Expression of RB C pocket fragments in HSF induces delayed cell cycle progression and sensitizes to apoptosis upon cellular stresses

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Abstract. The retinoblastoma protein (RB) plays an important role in growth suppression through the formation of multiple protein complexes with its target proteins using A/B and C pockets. Even though the A/B and C pockets co-operate for growth suppression, the function of RB in growth arrest is inhibited by the coexpression of RB C fragments with full length RB in the absence of p53, which implies that C pocket fragments are likely to act as a dominant-negative inhibitor of RB function. In contrast, the loss of the RB functions in the presence of p53 triggers a cell cycle arrest or apoptosis by p53-dependent pathways. Thus, it still remains to be elucidated whether the expression of RB C pocket fragments in the presence of p53 induces delayed cell cycle progression and sensitizes cells to apoptosis through p53-dependent pathways. Our results show that the expression of RB C pocket fragments not only induces delayed cell cycle progression, which is mediated by the down-regulation of cyclin A, cyclin E, and E2F-1, but also sensitizes cells to apoptosis through p53-dependent pathways.

INTRODUCTION

The retinoblastoma gene product (RB) has been implicated in the regulation of G1 cell cycle arrest, differentiation, and apoptosis (Szekely *et al.* 1992; Weinberg 1995; Smith & Pereira Smith 1996). RB consists of two distinct protein-binding pockets: the large A/B pocket, which interacts with E2F, and the C pocket, which interacts with the nuclear c-Abl tyrosine kinase or MDM-2 (Weintraub *et al.* 1992; Welch & Wang 1995; Wang 1997). Thus, it has been recognized that RB plays its role as a tumour suppressor protein by formation of multiple protein complexes.

The RB C pocket has been defined to be within the C-terminal region of RB that consists of amino acids 768–869. The role of the C pocket is growth suppression by co-operation with the A/B pocket (Whitaker *et al.* 1998). Interestingly, even though the RB C pocket itself has a growth

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suppression function, Welch & Wang (1995) reported that the growth suppression function of RB is inhibited by the coexpression of the C pocket fragments and resulted in cell cycle progression rather than augmentation in cell cycle arrest, which implies that C pocket fragments are likely to act as a dominant-negative inhibitor of RB function. Thus, it has recently been recognized that the RB plays a key role as molecular matchmaker for regulation of cell cycle progression, not only by inhibition of its target proteins by binding, but also by formation of the proper protein complexes.

However, previous studies have been done in the absence of p53^{WT} (p53) (Welch & Wang 1995; Whitaker *et al.* 1998). In the presence of p53, the loss of the RB functions triggers a cell cycle arrest or apoptosis by p53-dependent pathways, which serve intrinsic protective mechanisms for eliminating cells in which the RB pathway is deregulated (Howes *et al.* 1994; Morgenbesser *et al.* 1994; Wu & Levine 1994). Recent evidence also suggests that apoptotic pathways are activated by the disruption of intracellular signals that are involved in regulating the cell cycle progression (Lee *et al.* 1993; Hoffman & Liebermann 1994; White 1996). Therefore, it is interesting to investigate whether the inhibition of RB function by the expression of RB C pocket constructs in normal cells can trigger cell cycle arrest or apoptosis by means of p53-dependent pathways, rather than through progression in cell cycles that are caused by loss of the growth suppression function of RB.

We previously reported that human skin fibroblast (HSF) cells show a distinct G1 arrest by accumulation of p21^{WAF1} following an increase of p53 without increasing the subG1 population 24 h after etoposide treatment, implying HSF cells survive by means of a p53 pathway involved in defense mechanisms for genotoxic stress (Cho & Suh 2001). To determine the effect of the expression of RB C pocket constructs on the cell cycle progression and apoptotic pathways, HSF cells were infected with a retrovirus containing RB C pocket constructs (SE, SEA, and ME) or an empty vector (mock). Our results suggest that the expression of RB C pocket constructs in the presence of p53 not only induces delayed cell cycle progressions but also results in sensitizing to apoptotic signals by p53-dependent pathways.

MATERIALS AND METHODS

Plasmids and cells

Expression vectors for RB C pocket constructs (pCMV-SE, SEA, and ME) were kind gifts from Dr J. Y. J. Wang (University of California, San Diego; Welch & Wang 1995). We have established stable transfectants expressing RB C pocket fragments in human skin fibroblast (HSF) using PA317 packaging cell lines. Briefly, for production of ecotrophic retrovirus, plasmids were transfected into Bosc23 ecotrophic producing cell lines using the calcium phosphate method. Transfectant cells were selected subsequent to incubation in medium containing 800 µg/ml G418 (Calbiochem, San Diego, CA, USA) for 14 days and cells were continuously maintained in medium containing 400 µg/ml G418. For production of amphotrophic retrovirus, medium was harvested from confluent monolayers of the Bosc23 ecotrophic producing cell lines 24 h after a medium change. The medium was filtered through a 0.45 µm syringe filters and used immediately for infection. The diluted virus was added into PA317 packaging cell lines in 1 ml of Dulbecco's modified Eagle's medium (DMEM) with polybrene (4 µg/ml), and then cells were incubated in a 5% CO₂ incubator at 37°C. Cells were split 1 : 10 2.5 h after infection and have been selected with 800 µg/ml G418 for 14 days.

Next, HSF cells maintained in DMEM supplied with 10% fetal bovine serum (FBS) were infected with virus produced from PA317 packaging cell lines in 1 ml of DMEM with polybrene

Table 1. DNA sequences of PCR primers used in this study

Name	PCR primer sequences	Product size (bp)
GAPDH		300
Sense	5'-CGTCTTCACCACCATGGAGA-3'	
Antisense	5'-CGGCCATCACGCCACAGTTT-3'	
Cyclin A		615
Sense	5'-CAGAATGAGACCCTGCATTTGGCTG-3'	
Antisense	5'-CAGATTTAGTGTCTCTGGTGGGTTG-3'	
Cyclin D1		402
Sense	5'-ACCTGGATGCTGGAGGTCTG-3'	
Antisense	5'-GAACCTTCACATCTGTGGCACA-3'	
Cyclin E		638
Sense	5'-GGAAGGCCAAACGTGACCGTT-3'	
Antisense	5'-GGGACTTAAACGCCACTTAA-3'	
E2F-1		336
Sense	5'-ACGTGACGTGTCAGGACCTT-3'	
Antisense	5'-GTTCTTGCTCCAGGCTGAGT-3'	

(4 µg/ml) and selected by G418 (800 µg/ml) treatment for 14 days. Resistant clones were separately propagated and the expression of RB C pocket fragments was analysed by western blot.

Reverse transcription-polymerase chain reaction (RT-PCR)

As previously described (Cho *et al.* 2001), total RNA was isolated from cells using the RNAzolTM B (Biotecx Laboratories, Houston, TX, USA) and quantified by spectrophotometer. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara, Otsu, Japan). The primer pairs are described in Table 1.

Western blot analysis

We performed western blot analysis, as previously described (Cho *et al.* 2001). Briefly, cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 µg/ml), aprotinin (10 µg/ml), leupeptin (10 µg/ml), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. Lysates were quantified using the Bradford assay (Bio-rac Life Science, Hercules, CA, USA) with bovine serum albumin as a reference standard and resolved by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gels) and transferred to immobilon-P transfer membrane (Millipore, Bedford, MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science, Piscata, NJ, USA). Primary antibodies against Bax, p21, E2F-1, cyclin D1, E, A (Santa Cruz, Biotechnology, CA, USA), and p53 (Neomarkers, CA, USA) were applied at optimized concentrations. For detection of RB C fragments, we used a rabbit polyclonal anti-RB 851, which was kind gifted from Dr J. Y. J. Wang (Welch & Wang 1993).

Immunoprecipitation and histone H1 kinase assay

We performed the Histone H1 kinase assay, as previously described (Cho *et al.* 2001). Briefly, cells were lysed in ice-cold NET-N buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 100 mM NaF, 200 µM sodium orthovanadate, PMSF (10 µg/ml), aprotinin (10 µg/ml),

leupeptin (10 µg/ml), 5 mM phenanthroline, and 28 mM benzamidinium-HCl] for 20 min in ice. Lysates were clarified by centrifugation at 10,000 g for 20 min at 4 °C. After preclearing with protein A-sepharose beads (Sigma, St. Louis, MO, USA), the supernatants were then immunoprecipitated with the anti-CDK2 (cyclin dependent kinase; Santa Cruz) polyclonal antibody for 2 h and then protein A-sepharose beads for 1 h. The kinase reaction was initiated by resuspending the beads in 50 µl of kinase buffer [2.5 mM EGTA, 10 mM beta-glycerophosphate, 10 mM NaF, 5 µM ATP, Histone 1 µg, 0.37 MBq (10 µCi) (γ -³²P)ATP (NEN Life Science Products, Boston, MA)]. After incubation at 30 °C for 30 min with occasional mixing, the samples were boiled in SDS sample buffer and separated by electrophoresis. The signals were detected by autoradiography.

Induction of apoptosis

For treatment by etoposide (Sigma), cells (2×10^5) were firstly cultured in normal media for 2 days and then in the media containing etoposide (30 µM) for 1 day. For UVC treatment, cells (2×10^5) were cultured in normal media for 2 days, and growth media were then removed. Cells were exposed to 10 mJ/cm² in a UV-cross-linker (Ultra-LUM, Claremont, CA, USA) at 254 nm, and then new growth media were added.

Detection of apoptosis by propidium iodide (PI) staining

Cells were fixed with 95% cold-ethanol for 4 min and washed twice with PBS. After fixation, cells were stained with staining solution [propidium iodide (PI) (1 µg/ml), RNase A (0.1 mg/ml)] for 20 min at room temperature. Nuclear morphology of PI-stained cells was observed under fluorescence microscope (Olympus Optical, Tokyo, Japan). For cell cycle analysis, approximately 10^6 cells were fixed in 80% ethanol at -20 °C for 24 h. Ethanol-fixed cells were stained with staining solution [PI (50 µg/ml), RNase A (0.1 mg/ml), 0.1% NP-40, 0.1% trisodium citrate] for 1 h, and analysed using a FACS analyser (Becton-Dickinson Franklin Lakes, NJ, USA).

Enzymatic cleavage activity of caspase-3

Cells were lysed in lysis buffer (50 mM Tris (pH 7.5), 0.03% NP-40, 1.0 mM DTT) for 30 min on ice. After centrifugation at 10,000 g for 30 min at 4 °C, the supernatant was quantified using the Bradford assay kit with bovine serum albumin as a reference standard. Lysates (20 µg) were incubated with 0.2 mM ac-DEVD-pNA (Enzyme System Products, Livermore, CA, USA) in a total volume of 0.1 ml. Assays were performed twice, and the results were presented as the average increase in absorbance at 405 nm.

MTT assay

MTT (Sigma) stock solution (5 mg/ml) was added to each culture being assayed to equal 10% of the original culture volume and incubated for 3–4 h. At the end of the incubation period the media were removed and the converted dye was solubilized with DMSO (Dimethyl Sulfoxide, Sigma). Absorbance of converted dye is measured at a wavelength of 570 nm with background subtraction at 630–690 nm.

RESULTS

Determinations of RB C pocket constructs

We constructed stable transfectant cell lines, SE, SE delta (SEΔ), and ME cells producing RB C pocket constructs, SE, SEΔ, and ME, respectively. To determine the RB C pocket constructs

Figure 1. Schematic presentation of RB C pocket fragments (a) and western blot of RB C pocket genes SE, SE Δ and ME in human skin fibroblasts (b). A deletion of the amino acids from 785 to 806 is signified by Δ .

in stable transfectants, we performed western blot analysis using an anti-RB 851 antibody. The C pocket constructs were precisely detected as being 18–21 kDa proteins (Fig. 1).

Determination of G1 cell cycle regulatory proteins in stable transfectants

To investigate the effect of RB C pocket constructs on the expression levels of G1 cell cycle regulatory proteins, we performed western blot analysis using anti-E2F-1, cyclin D1, cyclin E, and cyclin A antibodies in stable transfectants. The expression levels of E2F-1, cyclin E, and cyclin A proteins, without there being notable down-regulation in mRNA levels, were decreased in the SE Δ cells as well as in the SE cells compared to mock cells (Fig. 2a and b). Cyclin D1 expression levels were similar in all cell lines. Consistent with these data, a remarkably down-regulated CDK2 (cyclin dependent kinase)-dependent kinase activity was observed in the SE Δ cells (Fig. 2c).

Effect of RB C pocket constructs on cell growth

The expression levels of the cyclins can modulate cell proliferation rate in most cells. The SE Δ cells showed markedly decreased expression levels of cyclin E, cyclin A, and E2F-1 compared to mock cells, but not the ME cells. To confirm this result, we performed viable cell counts daily using the trypan blue exclusion method. According to the growth curve, the SE Δ cells showed slower proliferation rate than the mock and ME cells (Fig. 3a). In addition, MTT assay was performed at 6–8 days of viable cell counts. Absorbance of SE Δ cells showed to be approximately 2-fold lower than those of mock and ME cells (Fig. 3b).

Modulation of apoptosis sensitivities to cellular stresses

In general, slowly growing cells show a higher survival rate than rapidly growing cells after some cellular stresses, such as genotoxic stress and serum starvation. In addition to the growing rate, the p53 status is also a key factor to decide cell death sensitivity by regulating the apoptotic pathways. The SE Δ cells showed a slower proliferation rate than mock cells. We investigated whether the slowly growing SE Δ cells showed decreased cell death rate after genotoxic stress and serum starvation in the presence of p53. Interestingly, the SE Δ cells showed a 2-fold increased cell death rate than mock cells after etoposide or UV treatment (Fig. 4a and b). When mock cells were cultured 5 days after serum starvation, mock cells showed an arrest in cell proliferation (Fig. 4c). In contrast to the mock cells, the SE Δ cells rapidly progressed to cell death 3 days after the serum starvation. However, the ME cells showed similar responses with the mock cells under cellular stresses.

Figure 2. Western blot (a) and RT-PCR (b) of E2F-1, cyclin D1, E and A in human skin fibroblasts and CDK2-associated kinase activity (c). Cell lysates were resolved by 12% SDS-PAGE and subjected to western blotting with anti-E2F-1, anticyclin D1, E and A antibodies (a). Cyclins and E2F-1 mRNA levels were determined by RT-PCR with specific primers (b). Note the dramatic decrease in the CDK2-associated H1 kinase activity in the SEΔ cells (c). Similar results were shown in two independent experiments.

Determination of apoptotic cell death

We investigated whether SEΔ cells show increased cell death rate through an apoptotic process after cellular stresses. According to flow cytometric analysis, the SEΔ cells showed a markedly increased subG1 population 24 h after the etoposide treatment, but the mock cells did not (Fig. 5a). Consistent with this result, SE delta cells showed higher caspase-3-like activity than that of mock cells (Fig. 5b). In Fig. 5c, the nontreated SE delta cells show normal nuclei appearing as homogeneously fluorescent oval discs, but the etoposide-treated SE delta cells showed a distinct fragmented nucleus under fluorescence microscopy, implying cells undergo apoptosis. In order to determine the p53 activation in this process, we performed western blot analysis using anti-E2F-1, p53, p21^{WAF1}, and Bax after the etoposide treatment. In Fig. 5d, the SEΔ cells

Figure 3. Effect of RB C pocket fragments on cell proliferation rate. Cells (10^5) were cultured for the indicated times and daily viable cell counts were done using trypan blue dye exclusion method (a). Cells (10^3) were also cultured for the indicated times in 96-well plates and the status of cell proliferation was measured by MTT assay (b). The data show the means of two independent experiments.

Figure 4. Cell death sensitivities of mock, SEA and ME cells after cellular stresses. Cells were cultured in the presence of etoposide ($30 \mu\text{M}$) for 24 h (a). Cells were cultured 24 h in normal media following UVC ($10 \text{ J}/\text{cm}^2$) treatment (b). Cells were cultured in the presence of serum to 70% confluency, the medium was then changed to serum-free medium and subsequently cultured in the absence of serum for the times as indicated (c). Note that SEA cells were more sensitive to cellular stresses than mock and ME cells. Relative index indicates the ratio of mock, SEA or ME/mock. The data show the means of three independent experiments.

showed markedly increased expression levels of E2F-1, p53, p21^{WAF1}, and Bax 24 h after the etoposide treatment.

DISCUSSION

RB has been regarded to play important roles in cell cycle control, apoptosis protection, chromosomal remodelling, and in the differentiation process (Weinberg 1995; An & Dou 1996; Bartek *et al.* 1996; Tan *et al.* 1997; Brehm *et al.* 1998; Magnaghi *et al.* 1998). In order to perform proper RB functions, it is essential to not only inhibit its target proteins by binding, but to also form the proper protein complexes, which indicates that RB plays a key molecular match-maker role in biologically important phenomenon (Weintraub *et al.* 1995; Welch & Wang 1995; Wang 1997).

The RB C pocket has been defined as being the binding site for c-Abl or MDM2 (Goga *et al.* 1995; Xiao *et al.* 1995; Wen *et al.* 1996). In a previous report, it has been found that both SE and SEA RB C pocket fragments can bind to c-Abl, and that only SE can also bind to E2F, but not to SEA (Welch & Wang 1993, 1995). In other words, SE and SEA fragments could inhibit correct complex formations of RB with other proteins. Another RB C pocket fragment, ME, can

Figure 5. Determination of apoptotic cell death in etoposide-treated SE delta cells. Cells were treated with etoposide (30 μ M) for 24 h, and apoptotic cell death was evaluated by flow cytometric analysis, measurement of caspase-3 like activity, and observation of nuclear morphology. Flow cytometric analysis was performed using propidium iodide (PI) staining solution in mock and SEA cells 24 h after etoposide treatment (a). Caspase-3-like activity was determined using ac-DEVD-pNA, synthetic substrate of caspase-3 (b). Assays were performed twice and results were presented as the average increase in absorbance at 405 nm. SE delta cells were stained with PI staining solution and observed under a fluorescence microscope (c; $\times$ 400). The expression levels of E2F-1, p53, p21 and Bax in SEA cells were confirmed by two independent experiments (d).

neither bind to c-Abl nor to E2F, which implies that ME could not inhibit the formation of proper protein complexes. It is therefore apparent that the expression of SE or SE Δ , but not the ME, competes with the endogenous RB in binding to the c-Abl tyrosine kinase, MDM2, or E2F, and thereby leads to the disruption of RB-assembled complexes.

In the present study, we have investigated whether the expression of the RB C pocket constructs (SE, SE Δ , and ME) could not only modulate the mechanisms underlying cell cycle progression, but also the apoptotic pathways in human skin fibroblast (HSF) cell lines. Our data indicate that expression of the RB C pocket fragments, either SE or SE Δ , induced delayed cell cycle progression by the down-regulation of E2F-1, cyclin E, and cyclin A levels. Furthermore, the SE Δ cells appeared to be prone to apoptotic cell death after cellular stresses compared to mock cells. In contrast to SE Δ , the ME fragment neither induced a modulation of cell cycle progression nor sensitized cells to apoptotic cell death. When the SE Δ fragment was cotransfected with p53, apoptotic cell death was found to be more prominent than in SE Δ alone (data not shown), but not with the mutant p53, implying p53-dependent apoptotic pathways play important roles in this process. Howes *et al.* (1994) also reported that the photoreceptors expressing the HPV-E7 gene in the presence of p53 progressed into apoptotic cell death and resulted in degeneration of retina, but not in a p53 nullizygous background.

Many of the most potent cytotoxic agents act by damaging DNA. Their toxicity is greater during the S phase of cell cycle. Thus, human neoplasms that proliferate rapidly tend to be more susceptible to cell cycle specific cytotoxic agents than slowly growing tumours (Hardman *et al.* 2001). In addition, cell cycle delay allows cells time to repair DNA damage, thus making them more resistant to ionizing radiation (Yarnold *et al.* 1996). However interestingly, our data showed that the disruption of the RB matchmaker function by the expression of RB C pocket fragments in the presence of p53 sensitized cells to apoptosis upon cellular stresses, such as etoposide or UV treatment, even though they were in a slowly growing state. This implies that our data could also provide an important insight for cancer chemo- or radio-therapy methods that use apoptotic cascade mechanisms.

Finally, we suggest that, when either SE or SE Δ is expressed in cells, cells undergo different pathways depending on their cellular contexts. Firstly, under normal conditions, cells show either a delayed growth rate as a result of the inhibition of G1 progression by SE and SE Δ itself activities (Whitaker *et al.* 1998) or a delayed G1 progression by the down-regulation of cyclin A, cyclin E, and E2F-1 expression levels. Secondly, under genotoxic stress, cells are prone to apoptotic cell death caused by a p53 activation that is amplified by the disruption of the RB matchmaker function. Thus, in the presence of p53, disruption of the RB matchmaker function will induce either a delayed G1 progression or a sensitizing apoptosis, thereby sparing the restriction point control time or elimination of abnormal cells that can lead to transformation. In contrast, in the absence of p53, the disruption of the RB matchmaker function will neither cause a delayed G1 progression nor a sensitizing apoptosis, thereby permitting an abnormal cell cycle progression and subsequent transformation.

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