVB-induced murine SCCs. The mechanism whereby UVB induces accumulation of cyclin D1 in skin is not known. Loss of matrix contact, thus allowing anchorage-independent survival and proliferation, is known to be a hallmark of neoplastic transformation [41]. Rapid down-regulation of cyclin D1 is triggered by GSK3β in epithelial cells deprived of matrix contact [34]. It is also known that

degradation of cyclin D1 leads to assembly of the represser complex of histone deacetylase–Rb–E2F thereby inhibiting transcription of insulin-like growth factor-1, leading to loss of Akt activity and apoptosis [34]. This feedback loop involving cyclin D1 further demonstrates the potential importance of cyclin D1 stability in the process of tumorigenesis. It has been proposed that cyclin D1 accumulation in epithelial cells prevents loss of cdk4 activity and apoptosis [28]. It was further suggested that loss of p16 is essential for maintaining cdk4, as well as cdk2, perhaps by sequestering p21 and p27 [30]. We have recently shown that UVB-irradiation of human epidermoid carcinoma A431 cells in which p53 is defective, leads to G1 arrest following down-regulation and degradation of cdk4/cyclin D kinases [24]. However, we also observed that the half-life of cyclin D1 was increased in A431 cells following UVB irradiation prior to degradation of the protein. These results are consistent with our findings showing cyclin D1 accumulation during UVB-induced tumorigenesis in a murine model [21]. It has been demonstrated that cyclins D1 and D3 accumulation occur in the breast cancer cell line MCF-7 as a result of their diminished degradation [22]. In addition, breast cancers (~15%) also show defects in cyclin D1 degradation capacity [22]. Similar to these results we observed an impairment in proteosomal degradation of cyclin D1 in UVB-induced murine SCCs, which may contribute to its accumulation during the neoplastic process.

In summary our results indicate that the accumulation of cyclin D1 in UVB-induced SCCs is associated with impairment of its ubiquitination and proteosomal degradation. Our data also indicate that phosphorylation of threonine 286 residues in cyclin D1 is crucial for its degradation in skin. Furthermore inactivation of GSK3β by inhibitory phosphorylation may in part be responsible for accumulation of cyclin D1 in UVB-induced SCCs.

Acknowledgments

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