

## Trichostatin A, a Histone Deacetylase Inhibitor, Potentiated Cytotoxic Effect of Ionizing Radiation in Human Head and Neck Cancer Cell Lines

Jin Ho Kim, M.D., M.S.\*; Jin Hee Shin, B.S.<sup>†</sup>, Eui Kyu Chie, M.D., M.S.\*; Hong-Gyun Wu, M.D., Ph.D.\*<sup>†</sup> ‡, Jae Sung Kim, M.D., Ph.D.\*<sup>†</sup> ‡, Il Han Kim, M.D., Ph.D.\*<sup>†</sup> ‡, Sung Whan Ha, M.D., Ph.D.\*<sup>†</sup> ‡, Charn Il Park, M.D., Ph.D.\*<sup>†</sup>, Wee-Saing Kang, Ph.D.\*<sup>†</sup>

\*Department of Radiation Oncology, <sup>†</sup>Cancer Research Institute, <sup>‡</sup>Institute of Radiation Medicine, Medical Research Center, Seoul National University College of Medicine, Seoul, Korea

**Purpose:** We have previously reported that human glioblastoma cells are sensitized to radiation-induced death after their exposure to trichostatin A (TSA), a histone deacetylase inhibitor (HDAC-I), prior to the irradiation. We aimed to measure the magnitude of the radiosensitizing effect of TSA in human head and neck cancer cell lines.

**Materials and Methods:** Human head and neck cancer cell lines, HN-3 and HN-9, were exposed to 0, 50, 100, and 200 nM TSA for 18 hr prior to irradiation. Then, the TSA-treated cells were irradiated with 0, 2, 4, 6, and 8 Gy, and cell survival was measured by clonogenic assay.

**Results:** Pre-irradiation exposure to TSA was found to radiosensitize HN-3 and HN-9 cell lines. In HN-9 cells, the fraction surviving after 2 Gy (SF2) was significantly reduced by treatment of TSA at concentration as low as 50 nM. However, a treatment with 200 nM TSA was required to significantly decrease SF2 in the HN-3 cell line. SER of pre-irradiation treatment with 200 nM TSA was 1.84 in HN-3 and 7.24 in HN-9, respectively.

**Conclusions:** Our results clearly showed that human head and neck cancer cell lines can be sensitized to ionizing radiation by pre-irradiation inhibition of histone deacetylase (HDAC) using TSA, and that this potentiation might well be a general phenomenon.

핵심용어: Trichostatin A, Histone deacetylase inhibitor, Radiosensitization, Head and neck cancer

### Introduction

Histones are building blocks of nucleosomes, and undergo a variety of post-translational modification, such as acetylation, methylation, and phosphorylation. Among these, acetylation of histones is regulated by two classes of counter-acting enzymes: histone acetyl transferase (HAT) and histone deacetylase (HDAC).<sup>1)</sup> Histone acetylation is one of regulatory mechanisms of gene transcription, and changes in acetylation

status of nucleosomal histones are associated with altered expression of specific genes.<sup>2~4)</sup> However, acetylation of non-histone proteins, such as p53, Rb, and E2F1, is also regulated by HAT and HDAC. Acetylation regulates biological functions of target proteins.<sup>5~9)</sup>

Cellular HDAC activity can be modulated by histone deacetylase inhibitor (HDAC-I).<sup>10,11)</sup> HDAC-I has various biological effects, i.e., morphological change,<sup>12)</sup> transcriptional change,<sup>2,13)</sup> cell differentiation,<sup>14)</sup> cell cycle arrest,<sup>15~17)</sup> anti-angiogenesis,<sup>18)</sup> and apoptosis.<sup>19~21)</sup> Notably, HDAC-Is have *in vitro* and *in vivo* anti-tumor activity against transformed cells of various histological origins.<sup>22~29)</sup> As their anti-tumor activity was shown against several human malignant cell lines, HDAC-Is have been investigated for toxicity and anti-tumor effect in clinical trials.<sup>30~35)</sup> Presently, several phase I/II clinical trials using HDAC-I are underway.

Anti-tumor activity of HDAC-I has been extensively

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Reprint request to Jae Sung Kim, Department of Radiation Oncology, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea  
Tel: +82-2-760-2528, Fax: +82-2-765-3317  
E-mail: ihkim@snu.ac.kr  
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investigated. However, studies on the combination of HDAC-I and ionizing radiation are limited. Recently, a few studies reported the *in vitro* radiosensitizing effect of HDAC-I in human malignant cell lines.<sup>36,37</sup> Previously, we reported that pre-irradiation treatment with TSA, the most potent HDAC-I discovered so far, radiosensitized human glioblastoma lines.<sup>38</sup> In this study, we extended our previous results, and tested the effect of TSA on radiosensitivity of two human head and neck cancer cell lines.

## Materials and Methods

### 1. Cell culture

HN-3 and HN-9, human head and neck cancer cell lines, were provided from Dr. EK Choi (Dept. of Radiation Oncology, Asan Medical Center, Seoul, Korea). HN-3 cell line was derived from squamous cell carcinoma of the larynx, and HN-9 from undifferentiated carcinoma of the parotid.<sup>39</sup> Cells were grown as attached monolayers in 25-cm<sup>2</sup> flasks in RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 12.5µg/ml gentamicin (Gibco). Cells were incubated at the exponential growth phase in humidified 5% CO<sub>2</sub>/95% air atmosphere at 37°C.

### 2. Combined treatment of trichostatin A and radiation

Survival of TSA-treated cells was determined by clonogenic assay.<sup>38</sup> Known numbers of HN-3 and HN-9 cells were plated in 25-cm<sup>2</sup> flasks, and treated with 0, 50, 100, and 200 nM TSA (Sigma, St. Louis, MO) for 18 hr. After TSA treatment, the TSA-containing medium was replaced with fresh medium. Cells were then irradiated with 4 MV X-ray from a linear accelerator (Clinac 4/100, Varian Medical Systems, Palo Alto, CA) at a rate of 2.46 Gy/min. Then HN-3 cells were incubated for 14 days, and HN-9 cells for 12 days. After incubation, colonies were fixed with methanol and stained with 0.5% crystal violet.

Since both TSA and ionizing radiation decrease cellular clonogenicity, surviving fraction (SF) of TSA-treated cells was obtained in two ways. First, SF of cells treated with  $x$  nM TSA and irradiated was calculated as follows:

$$\text{Equation1. } SF_{x \text{ nM}, D \text{ Gy}} = \frac{PE_{x \text{ nM}, D \text{ Gy}}}{PE_{0 \text{ nM}, 0 \text{ Gy}}}$$

where  $D$  is the radiation dose in Gy, and  $PE_{x \text{ nM}, D \text{ Gy}}$  is a plating efficacy of cells irradiated with  $D$  Gy following  $x$  nM TSA treatment for 18 hr. When  $D$  is zero, this formula allows for evaluation of cytotoxicity of TSA treatment. Second, SF of cells irradiated after TSA treatment was alternatively calculated as follows:

$$\text{Equation2. } SF_{x \text{ nM}, D \text{ Gy}} = \frac{PE_{x \text{ nM}, D \text{ Gy}}}{PE_{x \text{ nM}, 0 \text{ Gy}}}$$

where  $D$  is the radiation dose in Gy, and  $PE_{x \text{ nM}, D \text{ Gy}}$  is a plating efficacy of cells irradiated with  $D$  Gy following  $x$  nM TSA treatment for 18 hr. Compared to Equation 1, this formula corrects cell survival with TSA cytotoxicity, allowing for evaluation of radiation-induced cell killing only.

SER (sensitizer enhancement ratio) was defined as the ratio of the isoeffective dose in the absence of TSA to that in the presence of TSA. The radiation dose required to achieve a specific SF was calculated from a linear-quadratic model fitted to experimental data using a software (described in *Statistics*).

### 3. Statistics

Confidence intervals of SF were calculated using the  $\chi^2$  distribution. SF was obtained as mean of values from triplicate experiments. Comparison of the SF2 of TSA-treated cells and untreated cells was done using the t-test in SAS (SAS Institute Inc., Cary, NC). Radiation survival data were fitted to a linear-quadratic model by nonlinear regression using JMP5.0.1a (SAS Institute Inc.).

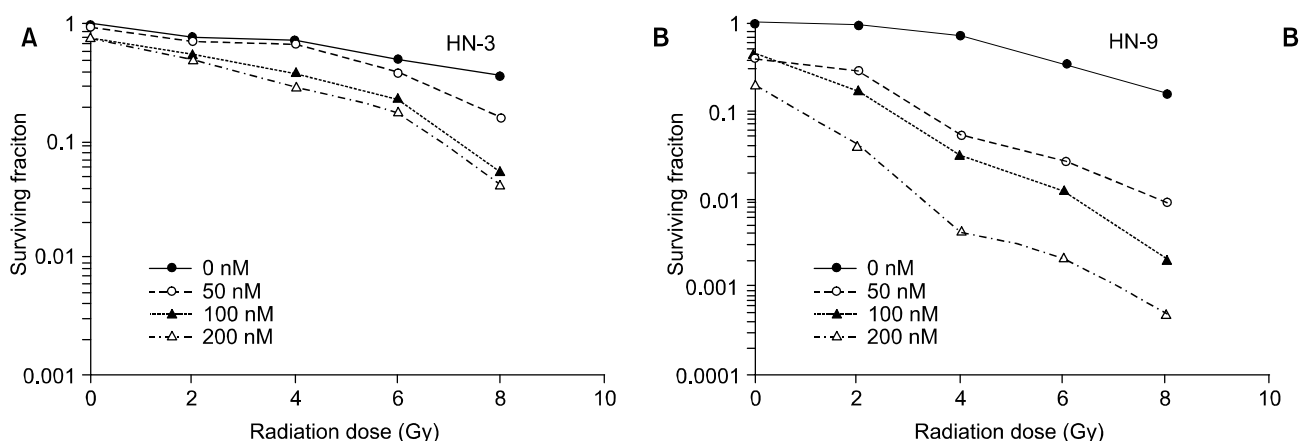
## Results

### 1. Surviving after irradiation alone and TSA effect on cellular clonogenicity

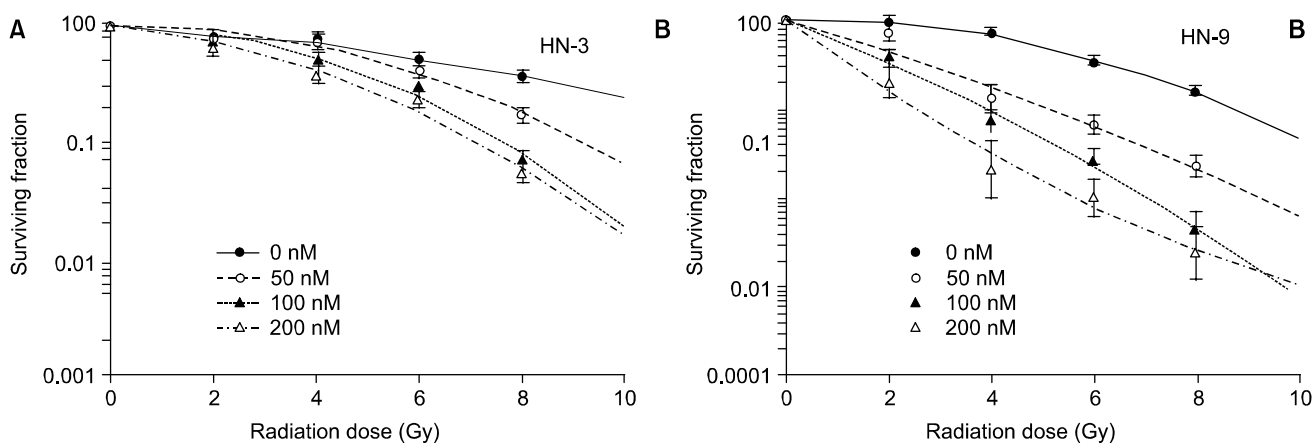
SF2s of untreated HN-3 and HN-9 cell lines were  $0.793 \pm 0.038$  and  $0.957 \pm 0.029$ , respectively (mean  $\pm$  standard deviation). Survival curves are represented in Fig. 1. Two cell lines differed in susceptibility to TSA as well as in radiosensitivity. After exposure to 200 nM TSA for 18 hr, SF was 0.793 in HN-3 and 0.205 in HN-9, respectively (Fig. 1, A and B).

### 2. TSA effect on radiosensitivity in human tumor cells

SF of the TSA-treated cells was corrected for TSA toxicity by Equation 2. Corrected radiation survival curves are



**Fig. 1.** Survival curves of the HN-3 (A) and HN-9 (B) cell lines. Cells were exposed to 0, 50, 100, and 200 nM TSA for 18 hr, and then irradiated with 4 MV X-ray. Surviving fraction was measured by clonogenic assay, but was not corrected by TSA cytotoxicity.



**Fig. 2.** Corrected survival curves of the HN-3 (A) and HN-9 (B) cell lines. Cells were exposed to 0, 50, 100, and 200 nM TSA for 18 hr, and then irradiated with 4 MV X-ray. Surviving fraction was measured by clonogenic assay, and corrected by TSA cytotoxicity. Error bars represent the 95% confidence intervals.

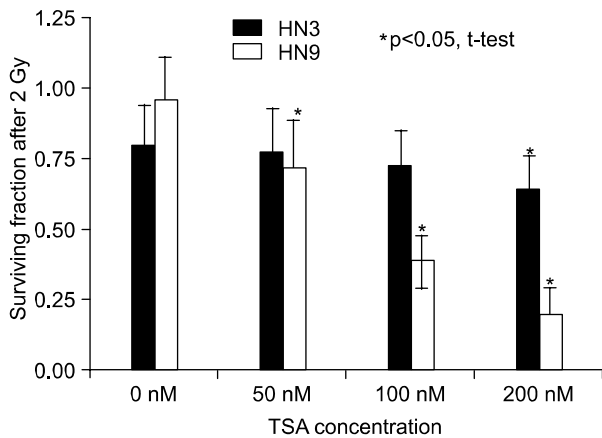
represented in Fig. 2, A and B. Survival curves showed that pre-irradiation TSA treatment sensitized both cell lines to radiation-induced cell killing. In HN-3 cell line, radiation survival curves of TSA-treated cells diverge from that of TSA-untreated control as radiation dose increases. Shift of radiation survival curves in TSA-treated cells was more prominent in HN-9 cell line even at as low radiation dose as 2 Gy. We analyzed difference of SF2 between TSA-treated cells and untreated cells (Fig. 3). SF2 of HN-9 cell line was significantly reduced by pre-irradiation TSA exposure to as low concentration as 50 nM. In HN-3 cell line, 200 nM TSA was required to significantly reduce SF2. SER to obtain SF of 0.5 was estimated in two cell lines (Fig. 4). SER of pre-irradiation treatment with 200 nM TSA was 1.84 in HN-3

and 7.24 in HN-9, respectively.

### Discussion

Our results for the first time showed that by pre-irradiation treatment with TSA at nanomolar concentrations, a potent HDAC-I, potentiated cytotoxic effect of radiation in human head and neck cancer cell lines.

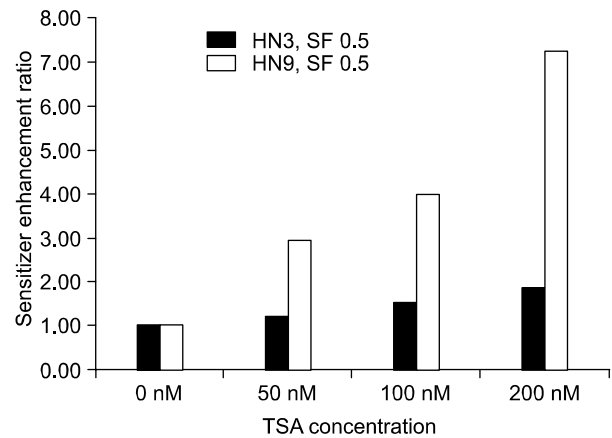
During the last decade, anti-tumor activity of HDAC-I has been a field of extensive investigations, including *in vitro* and animal experiments and clinical trials. However, studies regarding combined effect of HDAC-I and ionizing radiation are very limited. Recently, HDAC-I has been reported to radiosensitize human malignant cell lines. Chung and



**Fig. 3.** Surviving fraction after 2 Gy (SF2) of the HN-3 and HN-9 cell lines. Cells were exposed to 0, 50, 100, and 200 nM TSA for 18 hr, and then irradiated with 4 MV X-ray. Cell survival was measured by clonogenic assay. SF2s of TSA-treated cells were compared with those of untreated cells using t-test. Error bars represent the 95% confidence intervals.

colleagues reported that treatment with phenyl butyrate radiosensitized a nasopharyngeal cancer cell line.<sup>37)</sup> Biade and colleagues reported similar results using a colon cancer cell line and TSA.<sup>36)</sup> Extending their observations, we reported previously TSA radiosensitized human glioblastoma cell lines.<sup>38)</sup> However, a mechanism underlying radiosensitization by HDAC-I is presently an open question.

As there are few reports upon radiosensitizing effect of HDAC-I, we first investigated this effect in cell lines that have not been tested for combined effect of HDAC-I and radiation in previous studies, and our results clearly demonstrated that TSA enhanced radiosensitivity of human malignant cell lines. HN-3 and HN-9 cell lines were radiosensitized by pre-irradiation treatment with TSA (Fig. 2). As a next step, we performed experiments to determine TSA concentration required to sensitize these cell lines. In our experimental settings, minimum concentrations of TSA to significantly reduce SF2 were different between HN-3 and HN-9 (Fig. 3). From our results, we concluded that enhancement of radiosensitivity by TSA was characteristic of individual cell lines. Some points are remarkable in our observations of TSA-treated HN-9 cell line. SF2 of HN-9 cells was further significantly decreased by increasing TSA concentration, from 50 nM to 100 or 200 nM (Fig. 3). Radiation survival curves of HN-9 also suggest a dose-response relationship between TSA concentrations and radio-



**Fig. 4.** The SERs (sensitizer enhancement ratio) of TSA in the HN-3 and HN-9 cell lines. SER was calculated as the ratio of the radiation dose required to obtain surviving fraction (SF) of 0.5 in the absence of TSA to the radiation dose required to obtain the same SF in the presence of TSA. Cells were exposed to 0, 50, 100, or 200 nM TSA for 18 hr, and then irradiated with 4 MV X-ray. Cell survival was measured by clonogenic assay.

sensitization (Fig. 2). Although not explicit, HN-3 cell line showed similar trends. This dose-response relationship was clearly revealed in dependency of SER on TSA concentrations in both cell lines (Fig. 4). Others reported similar observations on dose-response relationships. In a report by Biade and colleagues, pre-irradiation exposure to 50 or 100 nM TSA for 16 hr had no radiosensitizing effect in human colon cancer cells. They reported that SF2 was significantly decreased at TSA concentrations of  $\geq 200$  nM.<sup>36)</sup>

Although the underlying mechanism is not known yet, several potential candidates might be suggested. Since DNA is the principal target of ionizing radiation, DNA repair machineries are closely related with cellular radiosensitivity. ATM and ATR are thought to act as sensors of DNA damage, and there are evidences that HDAC is associated with ATM and ATR *in vitro* and *in vivo*.<sup>40,41)</sup> Kao and colleagues reported that HDAC is involved in DNA repair and G2 checkpoint.<sup>42)</sup> So, it might be hypothesized that HDAC-I interferes HDAC-associated DNA repair machineries. p53, a downstream molecule of ATM, is one of key molecules in cellular response to ionizing radiation. DNA-damaging agents, such as etoposide and ionizing radiation, are known to induce acetylation as well as phosphorylation of p53. Acetylation of p53 enhances its stability and function, and is regulated by balance between HAT and HDAC.<sup>7)</sup> One study showed that

inhibition of p53 by silent information regulator (SIR2) is associated with enhanced cellular survival after DNA-damaging stress.<sup>43)</sup> SIR2 functions as an NAD-dependent HDAC, and its deacetylase activity is not inhibited by TSA.<sup>1)</sup> Another study showed that the abolishment of SIR2 activity significantly enhanced  $\gamma$ -ray-induced cellular lethality.<sup>44)</sup> These results suggest that p53 activity seems to be a key determinant of cellular survival after DNA damage, and that up-regulation of p53 activity by SIR2 inhibition might be a mechanism of enhanced cellular radiosensitivity. Based on such relations, we could postulate that there are at least two pathways of p53 deacetylation, which involve TSA-sensitive and TSA-resistant HDAC, respectively. In our experiment, we used TSA. So, at least one arm of the deacetylation mechanism of p53, possibly SIR2-related, was intact under our experimental conditions. Thus, it suggests that inhibition of both TSA-sensitive and TSA-resistant HDAC may enhance cellular radiosensitivity more effectively than inhibition of either one only.

During the last decade, HDAC-Is have been extensively investigated for potential anti-cancer agents, and some have been tested in clinical trials. Our results extended the scope of biological properties of HDAC-I by demonstrating that HDAC-I may be used as radiosensitizers as well as anti-tumor agents. Since several HDAC-Is are currently under active investigation, it seems quite timely to address the usefulness of HDAC-Is as potential radiosensitizers. Furthermore our results suggest that potentiation of radiosensitivity by addition of HDAC-I might be a general phenomenon. We keep doing our investigation to prove this hypothesis and to elucidate underlying mechanism.

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## 히스톤탈아세틸효소 억제제 Trichostatin A에 의한 인간 두경부암 세포주의 방사선 감수성 증강

서울대학교 의과대학 방사선종양학교실\*, 암연구소†, 의학연구원 방사선의학연구소‡

김진호\* · 신진희† · 지의규\* · 우홍균\*†‡ · 김재성\*†‡ · 김일한\*†‡ · 하성환\*†‡ · 박찬일\*†‡ · 강위생\*‡

**목 적:** 본 연구진이 기왕에 입증한 바 있는 히스톤탈아세틸효소 억제제 trichostatin A (TSA)가 나타내는 방사선 감수성 증강 작용이 두경부암 세포주에서 발생하는 정도를 실험적으로 확인하고자 하였다.

**대상 및 방법:** 인간 두경부암 세포주인 HN-3과 HN-9를 0, 50, 100, 200 nM의 TSA에 18시간 동안 전처리시킨 후 각각 0, 2, 4, 6, 8 Gy 방사선을 조사하였다. 세포생존곡선은 clonogenic assay를 이용하여 산출하였고 linear quadratic 모델에 따라 분석하였다.

**결 과:** 방사선 조사 전 TSA 처리는 HN-3과 HN-9 세포주의 방사선 감수성을 증강시켰다. 50 nM의 TSA로 처리된 HN-9 세포주에서 2 Gy 조사후 생존분획(SF2)은 유의한 수준으로 감소하였으나, HN-3 세포주는 200 nM의 TSA 처리 후 SF2가 유의하게 감소하였다. HN-3과 HN-9 세포주에서 200 nM TSA의 sensitizer enhancement ratio는 각각 1.84와 7.24였다.

**결 론:** 방사선 조사 전 히스톤탈아세틸효소 억제제는 인간 두경부암 세포주의 방사선 감수성을 증가시켰으며, 이 증강 작용이 암세포주에서의 일반적으로 관찰되는 현상일 가능성이 크다고 제안한다.

**핵심용어:** Trichostatin A, 히스톤탈아세틸효소 억제제, 방사선감수성, 두경부암 세포주