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Leptomycin B Increases Radiosensitization by Trichostain A in HeLa Cells

In Ah Kim, M.D., Ph.D.*†§, Jin Ho Kim, M.D.*†, Jin Hee Shin, BSc.*†, Il Han Kim, M.D., Ph.D.*†‡, Jae Sung Kim, M.D., Ph.D.*†§, Hong Gyun Wu, M.D., Ph.D.*†‡, Eui Kyu Chie, M.D.*†, Yong Ho Kim, M.D.*‖, Bo Kyung Kim, M.D.*¶, Semie Hong, M.D.*#, Sung Whan Ha, M.D., Ph.D.*‡ and Chan Il Park, M.D., Ph.D.*†

*Department of Radiation Oncology, †Cancer Research Institute, ‡Institute of Radiation Medicine, Seoul National University College of Medicine, §Department of Radiation Oncology, Seoul National University Bundang Hospital, ‖Department of Radiation Oncology, Soonchunhyang University Hospital, ¶Department of Radiation Oncology, Dankook University Hospital, #Department of Radiation Oncology, Konkuk University Hospital

Purpose: Histone deacetylase inhibitors (HDIs) are emerging as potentially useful components of anticancer therapy and their radiosensitizing effects have become evident. Specific HDIs are now available that preferentially inhibit specific HDAC classes; TSA inhibits Class I and II HDACs, and SK7041 inhibits Class I HDACs.
Materials and Methods: We tested the differential radiosensitization induced by two different classes of HDIs in HeLa cells. We next tested the hypothesis that p53 expression in cancer cells may influence the susceptibility to HDIs by using pharmacologic modification of the p53 status under an isogenic background.
Results: It is interesting that p53 expression in the HeLa cells clearly increased the degree of radiosensitization by TSA compared to that of the class I specific inhibitor SK7041. This suggests that p53 may, in part, be responsible for the mechanistic role for the greater radiosensitization induced by Class I & II inhibitors compared to that of the class I specific inhibitors. Thus, these studies are useful in distinguishing between events mediated solely by the Class I HDACs versus those events involving the other classes of HDACs as well.
Conclusion: The anticancer efficacy of targeting Class I and II HDACs, in conjunction with radiation therapy, may be further enhanced by the restoration of p53 expression.

Key Words: Histone deacetylase inhibitor, Radiosensitization, Leptomycin B, p53

Introduction

Histone deacetylases (HDACs) are intimately linked to chromatin condensation and subsequent gene expression.[1~6] There are three distinct classes of HDACs and abundant evidence suggests the individual HDACs are not redundant in function. Class I HDACs include HDAC 1, 2, 3, and 8 and are generally nuclear proteins homologous to the yeast protein Rpd3.[7,8] Class II HDACs include HDACs 4, 5, 6, 7, 9 and 10 and related HDA1.[7~10] The third class of HDACs are the nicotine adenine dinucleotide-dependent Sir 2 family of deacetylases, which differ from class I and II HDACs in that they are not inhibited by TSA, SAHA or related compound. Sir2p is an NAD+ dependent histone deacetylase required for chromatin-dependent silencing in yeast.[11,12]

Histone deacetylase inhibitors (HDIs) are emerging as potentially useful components of the anticancer therapy as well as useful tools to dissect mechanistic pathways.[1~6] Specific HDIs are now available that preferentially inhibit specific HDAC classes; while TSA inhibits Class I and II HDACs,

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Reprint request to Il Han Kim, Department of Radiation Oncology, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea
Tel: 02)2072-2528, Fax: 02)765-3317
E-mail: ihkim@snu.ac.kr

SK7041 inhibits Class I HDACs.[13] Here we tested the differential cytotoxicity and radiosensitization induced by two different classes of HDIs. More recently increasing evidence has demonstrated HDAC modification of nonhistone substrate[7,9,10] and an involvement in a broad array of biological events including apoptosis[3,14~18] and radiosensitivity.[19~24] Silencing HDAC4, one of the class II HDC via RNA interference decreased levels of p53 binding protein 1 (53 BP1), one of two proteins that interacted with the transactivation domain of p53 aborogated the DNA damage-induced G2 delay, and radiosensitized HeLa cells. They suggest HDAC4 is a critical component of DNA damage response pathway that acts through 53 BP1.[19] HDACs are involved in the regulation of a tumor suppressor protein p53, a key molecule in cellular response to DNA damage,[25~28] and its physical stability and functional activity are regulated by the acetylation which inhibit its ubiquitination and subsequent proteolysis.[29~33] It is also known that HDI augments and prolongs radiation-induced stabilization of p53 in human tumor cell lines.[33,34] We tested the hypothesis that p53 expression in cancer cells may influence susceptibility to HDIs by using pharmacologic modification of p53 status under isogenic background as a gain of function model.

Materials and Methods

1. Cell culture

HeLa Cell lines were purchased from the Korean Cell Line Bank. Cells were cultured at 37°C in water saturated 5% CO_2. Cultures were maintained in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum and 12.5 μg/ml gentamicin (Gibco).

2. Pharmacologic inhibitors

TSA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). SK-7041, a novel class I inhibitor, was kindly provided by Dr. Young Joo Bang, (Internal Medicine, Seoul National University). Leptomycin B, a specific inhibitor of CRM1-mediated nuclear export, was obtained from Sigma. Inhibitors were dissolved as concentrated stock solutions in DMSO, stored at −20°C, and diluted at the time of use in culture medium. Control cells were treated with medium containing an equal concentration of drug carrier, DMSO.

3. Clonogenic assays

A specified number of cells were seeded into each wells of six well culture plates and treated with HDIs (200 nM for TSA and SK-7041, respectively). After exposure for 18 hours, cells were irradiated with 4 MV X-ray from a linear accelerator (Clinac 4/100, Varian Medical Systems, Palo Alto, CA) at a dose rate of 2.46 Gy/min and were incubated for colony formation for 14~21 days. Colonies were fixed with methanol and stained with 0.5% crystal violet, the number of colonies containing at least 50 cells was determined and surviving fraction were calculated. Radiation survival data were fitted to a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software, Reading, PA, USA). Each point on the survival curves represents the mean surviving fraction of triplicate from the experiments repeated three times. Statistical comparisons for differences of the surviving fractions between TSA treated cells and SK7041 treated cells in the presence or absence of LMB was analyzed via ANOVA. Statistical differences in SF2 or SER of TSA treated- vs. SK7041 treated cells in the presence or absence of LMB was analyzed using the *t*-test in SAS v8.01 (SAS Institute Inc. Cary, NC, USA) respectively. Sensitizer enhancement ratio (SER) was defined as the ratio of the isoeffective dose at SF 0.5 in the absence of HDI to that in the presence of HDIs.

4. Western analysis

Cells were washed and scraped and resuspended in lysis buffer (iNtRON Biotechnology, Seoul, Korea). Proteins were solubilized by sonication and equal amounts of protein were seperated on SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk and probed with primary antibody directed against polyclonal rabbit anti-acetyl-histone H3 IgG (Upstate, Lake Placid, NY) at 1 : 1,000 dilution, monoclonal anti-(-tubulin antibody (Sigma, St. Louis, MO) at a 1 : 5,000 dilution and polyclonal rabbit p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed, and then incubated with secondary antibody consisting of peroxidase-conjugated goat anti-rabbit or mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a 1 : 2,000 dilution for 1 hour. Detection of antibody

binding was done using the ECL detection kit from Amersham using the appropriate secondary antibody supplied with the kit.

Results

While trichostatin A (TSA) inhibits both Class I and II HDACs, SK7041 inhibits Class I HDACs, we tested the effects of two different HDIs for radiosensitization of HeLa cell lines via clonogenic survival assays and tested the hypothesis that p53 expression in cancer cells may influence susceptibility to HDIs via pharmacologic modification of p53 expression under isogenic background. HeLa cells were treated with different HDIs either alone or concurrently with LMB, a specific inhibitor of CRM1-mediated nuclear export. Treatment of HeLa cells with 5 μg of LMB for 24 hours lead to a great increase in the transcriptional activity of p53 (shown in western analysis Fig. 1). Fig. 2. shows the level of TSA-induced radiosensitization was much greater in LMB treated HeLa cells (p53 proficient) compared to that of control cell (p53 deficient)($p<0.05$, ANOVA). In contrast, level of radiosensitization by SK7041 was not significantly affected by p53 expression status. Fig. 3. shows the SER of TSA in LMB (+) HeLa cells was higher than that in LMB (−) HeLa cell (2.2 vs. 1.3). SF2 of TSA treated cell was also significantly affected by p53 expression induced by LMB compared to that of SK 7041 treated cells ($p<0.05$, *t*-test)(Fig. 4).

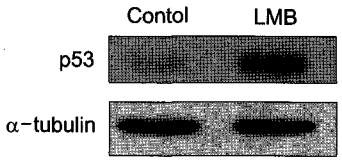

Fig. 1. Leptomycin B leads to a great increase in the transcriptional activity of p53. Treatment of HeLa cells with 5 μg of Leptomycin B (LMB) for 24 hours lead to a great increase in the transcriptional activity of p53 compared to mock treated control cells.

This LMB-mediated p53 expression increased the degree of radiosensitization by TSA. Interestingly, SK7041 treated cells showed more intensive hyperacetylation of histone H3 com-

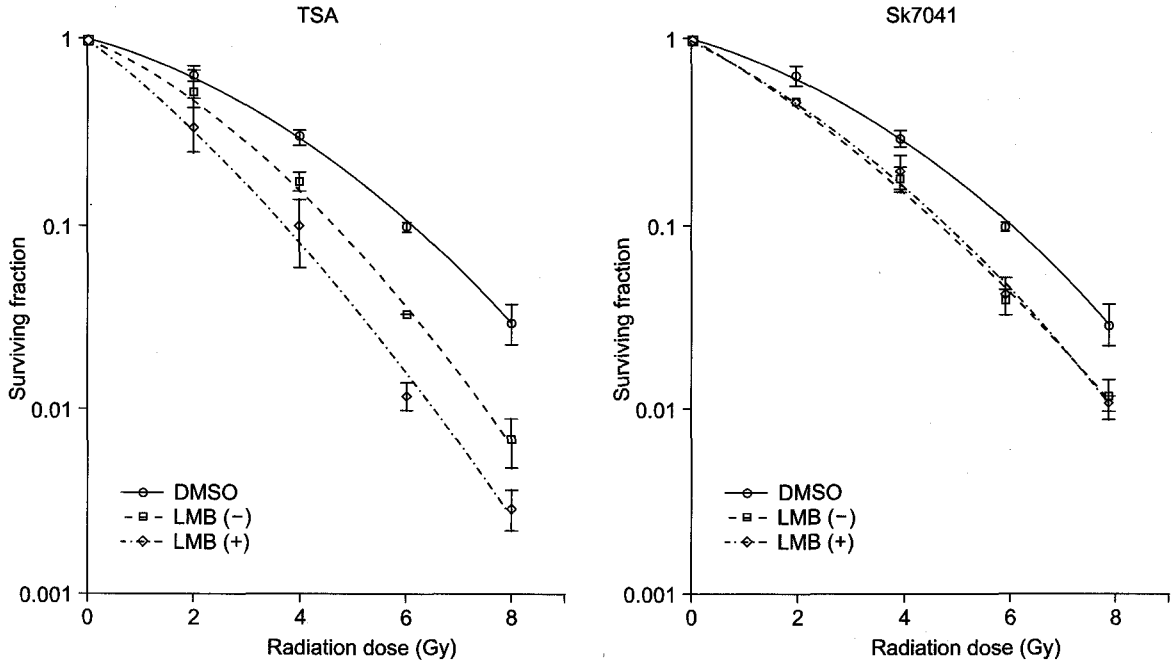

Fig. 2. Clonogenic survival curves for combined treatment of radiation and histone deacetylase inhibitors with or without pretreatment of Leptomycin B. A specified number of cells were seeded into each wells of six well culture plates and treated with HDIs (200 nM for TSA and SK-7041, respectively). After exposure for 18 hours, cells were irradiated and were incubated for colony formation. The level of TSA-induced radiosensitization was much greater in LMB treated HeLa cells (p53 proficient) compared to that of mock treated control cell (p53 deficient). In contrast, level of radiosensitization by SK7041 was not significantly affected by p53 expression status. Each point on the survival curves represents the mean surviving fractions of triplicate from the experiments were repeated three times.

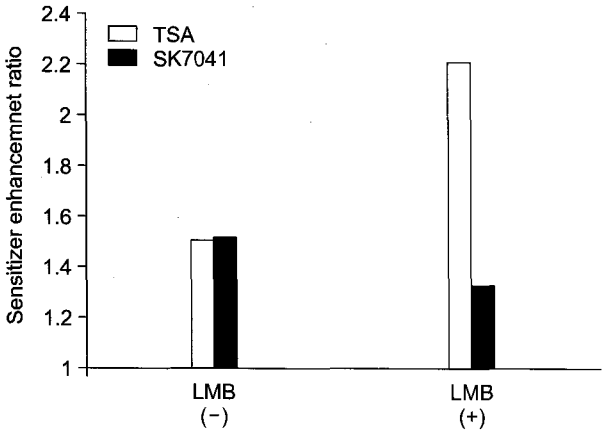

Fig. 3. Sensitizer enhancement ratio. Sensitizer enhancement ratio (SER) was defined as the ratio of the isoeffective dose at SF 0.5 in the absence of HDIs to that in the presence of HDIs. SER of TSA in LMB (+) HeLa cells was higher than that in LMB (+) HeLa cell. In contrast, LMB did not significantly affect SER of SK7041. The experiments were repeated three times. Statistical differences in SER of TSA- treated vs. SK7041-treated cells in the presence or absence of LMB was analyzed using *t*-test.

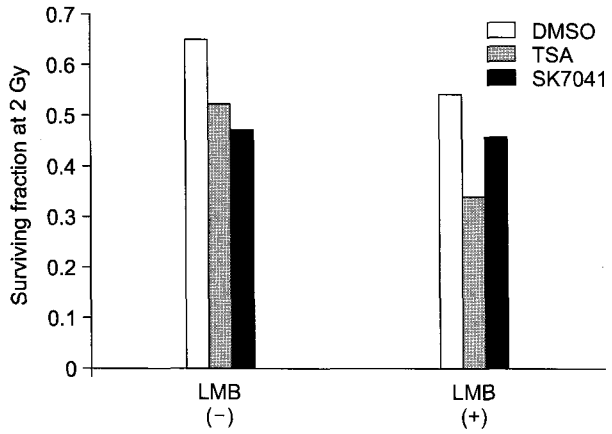

Fig. 4. Surviving fraction at 2 Gy. SF2 of TSA-treated cell was significantly affected by p53 expression induced by LMB compared to that of SK 7041-treated cells. The experiments were repeated three times. Statistical differences in SF2 of TSA-treated vs. SK7041-treated cells in the presence or absence of LMB was analyzed using *t*-test.

pared to TSA. These findings suggest that TSA and Class I specific inhibitor, SK7041 likely have overlapping but nonidentical targets such as nonhistone substrates possibly related to radiosensitivity (Fig. 5).

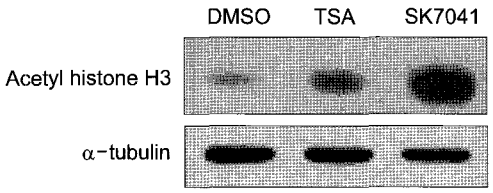

Fig. 5. TSA and SK 7041 leads to hyperacetylation of histone H3 in HeLa cells. SK7041 treated cells showed more intensive hyperacetylation of histone H3 compared to TSA. These findings suggest that TSA and Class I inhibitors, SK7041 likely have overlapping but nonidentical targets such as nonhistone substrates possibly related to radiosensitivity.

Discussion

Chromatin structure plays a major role in regulating the expression of the genetic information encoded in DNA. DNA is packaged into nucleosomes, the repeating units of chromatin, composed of -146 base pairs of two superhelical structure turns of DNA wrapped around an octamer core of pairs of histones H2A, H2B, H3 and H4. The posttranslational modifications in gene regulation include acetylation of lysines, methylation of lysine and phosphorylation of serine. Accumulation of acetylated histones is associated with a neutralization of lysine-positive charge leading to an alteration in chromatin conformation, which may provide for greater access to promoter regions of genes for transcription factor complexes. The pattern of acetylation of histone as well as the pattern of methylation and phosphorylation of histones may represent a "code" that is recognized by nonhistone complexes involved in the regulation of gene expression.[5,6,9,35]

HDACs belong to a deacetylase superfamily that can be divided into three distinct classes.[7~8] Genetic studies using knockout in yeast and RNA interference in mammalian cells, have indicated that the class I HDACs are essential to cell proliferation and survival.[36,37] Class II HDACs differ from class I proteins depending on their tissue expression, subcellular localization and consequently biological roles. Class I HDACs are ubiquitously expressed, whereas class II enzymes display tissue-specific expression in human and mice. Class I HDACs are found almost exclusively in the nucleus, whereas class II HDACs shuttle between nucleus and cytoplasm on certain cellular signals.[7~10] The deacetylase activity of Sir2p role in mammalian cells appears to involve deacetylation of other proteins or transcriptional factors, e.g., p53 rather than

histones. Deacetylation of p53 by Sir2 can induce downregulation of transcriptional and proapoptotic activities of p53 in response to DNA damage.[11,12]

In response to DNA damage, the activity of p53 tumor suppressor is modulated by protein stabilization and post-translational modification including ubiquitination, phosphorylation and acetylation. Both acetylation and ubiquitination can modify same lysine residues at C-terminal regulatory domain of p53 implicating the role of acetylation in the regulation of p53 stability. There is a direct evidence that p53 can be stabilized through its acetylation which inhibit its ubiquitination and subsequent proteolysis induced by mdm2.[32] Endogeneous p53 can be fully acetylated in response to DNA damage when both histone deacetylase complex 1 (HDAC1) - and Sir2-mediated deacetylation are inhibited, indicating dynamic p53 acetylation and deacetylation events during the DNA damage response. Acetylation of endogeneous p53 significantly arguments its sequence specific binding to endogeneous target genes and p53 acetylation levels correlate well with p53-mediated transcriptional activation both in vitro and in vivo.[33] Henderson et al demonstrated that the susceptibility to TSA- and SAHA-induced cell death is regulated by p53.[38] Joseph et al also recently reported that NaB suppresses the growth of WT p53 containing cells more efficiently. NaB treatment lead to a major G (2)/M arrest of cells in the presence of p53, while cells without wild-type p53 accumulated mainly in G (1) phase of the cell cycle. Furthermore, apoptosis induction by NaB is greatly reduced in the absence of p53. These results suggest that p53 pathway mediates in part growth suppression by NaB and the p53 status may be an important determinant of chemosensitivity in HDI based cancer chemotherapy.[39]

Leptomycin B (LMB) is thought to function primarily as an inhibitor of the export of proteins from the nucleus to cytoplasm because of its ability to interact with and impair the function the nuclear export factor CRM-1.[40,41] Although other effects of LMB that could lead to a DNA damage response cannot be excluded, LMB is likely to be a potent inducer of the p53 response, which does not act directly through the DNA damage pathway.[42] p53 induces the expression of an ever-growing number of genes that promote cell cycle arrest, or genes that cause cell death by apoptosis. Since p53 activity has both cytostatic and cytotoxic effect, its levels and activity must be very tightly controlled. This regulation occurs primarily at the level of degradation. In normal non-stressed cells, the half-life of p53 is very short and this is due to an autoregulatory feedback-loop mechanism in which the mdm2 protein plays a key role. The binding of mdm2 to p53 may interfere with the interaction of p53 with the transcription machinery and mdm2 can act as a ubiquitin E3 ligase on p53 and target it for degradation by the proteasome.[33,43,44] LMB was shown to specifically inhibit the function of CRM1 exportin. Treatment of cells with LMB leads to a great increase in the transcriptional activity of p53 in several cell types.[43,45,46] How does LMB affect p53 level and activity? The p53 sequence contains two nuclear export signals that mediate its interaction with CRM1. LMB also decreases the levels of ubiquitinated p53 and it has been suggested that ubiquitination can occur in the nuclear compartment.[47,48]

In this study, TSA-induced radiosensitization was greater in p53 proficient HeLa cell induced by leptomycin B compared to p53 deficient control HeLa cell. In contrast, radiosensitization by SK7041 was not significantly affected by p53 expression status. This suggests that p53 may, in part, be responsible for the mechanistic role in the greater radiosensitization induced by TSA, Class I & II inhibitor compared to that of class I specific inhibitor. Taken together, targeting different classes of HDACs may have different effects for radiosensitization. These studies are therefore useful in distinguishing between events mediated solely by Class I HDACs versus those involving other classes of HDACs as well. The anticancer efficacy of targeting Class I and II HDACs in conjunction with radiation therapy may be further enhanced by restoration of p53 expression.

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국문초록

HeLa세포주에서 Leptomicin B에 의한 Trichostain A의 방사선 감작효과의 증가

서울대학교 의과대학 방사선종양학교실*, 암연구소†, 의학연구원 방사선의학연구소‡,
분당서울대학교병원 방사선종양학과§, 순천향대학교병원 방사선종양학과‖,
단국대학교병원 방사선종양학과¶, 건국대학교병원 방사선종양학과#

김인아*†§ · 김진호*† · 신진희*† · 김일한*†‡ · 김재성*†§ · 우홍균*†‡
지의규*† · 김용호*‖ · 김보경*¶ · 홍세미*# · 하성환*† · 박찬일*†

목 적: 히스톤탈아세틸화효소 억제제는 그 자체의 항암효과뿐만 아니라 방사선 감작제로서의 효과가 점차 분명해져가고 있다. 최근 Class I 특이적인 히스톤탈아세틸화효소 억제제의 개발로 계층 특이적인(class specific) 연구가 가능해짐에 따라, 본 연구에서는 서로 다른 히스톤탈아세틸화효소억제제의 방사선감작효과를 비교함과 동시에 p53 발현도의 차이가 히스톤탈아세틸화효소억제제의 방사선 감수성에 미치는 영향을 알아보고자 하였다.

대상 및 방법: 이를 위해 p53 발현도가 매우 낮은 HeLa 세포에 p53의 핵 외 수송을 억제하여 세포질 내 분해를 차단하는 Leptomycin B를 처리하여 p53의 발현도를 현저하게 높인 후, Trichostatin와 SK7041의 방사선 민감도를 비교 관찰하였다.

결 과: 세포생존곡선, SER 및 SF2를 비교 분석 시, p53의 발현이 높은 Leptomycin B 처리군에서 Trichostatin A가 Class I HDAC만을 억제하는 SK7041에 비해 유의하게 높은 방사선 감작효과를 나타내었다. 이는 p53이 Class I 특이적 억제제인 SK7041과 Class I과 II를 모두 억제하는 TSA의 방사선감작효과에 미치는 영향의 차이에 기전적으로 관여함을 시사한다.

결 론: Leptomycin B에 의해 유도된 p53의 발현증가는 Class I과 Class I과 II를 모두 억제하는 TSA의 방사선 감작효과를 증강시킨다.

핵심용어: Histone deacetylase inhibitor, Radiosensitization, Leptomycin B, p53