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TGF-β1 accelerates the DNA damage response in epithelial cells via Smad signaling

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ABSTRACT

The evidence suggests that transforming growth factor-beta (TGF-β) regulates the DNA-damage response (DDR) upon irradiation, and we previously reported that TGF-β1 induced DNA ligase IV (Lig4) expression and enhanced the nonhomologous end-joining repair pathway in irradiated cells. In the present study, we investigated the effects of TGF-β1 on the irradiation-induced DDRs of A431 and HaCaT cells. Cells were pretreated with or without TGF-β1 and irradiated. At 30 min post-irradiation, DDRs were detected by immunoblotting of phospho-ATM, phospho-Chk2, and the presence of histone foci (γH2AX). The levels of all three factors were similar right after irradiation regardless of TGF-β1 pretreatment. However, they soon thereafter exhibited downregulation in TGF-β1-pretreated cells, indicating the acceleration of the DDR. Treatment with a TGF-β type I receptor inhibitor (SB431542) or transfections with siRNAs against Smad2/3 or DNA ligase IV (Lig4) reversed this acceleration of the DDR. Furthermore, the frequency of irradiation-induced apoptosis was decreased by TGF-β1 pretreatment *in vivo*, but this effect was abrogated by SB431542. These results collectively suggest that TGF-β1 could enhance cell survival by accelerating the DDR via Smad signaling and Lig4 expression.

1. Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates the growth, differentiation, migration, adhesion, and apoptosis of various cell types [1]. TGF-β transduces signals through two distinct serine-threonine kinase receptors, termed type I (TβRI) and type II (TβRII). Upon ligand binding, TβRII activates TβRI, which then phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3, which interact with the common mediator Smad, Smad4, and move to the nucleus [1,2]. These nuclear Smad complexes interact with various transcription factors and transcriptional coactivators to regulate the transcription of target genes [3].

Radiotherapy is one of the best therapeutic choices for cancer treatment [4]. The cellular response to radiation-induced DNA damage is a complex process involving a wide and integrated array of signal transduction pathways [5]. The primary transducer of genotoxic stress caused by γ-radiation is the nuclear protein kinase, ataxia telangiectasia mutated (ATM) [6]. ATM, which is activated by the double-strand breaks (DSBs) caused by ionizing radiation, phosphorylates numerous substrates, including p53, NBS1, histone 2AX (H2AX), BRCA1, and Chk2 [7–10]. These DNA damage response (DDR) proteins activate a complex program that controls cell cycle checkpoints, apoptosis, and genomic integrity.

Several recent studies have begun to unravel the relationship between TGF-β1 and the irradiation-induced DDR, addressing the ability of TGF-β1 to act as either a radiosensitizer or a radioprotector. As a radiosensitizer, TGF-β1 reportedly mediates radiation-induced apoptosis in different cell types [11,12]. The TGF-β1-targeted signaling molecule, Smad3, has been shown to obstruct the repair of damaged DNA [13] and modulate pro-survival ERK-MAPK signaling [14]. Moreover, the inhibitory Smad, Smad7, has been shown to play a crucial role upstream of ATM and p53 in protecting the genome from γ-irradiation [15]. In the context of TGF-β1 as a radioprotector, studies have shown that TGF-β1 treatment significantly enhances clonogenic survival, reduces DNA strand breaks, and increases p27 expression by inducing G1 arrest [16,17]. TGF-β1 was shown to enhance DNA repair activity through Smad signaling, thereby protecting cells from hyperoxic stress [18]. In addition, inhibition of TβRI was shown to induce radiosensitivity

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[19,20] and attenuate the activation of ATM [21]. Finally, we recently showed that TGF-β1 protects cells against γ-irradiation by enhancing nonhomologous end-joining (NHEJ) repair [22,23]. Despite the existing pool of knowledge, however, it remains unclear exactly how TGF-β signaling regulates the DDR network.

To address this open question, we herein investigated whether TGF-β1 plays a role in the γ-radiation-induced DDR of the epithelial cell lines, HaCaT and A431. Here, we report that TGF-β1 accelerates the DDR, as exhibited by the levels of phospho-ATM, phospho-Chk2, and γH2AX. In addition, we report that the TGF-β1-accelerated DDR is dependent on Smad signaling and DNA ligase IV (Lig4) expression.

2. Materials and methods

2.1. Cell culture, chemicals, and antibodies

The HaCaT (human keratinocyte) and A431 (human epidermoid carcinoma) cell lines (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1X Zell shield (Minerva Biolabs). Cells were pretreated with the TβRI inhibitor, SB431542 (0.5 μM; Tocris) for 1 h, incubated with 0.5 ng/ml TGF-β1 (R&D Systems) for 24 h, and irradiated with 8 Gy using a Gamma-cell 3000 (Atomic Energy) or a BIOBEAM 8000 (Gamma-Service Medical GmbH) with a [^{137}Cs] source. The antibodies against pATM, ATM, pChk2, Chk2, p21, phospho-H2AX (γH2AX), Smad2, and Smad3 were purchased from Cell Signaling; those against Lig4 and β-actin were purchased from Santa Cruz; and that against PCNA was purchased from Dako.

2.2. Immunoblot analysis

Cells were extracted using 1X SDS sample buffer. Equal amounts of proteins were resolved, and immunoreactive proteins were detected using ECL reagents (Amersham Pharmacia Biotechnology) and X-ray films (AGFA).

2.3. siRNA transfection

siRNA transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The cells were transfected with control siRNA, Smad2/3 siRNA (Santa Cruz Biotechnology), or Lig4 siRNA (Bioneer Inc.) at a final concentration of 10 nM.

2.4. In vivo study

Female athymic mice (6 weeks old) were inoculated subcutaneously in the right thigh with 2×10^6 A431 cells/0.2 ml PBS. The tumor volume was calculated using the formula, (length × width^2)/2. When tumors reached a size of 100 mm^3, the mice were randomized into the following four groups (n = 6 mice/group): Group 1 was injected with 20% dimethyl sulfoxide (DMSO) in PBS; Group 2 was injected with 20 μg SB431542/mouse; Group 3 was injected with 15 ng TGF-β1/mouse; and Group 4 was injected with SB431542 (20 μg/mouse) 1 h before injection with TGF-β1 (15 ng/mouse). Half of the animals in each group were locally irradiated with 8 Gy delivered by a Theratron 780 (Atomic Energy of Canada) with a [^{60}Co] source. Twenty-four hours after irradiation, the tumors were removed, fixed with 10% neutral-buffered formalin at RT, and embedded in paraffin. All animal procedures were reviewed and approved by the Institutional Animal Care and Ethics Committee of Korea Institute of Radiation and Medical Sciences.

2.5. TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays were performed using an ApopTag kit (Millipore) in accordance with the manufacturer's instructions. Apoptotic bodies were visualized using a DAB reagent set (KPL).

2.6. Immunohistochemistry

Tissue sections were deparaffinized in a xylene-to-ethanol gradient and rinsed with PBS. The slides were placed in a domestic pressure cooker containing 1 mM EDTA buffer (pH 8.0) and boiled for 15 min. Endogenous peroxidase was blocked by incubating the sections for 10 min with 3% (v/v) H_2O_2 in methanol. The tissues were then rinsed with PBS, blocked with PBS containing 20% (v/v) horse serum, and incubated overnight at 4 °C with anti-PCNA. After a 30-min incubation with diluted biotinylated secondary antibody, immunoreactivity was detected using a Vectastain ABC kit (Vector Laboratories). Sections were washed with PBS, antibody binding was visualized using a DAB reagent set, counterstaining was performed with hematoxylin, and the results were examined using a light microscope.

2.7. Statistical analysis

All data were analyzed using Microsoft Office Excel (Microsoft Corp.) and are presented as means ± standard deviations (SDs). A *p*-value (Student's *t*-test) less than 0.05 was considered significant.

3. Results

3.1. TGF-β1 pretreatment accelerates the γ-radiation-induced DDR

To investigate the mechanism of the DDR, we examined the levels of phospho-ATM, phospho-Chk2, and γH2AX after irradiation. ATM, which is a serine/threonine protein kinase required for the rapid response to γ-radiation-induced DNA damage, can directly phosphorylate Chk2 and H2AX in response to irradiation [24]. As shown in Fig. 1A, the irradiation of epithelial cells increased the phosphorylations of ATM, Chk2, and γH2AX, regardless of TGF-β1 pretreatment. At 30 min after irradiation, however, TGF-β1-pretreated cells exhibited lower levels of phospho-ATM, phospho-Chk2, and γH2AX. Irradiation did not change the total protein levels of ATM or Chk2. The effect of TGF-β1 was confirmed by the expression of p21, a downstream effector of TGF-β signaling.

To further understand the effect of TGF-β1 on irradiated epithelial cells, we investigated whether the TβRI inhibitor, SB431542, could affect γ-radiation-induced DDR. SB431542 alone did not affect the γ-radiation-induced DDR; however, it did abrogate the TGF-β1-induced reductions in phospho-ATM, phospho-Chk2, and γH2AX (Fig. 1B). These results show that TGF-β1 accelerated the radiation-induced DDR, and that this effect was TGF-β receptor-dependent.

3.2. The TGF-β1-accelerated DDR is Smad-dependent

We speculated that the R-Smad proteins, which are well-known downstream molecules of TGF-β1, could be involved in the ability of TGF-β1 pretreatment to accelerate the γ-irradiation-induced DDR. To test this, we examined whether Smad2/3 knockdown interfered with the effect of TGF-β1 on the DDR. Cells were transfected with Smad2/3 siRNAs, pretreated with TGF-β1, and exposed to γ-radiation. As shown in Fig. 2 and consistent with the above-described results, immunoblot analyses showed that TGF-β1 pretreatment resolved the DNA damage within 30 min after irradiation. However,

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