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A Novel Role for Thyroid Hormone Receptor Beta in Cellular Radiosensitivity

Michiko MATSUSE[1], Vladimir SAENKO[2]*, Ilya SEDLIAROU[1], Tatiana ROGOUNOVITCH[1], Yuka NAKAZAWA[1], Norisato MITSUTAKE[1], Natallia AKULEVICH[1], Hiroyuki NAMBA[1] and Shunichi YAMASHITA[1, 2]

Thyroid hormone receptor beta/Radiosensitivity/Clonogenic survival/Cellular senescence.

Thyroid hormone receptors (THRs) widely govern cell growth, differentiation and metabolism acting in a ligand- and cofactor-dependent manner to modulate tissue-specific gene expression. Given a large variety of genes regulated by THRs and multiplicity of cellular processes potentially influenced by THRs, we addressed the role of THRB (thyroid hormone receptor beta) in cellular radiosensitivity. Wild-type and mutant THRB were overexpressed in several cell lines using an adenovirus-mediated gene delivery and their effects were examined after cell exposure to γ-rays. Wild-type THRB decreased clonogenic survival of the cell lines with low levels of endogenous THRB, retarded their growth and synergized with radiation in decreasing proliferative potential and promoting cellular senescence. These changes were accompanied by the accumulation of p21 (CDKN1A, CIP1, WAF1) and p16 (CDKN2A, INK4a) inhibitors of cyclin-dependent kinases and by the decrease of Rb (retinoblastoma protein) phosphorylation. Mutant THRB produced a radioprotective effect, attenuated radiation-induced growth inhibition and cellular senescence. The results suggest that THRB may modulate cellular radiosensitivity and stress-induced senescence.

INTRODUCTION

The thyroid hormone receptors (THRs) are transcription factors belonging to the steroid/thyroid/retinoid nuclear receptor superfamily. Depending on the binding to thyroid hormone, THRs can activate or suppress gene expression in a tissue-specific manner through heterodimerization with retinoid X receptors and interaction with the positive or negative response elements in the regulatory regions of target genes.[1)] Suppression of gene expression by THRs also involves various corepressor proteins that form chromatin modifying complexes.[2)] Thyroid hormone plays an essential role in the development, differentiation, metabolic activity and maintenance of tissue homeostasis implemented through the THR interface.[3,4)]

Besides germline mutations in THR-encoding genes in the syndromic states cumulatively referred to as resistance to thyroid hormone,[5)] somatic alterations of these genes and their loci have been described in a variety of human cancers. For instance, somatic mutations of THR genes were reported in thyrotropin-secreting pituitary tumors,[6–8)] hepatocellular carcinoma,[9,10)] renal clear cell carcinoma[11)] and papillary thyroid carcinoma.[12)] Loss of heterozygosity of THR genes was reported in lung[13–15)] and breast cancer[16–20)] and uveal melanoma.[21)] The reduction of *THRB* mRNA levels has been found in thyroid[22)] and pituitary tumors.[23)] The diminished expression of THRB protein has been shown in poorly differentiated osteosarcoma cell lines.[24)] The abnormal intracellular localization of THRs has been detected in breast carcinoma.[25)] Collectively, these data indicate possible involvement of THRs in carcinogenesis.

Cellular response to radiation varies broadly among cell types and is strongly affected by the spectrum of expressed genes. THRs have been shown to regulate transcription of different target genes[4,26)] and several previous studies suggested that THRs may play a tumor suppressor role.[13,14,27)] So far, a number of tumor suppressor proteins, like p53, have been shown to modulate cellular response to DNA-damaging agents. Accordingly, we hypothesized that THRs may also influence cellular radiosensitivity. To the best of our knowledge, relationships between THRs and radiation have not been established yet. This investigation was set out to determine the effects of THRB on outcomes of cell

*Corresponding author: Phone: +81-95-849-7122,
Fax: +81-95-849-7169,
E-mail: saenko@net.nagasaki-u.ac.jp
[1]Department of Molecular Medicine and [2]Department of International Health and Radiation Research, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
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irradiation and to elucidate mechanisms involved in the THRB-mediated modulation of radiation response in several cell lines.

MATERIALS AND METHODS

Antibodies

The antibodies to PARP, cyclin D1, Chk1, phospho-Chk1(Ser345), Chk2, phospho-Chk2 (Thr68), Cdc2, phospho-Cdc2 (Tyr15), Rb, phospho-Rb (Ser780), cleaved caspase-3 and -9, and secondary anti-mouse and anti-rabbit HRP-conjugated antibodies were obtained from Cell Signaling (USA); the antibodies to THRB and β-actin were from Santa Cruz (USA); anti-p21(CDKN1A, CIP1, WAF1) and anti-p16 (CDKN2A, INK4a) were from Calbiochem (USA).

Cell cultures

Human anaplastic thyroid carcinoma cell lines ARO and FRO, papillary thyroid carcinoma cell line NPA and TPC-1 were originally provided by J. A. Fagin (Memorial Sloan-Kettering Cancer Center, New York, USA); human breast cancer cell line MCF-7, monkey kidney COS7 and human embryonic kidney HEK293 cell lines were purchased from the American Type Culture Collection (USA). ARO and FRO cells were grown in RPMI1640 medium, other cells in DMEM, supplemented with 5% FBS (Invitrogen/Life Technologies Inc., UK), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma, USA). Cultures of primary human thyrocytes were established and maintained as described previously.[28]

For some experiments, FBS was treated with Dowex 1 × 8 resin (Fluka, Germany) to deplete thyroid hormones. This procedure reduced T3 and L-thyroxine concentration to undetectable levels.[29,30]

Recombinant adenoviruses

Full length coding portions of wild-type (*wtTHRB*) and K411E mutant THRB (*mutTHRB*) and Green Fluorescent Protein (*GFP*) cDNA were inserted into pAdHM4CMV according to described methodology.[31] A K411E mutation in the ligand-binding domain of THRB was introduced beforehand using QuikChange XL site-directed mutagenesis kit (Stratagene, USA). This mutant form of *THRB* has been described previously in human papillary thyroid carcinoma.[32] In the cited work[32] and in our preliminary experiments (Sedliarou *et al.*, in press) it was determined that the K411E mutTHRB protein displays less than 40% of wtTHRB transactivation activity. The plasmids were linearized with *Pac*I endonuclease (New England Biolabs, USA) and transfected into HEK293 cells for packaging and propagation. Adenoviruses (Ad) were purified by $CsCl_2$ density-gradient centrifugation.[33] The concentration of viral particles was determined by measuring the absorbance at 260 nm.[34]

To evaluate the transduction efficacy, cells were infected with *GFP*-Ad at 10–1000 multiplicities of infection (MOI). The expression of GFP in infected cells was evaluated using an Eclipse TE2000-U fluorescent microscope (Nikon, Japan). Viral dose yielding GFP expression in at least 90% of infected cells without evidence of cytotoxicity was determined (50 MOI for ARO, MCF-7, COS7; 400 MOI for TPC-1 and NPA; and 1000 MOI for FRO, data not shown).

Clonogenic survival assay

The effects of THRB on the ability of irradiated cells to form colonies were assessed in a standard clonogenic assay. The cells were infected with *wtTHRB*-Ad, *mutTHRB*-Ad and *GFP*-Ad at the above specified MOI, incubated for 24 h to allow the target gene expression, plated at clonogenic density and irradiated with a single acute dose of 0–10 Gy of γ–rays (^{137}Cs source, dose rate of 1.0 Gy/min, PS-3100SB, Pony, Japan) at room temperature. The dishes were incubated for 10–14 days, then cells were fixed with methanol, stained with Giemsa, and the colonies of more than 50 cells were counted as survivors. Non-irradiated cells infected with corresponding adenoviral construct served as a control determining 100% survival.

Western blotting

Cells were infected with *wtTHRB*-Ad, *mutTHRB*-Ad and *GFP*-Ad, incubated for 24 hours to allow the expression of target gene and irradiated with 0–10 Gy of γ-rays. Cell lysates were collected 6–120 h after irradiation. As a control, non-irradiated cells were used. Forty micrograms of protein were resolved in SDS-PAGE and transferred onto BioTrace NT nitrocellulose membrane (Pall Corp., USA). After the incubation with an appropriate primary antibody, the antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and a chemiluminescence substrate (Nacalai, Japan). Detection was performed using a LAS3000 imaging system (Fujifilm, Japan). For the quantitative estimation of band intensities image analysis was done with Gel-Pro Analyzer ver.3.1 software (Media Cybernetics, USA).

Cell growth assay

The effect of THRB on the growth of irradiated cells was estimated using Cell Counting Kit-8 (Dojindo, Japan) which employs water soluble tetrasolium method. Cells were seeded on the 96-well plates (2×10^3 cells/well) and infected with appropriate adenoviruses. Twenty-four hours later, the cells were irradiated with the dose of 5 Gy and allowed to grow for 120 hours. The reagent was added to the medium and optical density was read at 450 nm in a microplate reader ImmunoMini NJ-2300 (System Instruments, Japan).

PKH-2 staining

To determine cell division dynamics, the PKH-2 fluorescent dye (Sigma Chemical Co., Tokyo, Japan) was used as described before.[35,36] Briefly, cells were labeled with PKH-2 according to the manufacturer's protocol, infected with adenoviral vectors and irradiated 24 h later. PKH-2 fluorescence was assessed 120 h after irradiation using a FACScan flow cymeter (BD Biosciences, USA). Twenty thousand events were collected for each data point.

Senescence-associated beta-galactosidase (SA-β-gal) staining

The senescence-associated β-galactosidase staining was performed as described elsewhere.[36–38] Briefly, the cells were infected, irradiated with the dose of 5 Gy and fixed on day 5. SA-β-gal positive cells were detected by bright-field microscopy. The number of SA-β-gal positive cells was determined in 4 random fields for each experimental point by scoring 200–400 cells/field for each sample.

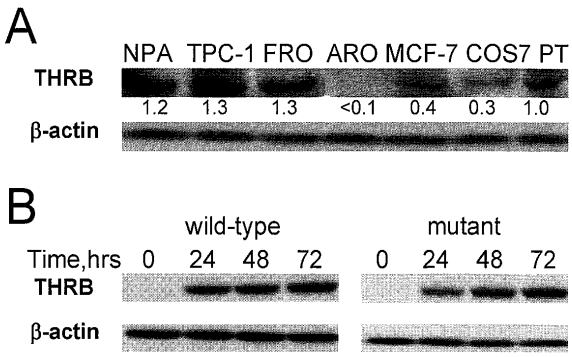

Fig. 1. **(A)** The endogenous THRB levels in well-differentiated (NPA and TPC-1) and anaplastic (FRO and ARO) thyroid cancer cell lines, breast cancer MCF-7 cells, green monkey kidney cell line COS7 and primary human thyrocytes (PT). Expression levels in relation to that in thyrocytes as determined by image densitometry are shown below the bands. **(B)** Forced expression of wtTHRB and mutTHRB after infection with corresponding adenoviral vectors in MCF-7 cells. Similar dynamics of THRB accumulation was found in all other cell lines tested. Note that endogenous THRB could not be visualized using short exposure time because of the high abundance of exogenous THRB. β–actin was used as a loading control.

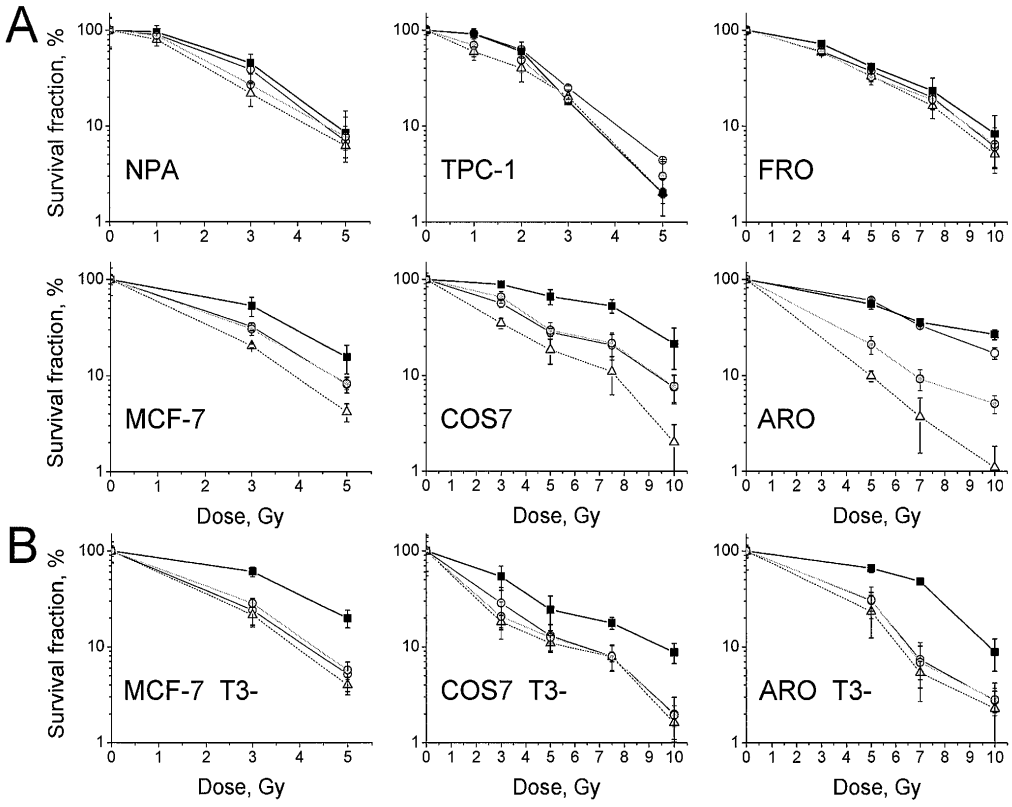

Fig. 2. Forced THRB expression modulates clonogenic properties of the cells with low levels of endogenous THRB protein in a T3-dependent manner. **(A)** Clonogenic survival of NPA, TPC-1 and FRO cells which have a high level of endogenous THRB was not affected by the infection with any adenoviral vector (upper row). ARO, MCF-7 and COS7 cells with a low level of endogenous THRB were radiosensitized by transduction with wtTHRB and protected by overexpression of mutTHRB (lower row). **(B)** Wild-type THRB does not change clonogenic properties of cells with low endogenous THRB in the absence of T3. Serum was depleted of thyroid hormone using Dowex resin. Clonogenic assay was done in the medium without T3. In **(A)** and **(B)**, ○- intact cells; ◍- cells infected with *GFP*-Ad; △ -cells infected with *wtTHRB*-Ad; ■- cells infected with *mutTHRB*-Ad. Each experiment was done at least three times in triplicates. Data are reported as the mean and standard deviation.

Statistical analysis

Statistical significance was evaluated using one-way analysis of variance (ANOVA) or unpaired t-test as appropriate. A P value not exceeding 0.05 was considered significant.

RESULTS

Levels of endogenous THRB protein in the cell lines

The expression of endogenous THRB protein was evaluated in NPA, TPC-1, FRO, MCF-7, COS7 and ARO cells and primary human thyrocytes by Western blotting. Compared to thyrocytes, which were used as a reference, the level of THRB was relatively high in NPA, TPC-1 and FRO cells. On the contrary, THRB was under-expressed in MCF-7 and COS7 and nearly absent in ARO cells (Fig. 1A).

Adenoviral infection with *wtTHRB*-Ad, *mutTHRB*-Ad and *GFP*-Ad led to the efficient accumulation of target proteins 24 h after infection reaching the maximum at 72 h (Fig. 1B). The proteins were fairly detectable even on day 5 (see below). Based on GFP fluorescence, more than 95% of cells were transduced under the conditions used without signs of cytotoxicity.

Wild-type and mutant THRB modulate clonogenic survival of irradiated cells

To investigate the relationship between cellular radiosensitivity and forced expression of wild-type and mutant THRB, the clonogenic assay was done. The infection with any adenoviral construct did not bring about difference in the number of colonies in NPA, TPC-1 and FRO cells (Fig. 2A). Of note, these three cell lines have a high level of endogenous THRB. In contrast, the transduction with wtTHRB of MCF-7, COS7 and ARO cells, which have low endogenous THRB, resulted in a significant reduction in the number of colonies indicative of cell sensitization to ionizing radiation. The infection with *mutTHRB*-Ad increased clonogenic survival of these cell lines compared to the cells infected with *GFP*-Ad.

In the thyroid hormone-depleted medium, clonogenic survival of cells infected with *wtTHRB*-Ad did not change whereas untreated cultures and cells infected with *GFP*-Ad produced the lower number of colonies which was nearly equal to that observed in the wtTHRB-transduced cells (Fig. 2B). The clonogenic survival of cells infected with *mutTHRB*-Ad also tended to decrease but the net radioprotective effect of mutTHRB overexpression remained significant. Reconstitution of T3 to the original levels in diluted serum or higher (up to 100 nM) restored the survival of uninfected and *GFP*-Ad-infected cells making radiosensitizing effect of *wtTHRB*-Ad infection evident (data not shown).

To investigate the mechanisms of radiosensitization, we further focused on the effects in the three cell lines with low endogenous THRB, *i.e.* MCF-7, COS7 and ARO.

Wild-type THRB suppresses cell growth after irradiation

The reduction of clonogenic survival may be caused either by cell death or irreversible growth arrest. In the infected and infected/irradiated cell cultures we did not observe notable cell detachment and/or generation of cleaved forms of PARP and caspase-3 and -9 on Western blotting (data not shown) suggestive of the negligible cytotoxic effect of the treatments. Furthermore, in the plates used for clonogenic studies, we noticed a radiation dose-dependent accumulation of single cells and small colonies of a few cells that looked alive. Therefore, we examined whether the diminished clonogenic survival was associated with growth arrest.

First, cell growth assay was done. As determined by the water-soluble tetrazolium-based method, cell growth was significantly suppressed in irradiated MCF-7, COS7 and ARO cultures pre-infected with *wtTHRB*-Ad (Fig. 3). Significant

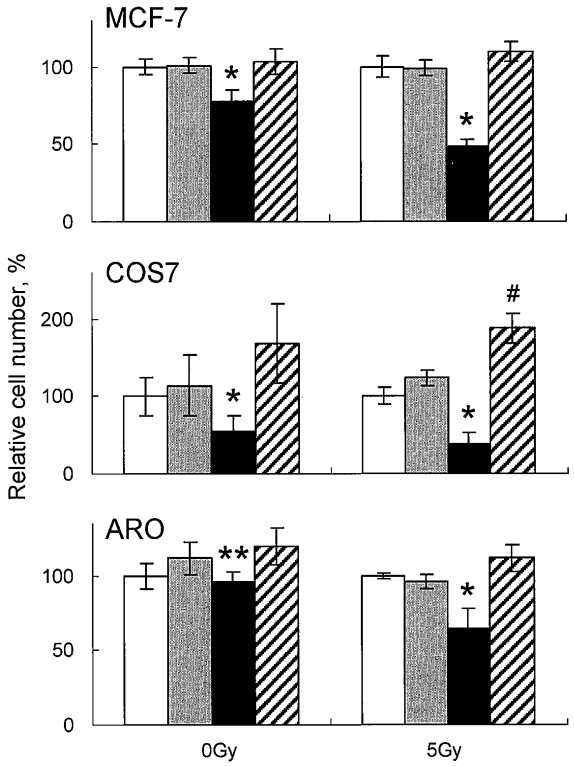

Fig. 3. Wild-type THRB retards cell growth. Cells were infected and irradiated as described in Materials and Methods and the cell growth assay was done 120 hours after irradiation. Results are expressed as the average percentage of optical density relative to that of uninfected cells. Experiment was reproduced three times using 6 wells for each vector. □- intact cells; ▒- cells infected with *GFP*-Ad; ■- cells infected with *wtTHRB*-Ad; ▨- cells infected with *mutTHRB*-Ad. Each bar represents the mean and standard deviation. * - $P < 0.05$ for pairwise comparison of THRB effect with any other culture. ** - $P < 0.05$ *vs.* ARO cells transduced with GFP and mutTHRB; no significant difference was found *vs.* intact cells. # - $P < 0.05$ for pairwise comparison with any other irradiated COS7 culture; there was no significant difference in mutTHRB effects in other cells.

growth inhibition was also observed in non-irradiated wtTHRB-transduced cells, although it was less pronounced. Ectopically expressed mutTHRB and GFP did not retard cell growth.

Second, cell division dynamics was evaluated using a lipophilic fluorescent compound PKH-2. PKH-2 incorporated into the plasma membrane is evenly divided amongst subsequent progeny, allowing discrimination of cells that undergo different number of divisions. FACS analysis demonstrated that after irradiation the subpopulation with a high PKH-2 (PKH-2^{hi}) signal, which represents the cells that have not divided or divided less times, was greater in *wtTHRB*-Ad-infected cells than in *mutTHRB*-Ad- and *GFP*-Ad-infected cultures (Fig. 4A and 4B). Considering individual changes produced by radiation or *wtTHRB*-Ad infection in parental cells, the effect of wtTHRB transduction in irradiated cells was more than additive in all three cell lines tested. On the contrary, mutTHRB decreased PKH-2^{hi} subpopulation in irradiated cells compared with the cells treated with radiation only or with a combination of radiation and infection with any other adenoviral vector.

Wild-type THRB promotes and mutant THRB attenuates cellular senescence after radiation exposure

As shown in Fig. 4A and 4C, a substantial part of growth-retarded PKH-2^{hi} cells also displayed elevated side light scatter (SSC^{hi}) on flow cytometry. The largest number of PKH-2^{hi}/SSC^{hi} cells was observed in the cultures transduced with wtTHRB and then exposed to radiation. Similarly to the suppression of cell division described above, the effect was more than additive in all three cell lines tested. In our previous work it has been demonstrated that the PKH-2^{hi}/SSC^{hi} phenotype strongly correlated with senescence-like terminal growth arrest.[36] Therefore, we examined SA-β-gal activity and found that the number of positively stained cells was significantly higher in the irradiated cultures overexpressing wtTHRB than in those treated by any singular agent or by any other combination (Fig. 5). Exposure to radiation by itself increased the number of SA-β-gal-positive cells, but the effect of the combination of radiation with *wtTHRB*-Ad

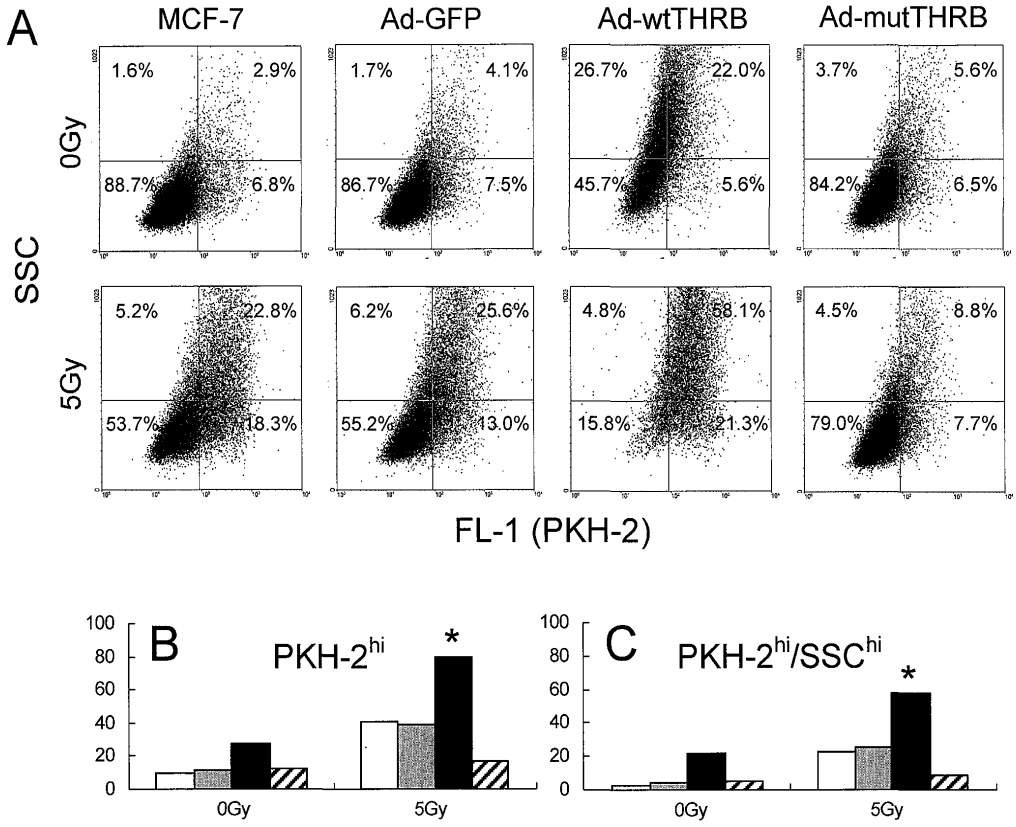

Fig. 4. Effect of THRB on cell division. **(A)** Representative dot plot diagrams demonstrating changes in the PKH-2 fluorescence (X axis) and side scatter (Y axis) of MCF-7 cells. Live cells were stained with PKH-2, infected with adenoviral vectors and irradiated with 5 Gy of γ–rays. Flow cytometry assay was done 120 hours after irradiation. **(B)** and **(C)** The sizes of PKH-2^{hi} and PKH-2^{hi}/SSC^{hi} subpopulations, respectively, derived from the graph above. □- intact cells; ▒- cells infected with *GFP*-Ad; ■- cells infected with *wtTHRB*-Ad; ▨- cells infected with *mutTHRB*-Ad. * - Effect of *wtTHRB*-Ad on the accumulation of both PKH-2^{hi} and PKH-2^{hi}/SSC^{hi} subpopulations was more than additive in irradiated cells; note that infection with *mutTHRB*-Ad substantially diminished the size of non-dividing cells subpopulations. Similar results were obtained in ARO and COS7 cells (data not shown). The experiment was reproduced two times for the three cell lines.

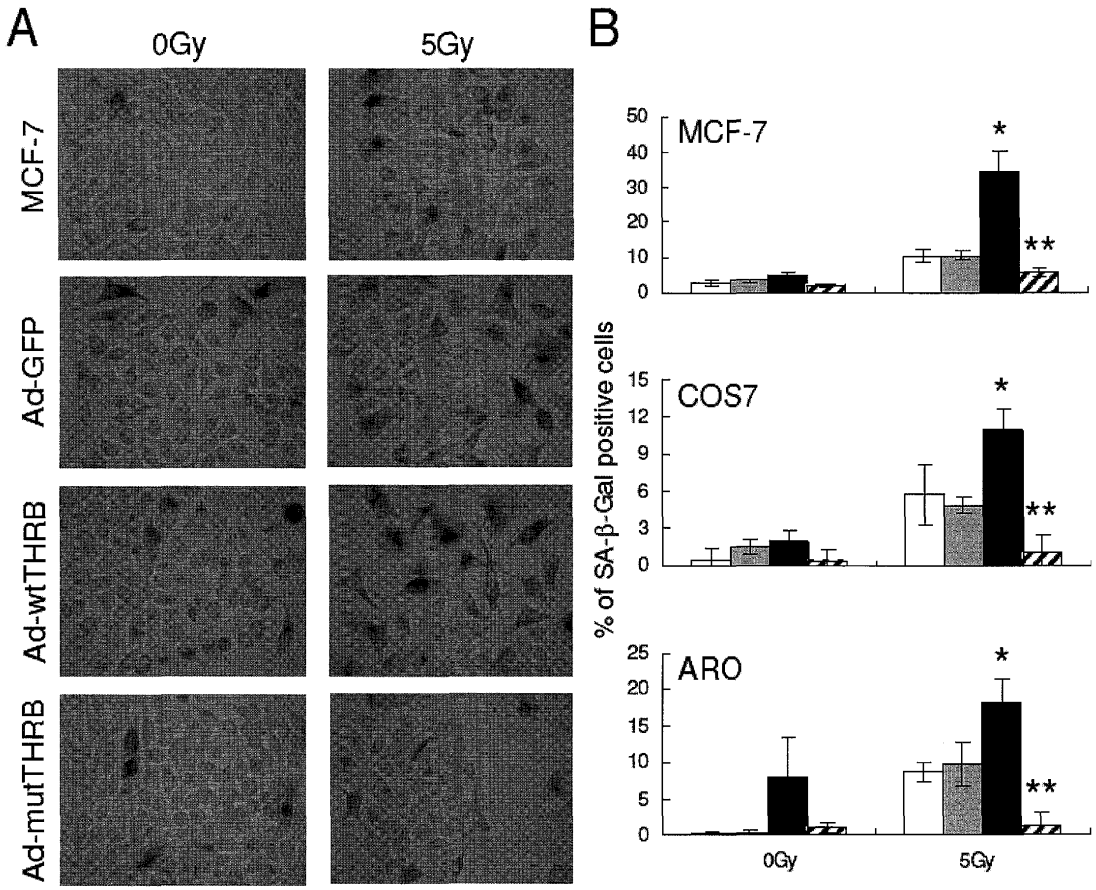

Fig. 5. Wild-type THRB promotes the accumulation of senescence-associated β-galactosidase (SA-β-gal)-positive cells in irradiated cultures. **(A)** Typical SA-β-gal staining of non-infected and infected with various adenoviral vectors MCF-7 cells in non-irradiated and irradiated cultures on day 5 after exposure. **(B)** The percentage of SA-β-gal-positive cells in the infected and infected/irradiated cultures. * - $P < 0.05$ for pairwise comparison of THRB effect with any other culture. Effect of *wtTHRB*-Ad on the accumulation of SA-β-gal-positive cells was more than additive in irradiated MCF-7 and COS7 cultures, and additive in ARO. ** - Infection with *mutTHRB*-Ad significantly decreased the number of SA-β-gal-positive cells in irradiated ARO and COS7 cultures, $P < 0.05$ for any pairwise comparison; in MCF-7 cells significant difference was observed only in comparison with the effect of *wtTHRB*-Ad infection. □- intact cells; ▒- cells infected with *GFP*-Ad; ■- cells infected with *wtTHRB*-Ad; ▨- cells infected with *mutTHRB1*-Ad. Each bar represents the mean and standard deviation. The experiment was reproduced three times in duplicates for each type of viral infection or with the control cells.

infection was more than additive in MCF-7 and COS7 cells and additive in ARO. Infection with *mutTHRB*-Ad significantly decreased the number of SA-β-gal-positive cells in irradiated COS7 and ARO cultures compared to any other combination treatment. In irradiated MCF-7 cells significance of difference was reached only with the cells transduced with wtTHRB. In the cell lines with high levels of endogenous THRB infection *wtTHRB*-Ad did not significantly changed the number of SA-β-gal-positive cells in both irradiated and non-irradiated cultures (data not shown).

Wild-type and mutant THRB affect the expression of cell cycle regulators

To explore the molecular mechanisms associated with infections with different adenoviral vectors in irradiated cells, we analyzed the levels and activation status of several key proteins involved in cell cycle regulation. The experiments revealed the variable dynamic changes among the cell lines at different time points after radiation exposure which are probably due to the different histogenetic and oncogenic backgrounds of the cells tested. Yet, common alterations were identified. Twenty-four hours after radiation exposure, *i.e.* relatively shortly after the cells have recovered from the radiation-induced cell cycle arrest, the level of cyclin D1 was elevated in the cells transduced with mutTHRB (Fig. 6A). Cells transduced with wtTHRB accumulated p16 and p21, the inhibitors of cyclin-dependent kinases which affect mostly the progression through the G_1 phase of the cell cycle, and displayed a decreased level of phospho-Rb (retinoblastoma protein). Of note, changes in the expression of these factors in the cultures infected with *mutTHRB*-Ad were opposite, *i.e.* there was an elevated level of cyclin D1,

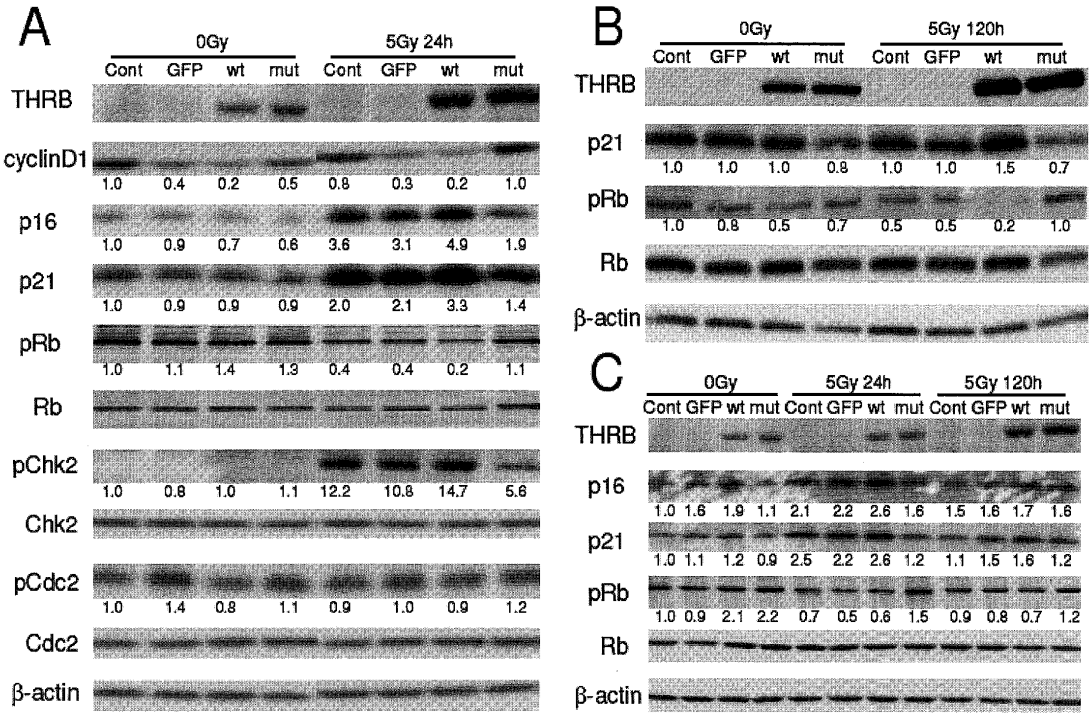

Fig.6. Changes in the expression of cell cycle-regulating proteins after adenoviral infections and exposure to radiation in MCF-7 cultures. Cells were infected with appropriate viral constructs and irradiated with 5 Gy of γ-rays in the medium with (**A** and **B**) or without T3 (**C**). Cell lysates were collected at different time points and subjected to Western blotting analysis. The expression levels determined by densitometry in relation to the control non-irradiated cells are shown below the bands. β–actin was used as a loading control. Images are representative of at least three independent experiments. Similar results were obtained in COS7 and ARO cells.

decreased levels of p16 and p21 and increased phospho-Rb content. Among the G_2 phase regulators examined (Chk2 and Cdc2), phospho-Chk2 level was decreased in the cells treated with *mutTHRB*-Ad possibly indicative of the attenuated G_2/M checkpoint in them in addition to the facilitated G_1/S transition. In the cells transduced with adenoviral constructs other than *mutTHRB*-Ad, the G_2/M checkpoint appeared to be preserved as seen from the rather high levels of phospho-Chk2. Taking into account, however, that Chk2 can affect not only the G_2/M checkpoint but also the G_1/S transition,[39–42] the elevated levels of its phosphorylated form might also reflect the extension of G_1 phase in such cultures. Phospho-Cdc2 levels did not undergo substantial alterations short time after radiation exposure and further on.

One hundred-twenty hours after irradiation, *i.e.* when growth inhibition and senescence-like changes have already taken place in the cultures, cultures infected with *wtTHRB*-Ad consistently displayed the decreased phosphorylation of Rb, suggestive of cell accumulation in the G_1 phase (Fig. 6B). Concordantly, these cells also had elevated p21. On the contrary, in the cells infected with *mutTHRB*-Ad, level of p21 was decreased and Rb phosphorylation was rather strong. These findings were in line with the above described properties of the cultures overexpressing wtTHRB to display an increased number of non-dividing cells and enhanced acquisition of senescence-like phenotype while mutTHRB exerted an opposite effect.

In the absence of T3, levels of p16, p21 and phospho-Rb were rather comparable in non-infected control cells and in the cells treated with *GFP*-Ad or *wtTHRB*-Ad (Fig. 6C) that corresponded well to their nearly identical clonogenic survival. By contrast, cultures infected with *mutTHRB*-Ad accumulated lower levels of p16 and p21 and elevated level of phospho-Rb, especially pronounced short time after irradiation. These changes were consistent with the higher radioresistance of the mutTHRB-transduced cells observed in thyroid hormone-free medium.

DISCUSSION

In the present work we found that different cell lines express different levels of endogenous THRB protein. This difference may probably stem from their distinct histogenetic origin or be the result of immortalization and long-term culturing. Based on the endogenous THRB level, cell lines could be conditionally subdivided into two groups, with the low and high expression of THRB. Such diverse levels provide a model for studying THRB effects in various settings, including radiation exposure. To examine THRB influence on the cellular reaction to radiation exposure, cultures were infected with recombinant adenoviruses encoding wild-type and mutant THRB and GFP and subjected to clonogenic

assay. Forced expression of the three proteins did not produce changes in clonogenic survival of NPA, TPC-1 and FRO cells after irradiation, in which the levels of endogenous THRB are high (Fig. 1 and 2A). In contrast, cells with low endogenous THRB, *i.e.* MCF-7, COS7 and ARO, pre-infected with wtTHRB displayed a significant reduction in the number of colonies while the effect of mutTHRB was rather radioprotective (Fig. 2A). In the absence of T3, surviving fraction of MCF-7, COS7 and ARO cells infected with any adenoviral vector but *mutTHRB*-Ad tended to decrease (Fig. 2B). The extent of decrease in survival of non-infected cells and of those infected with *GFP*-Ad was so pronounced that the difference with wtTHRB-transduced cell survival was lost. Such a change could probably be explained in part by the ability of the unliganded endogenous THRB to delay cell progression into the S phase of the cell cycle resulting in retarded proliferation.[43]

Radiation doses used in our study did not cause massive death in MCF-7, COS7 and ARO cultures. This is consistent with the notion that apoptosis may not be the major mode of death after exposure to radiation in epithelial and stromal-derived cells.[44,45] We found that growth was significantly retarded in the cultures infected with wtTHRB compared to those treated with any other adenoviral vector (Fig. 3). The effect of wtTHRB on the cell division blockage in irradiated cultures was more than additive as seen from the experiments on cultures labeled with a cell tracker PKH-2 (Fig. 4). MutTHRB had the opposite influence manifested in the decrease of the number of non-dividing cells after irradiation in all cultures.

The decreased proliferative potential (Fig. 3) and increased content of the PKH-2^{hi}/SSC^{hi} subpopulation (Fig. 4) in irradiated cells pre-infected with *wtTHRB*-Ad prompted us to evaluate SA-β-gal activity, a marker of senescent cells. The number of positively stained cells was found to be significantly different among irradiated cells transduced with different proteins being the highest in the cultures infected with *wtTHRB*-Ad and the lowest in the cultures treated with *mutTHRB*-Ad (Fig. 5). It should be noted however, that the number of SA-β-gal positive cells was somewhat lower than it would be expected from the size of the PKH-2^{hi}/SSC^{hi} subpopulation determined by flow cytometry (Fig. 5 and 4, respectively). Perhaps the determination of PKH-2^{hi}/SSC^{hi} cells, despite it requires special instrumentation, is a preferable technique for assessing an association between cellular senescence and survival/proliferation of irradiated cells. A good correlation between the numbers of PKH-2^{hi}/SSC^{hi} and SA-β-gal positive cells was nevertheless seen in all experiments attesting to the validity of SA-β-gal activity determination as of an indicator of the extent of cellular senescence even though some concerns have been raised regarding the specificity of this marker.[35,46]

Previously it has been shown that enlarged and flattened cell shape and increased cell granularity, permanently elevated p21 and SA-β-gal activity were the hallmarks of cellular senescence in irradiated cell lines derived from different types of thyroid tumors.[47] Our observations of the accumulation of negative cell cycle regulators, including increased levels of p21 and attenuated phosphorylation of the Rb protein both short and long time after irradiation (Fig. 6A and B), are in agreement with the reports on their essential role in the growth arrest and maintenance of the senescent state.[48–50] Rb phosphorylation is known to be a critical modification to enable E2F-dependent transcription of genes required for the S phase.[51–53] Thus, terminal growth arrest in the *wtTHRB*-Ad-infected/irradiated cultures was likely due to the accumulation of cells which did not progress through the G_1 phase of the cell cycle. Cell transduction with mutTHRB evoked the opposite changes in the levels and phosphorylation status of the cell cycle regulators (Fig. 6A and B) which were paralleled by the facilitated cell division and diminished SA-β-gal activity in irradiated cultures (Fig. 4 and 5). Hence, the difference in the effects of wtTHRB and mutTHRB implies the existence of a mechanism by which wtTHRB promotes the acquisition of senescence-like phenotype in irradiated cells.

Senescence is an emerging concept of the alternative to cell death mechanism of growth restriction.[54–56] Terminal growth arrest and senescence have been shown to take place in the absence of apoptosis after irradiation.[36,44,45] It has been also proposed that immortalized cells challenged by chemotherapeutic agents that induce DNA damage, oxidative stress and epigenetic alterations may initiate a latent senescence program.[57,58]

However, molecular changes leading to the induction of cellular senescence after radiation exposure are still far from being understood. The results of our study demonstrate that THRB may be used as a probe to reveal these mechanisms. In the cells with the high levels of endogenous THRB further increase of its expression does not alter clonogenic survival. Perhaps such cells are "adjusted" to the sufficiency of this transcription factor in terms of the spectrum of expressed genes and corresponding intracellular signaling machinery. On the contrary, in the cells with low levels of THRB, its restoration induces changes leading to cell radiosensitization in which post-radiation senescence plays a perceptible role.

Of note, effects of wtTHRB are apparently p53-independent since both ARO that harbor mutant *TP53*[59] and MCF-7 cells expressing wild-type *TP53*[60] (status of the orthologous gene encoding p53 protein in COS7 cells is undetermined to our knowledge) were radiosensitized and displayed elevated numbers of senescent cells after irradiation. This universality may be important in view of the high prevalence of the *TP53* gene mutations in human cancers. Involvement of p53 in cellular senescence has been demonstrated in a large number of experimental works which employed various approaches to manipulation with p53 level and activity in cells of different histogenetic origin. At the same time, exact

mechanisms leading to p53-mediated irreversible growth arrest remain unclear (ref. 61 for review). Recently, evidence of p53-independent pathways potentially contributing to irreversible cell cycle arrest started to accumulate. For example, it has been claimed that induction of senescent state in HeLa cells relied on the activation of the Rb family members but not on p53.[62] Rb activation, however, is not the case in THRB-mediated radiosensitization described in the present work as overexpression of wtTHRB reduced Rb phosphorylation (Fig. 6). In senescent prostate cancer cells, several upregulated genes have been identified, none of which is known to be p53-regulated.[63] In addition, our earlier work demonstrated that senescence-like phenotype could be induced in irradiated thyroid cancer cells with mutant p53.[36] The present investigation adds to this knowledge, suggesting that some pathways influencing radiation-induced senescence may be THRB-dependent in some types of cells regardless of *TP*53 status.

As a whole, our study identifies a previously unknown virtue of THRB to modify radiosensitivity of different cell lines whose common property is a low level of endogenous THRB. Such cell lines transduced with wtTHRB and mutTHRB display a decreased and increased clonogenic survival after irradiation, respectively. Radiosensitization of the cells transduced with wtTHRB is not due to cell death but rather to the decrease in the proliferative potential and acquisition of senescent phenotype. Accordingly, it is tempting to speculate that insufficiency of endogenous THRB or its inactivation due to loss-of-function mutation might be a factor that contributes to cell radioresistance. Our findings also imply that exploration of the changes in gene expression profiles produced by wtTHRB and mutTHRB in the cells with low levels of endogenous THRB may provide further clues to the underlying mechanisms of radiosensitization and radiation-induced cellular senescence.

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