Regulation of radiosensitivity by HDAC inhibitor trichostatin A in the human cervical carcinoma cell line Hela

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Summary

Histone deacetylase (HDAC) inhibitors play an important role in inducing growth arrest, differentiation, and/or apoptosis in cancer cells. Given their ability to disrupt critical biological processes in cancer cells, these agents are emerging as potential therapeutics for cancer. Recently, it has been identified that HDAC inhibitors can also efficiently enhance the radiation sensitivity of cells, both in vitro and in vivo. In this study, we investigated whether the potent HDAC inhibitor, Trichostatin A, modulates the radiation sensitivity of the human cervical carcinoma cell line Hela under hypoxic conditions. We concluded that TSA could significantly inhibit the proliferation of Hela cells in a dose-and time-dependent manner under normoxic and hypoxic conditions. Hypoxia resulted in the cervical carcinoma Hela cells resistant to TSA. The findings from clonogenic survival assays indicate that incubation with TSA for 24 hours prior to irradiation enhances the radiation sensitivity of Hela cells under hypoxic conditions. More generally, we found Hela cells under hypoxic conditions treated with TSA could significantly down-regulate the expressions of HIF-1α and VEGF proteins. Taken together, our results demonstrated that TSA acts as a powerful radiosensitizer in Hela cells under hypoxic conditions probably by down-regulated expression of HIF-1α and VEGF proteins.

Key words: Tumor cell; Hypoxia; Radiosensitivity.

Introduction

Hypoxic regions in tumors are a major cause of radiotherapy and chemotherapy failure in solid tumors (chemotherapy and radiotherapy resistant) [1-5]. The hypoxic cells account for 10%-50% in solid tumors and their tolerance to radiation and chemotherapy is 2.5-3 times stronger than that of aerobic cells, which becomes one of the important factors making cancer difficult to cure, and easy to recur and metastasize [6]. Therefore, utilizing cytotoxic drugs in combination with chemoradiotherapy is an important regimen for cancer treatment [7, 8].

Histone acetylation is regulated by a balance between histone acetyltransferase (HAT) and histone deacetyltransferase (HDAC) [9]. While HAT activity relaxes chromatin and promotes transcription by allowing access of transcription factors to DNA, HDAC activity condenses chromatin, leading to transcriptional repression. Both HAT inactivity and HDAC overactivity have been associated with tumorigenesis, presumably because of transcriptional repression of tumor suppressor genes. In the last few years, therapeutic interest on histone deacetylase (HDAC) inhibitors has been rapidly increasing and it has been demonstrated that these drugs, including trichostatin A (TSA), induce growth arrest, differentiation, and/or apoptosis in cancer cells [10-13]. Similar to other anticancer drugs, HDAC inhibitors selectively induce apoptosis in tumor cells: in vitro, the studies show that these cells may be tenfold more sensitive to HDAC inhibitors compared with normal cells. Trichostatin A (TSA), initially isolated as fungistatic antibiotics from streptomyces hygroscopicus, is a potent inhibitor of HDAC activity at nanomolar concentrations [14]. TSA has been suggested to block the catalytic reaction by chelating a zinc ion in the active site pocket through its hydroxamic acid group [15]. Although TSA has been demonstrated to induce apoptosis of various cancer cells and enhance their chemoradiotherapy [16-19], but their effect on the human cervical carcinoma cell line Hela under hypoxic conditions is not clearly known yet. In this study, the human cervical carcinoma cell line Hela was used as an in vitro model to explore the expression differences of hypoxia inducible factor lα (HIF-1α) and vascular endothelial growth factor (VEGF) in Hela cell lines under normoxic and hypoxic conditions and the impact of TSA on the expression. In addition detection of the radiation sensitizing effects of TSA on Hela cell lines under hypoxic conditions by clonogenic survival assays was undertaken so as to provide a new sensitizing means for clinical radiotherapy of cervical carcinoma.

Materials and Methods

Drugs and reagents

TSA was purchased from Sigma Chemical Co. (St Louis, MO, USA); methlthiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were abtained from Sigma Chemical Co.; RPMI-1640 medium was purchased from Gibco (Invitrogen, CA, USA); calf serum from ShangBao Bio-Engineering Co. (Shangbao, Shanghai, China); Mouse anti-human vascular endothelial growth factor monoclonal antibody and KIT-9710 kit from Maixin Bio- Development Co., Ltd. (Maixin, Fuzhou, China); Rabbit anti-human hypoxia-inducible factor-1 alpha polyclonal antibody from Boster Bio-Engineering Co., Ltd. (Boster, Wuhan, China).

Cells and culture conditions

The human cervical carcinoma cell line Hela was kindly provided by Tongji Medical College, Huazhong University of

Revised manuscript accepted for publication July 18, 2011

Science and Technology. Cells were cultured in RPMI1640 culture medium containing fetal calf serum (10%), penicillin (100 u/ml) and gentamicin (40 u/ml) under conventional conditions (37, 5% CO_2); for hypoxic culture, conventionally cultured cells were placed in a hypoxic chamber containing 99.9999% N_2. Cells were preconditioned in hypoxic conditions according to the experimental group. All experiments were carried out in the cell exponential growth phase.

Proliferation assays

Cells under normoxic and hypoxic conditions in the exponential growth phase were collected, adjusted to a cell concentration of 2×10^4/ml with RPMI-1640 medium containing 10% fetal calf serum, and were inoculated onto a 96 well plate, 0.1 ml/well. After cell adherence, different concentrations of TSA (a final concentration of 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 μmol/l) were added, adding the same amount of dimethyl sulfoxide (DMSO) in the control group; the drug of each concentration was inoculated into five holes. After 12, 24, 48 and 72 h under normoxic and hypoxic conditions, 20 μl MTT (5 mg/ml) was added, supernatant was discarded after a 4 h incubation at 37°C, and added with 150 μl DMSO. After full blending, the Multiskan Ascent enzyme-labeled instrument was used to measure the absorbance value at 492 nm wavelength and the cell viability was calculated by the following formula using application software of SPSS13.0 to calculate the 50% inhibition concentration (IC50) and 10% inhibition concentration (IC10). Each experiment was repeated three times.

Cell viability = $(A_{\text{test group}} A_{\text{blankgroup}}) / (A_{\text{control group}} - A_{\text{blank group}}) \times 100\%$.

Clonogenic survival assays

Cells under normoxic and hypoxic conditions in the exponential growth phase were collected and inoculated in a dish 60 mm in diameter, sub into the normoxic irradiation group, hypoxic irradiation group, and hypoxic drug-added irradiation group. After 12 h cell adherence, with a dose rate of 2.5 Gy/min 6MV X-ray irradiation under room temperature, radiation doses were 0, 1, 2, 4, 6 and 8 Gy. After irradiation, the cells were collected immediately with 0.25% trypsin-EDTA, counted and inoculated in a dish 60 mm in diameter by adding 4 ml RPMI-1640 medium containing 10% fetal calf serum and cultured for 14 days. The medium was discarded and the cells were fixed with 95% ethanol for 20 min, stained by solution with crystal violet (0.5%) for 15 min, and then the clone numbers in each dose group were counted (for colonies with more than 50 cells) and the cell survival fraction (SF) was calculated. The experiment was repeated three times. The singlehit multitarget mathematical model was used to draw a dose-survival curve, D_o, D_q and radiosensitization ratios (SER) were obtained. The radiosensitivity of each group was analyzed and compared.

Cell immunocytochemistry

Sterile coverslips were put into a 24-well plate, 1×10^5 cells/well, when the confluence reached approximately 70%. Cells were put into a hypoxic condition and treated with IC10 TSA for 24 h, with cells under a normoxic condition as the control. The coverslips were put into 4% paraformaldehyde for 30 min, then fixed on the slides with glue and blocked for endogenous peroxidase and avidin/biotin.The cells were permeabilized in PBS, incubated in mouse anti-human monoclonal VEGF antibody and rabbit anti-human polyclonal HIF-1α antibody overnight at 4°C. After washing with PBS, cells were stained with secondary antibody for 10 min at room temperature, washed with PBS, and added into horseradwash peroxidase-conjugated avidin. After washing with PBS, DAB was used as the chromogen for active HIF-1α and VEGF cytochemistry. Cell slides were stained with hematoxylin, dewatered conventionally, treated with transferase and sealed. Cell slides were observed and photograph were taken. The location expression of HIF-1α was cytoplasm and (or) nucleus, and VEGF was cytoplasm. There were three cell slides in each group, and immunocytochemistry studies were performed in triplicate.

Immunocytochemical evaluation

The immunocytochemical evaluation was calculated by combining an estimated percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score), as follows [20]: no staining was scored as 0, 1-10% of cells stained were scored as 1, 11-50% as 2, 51-80% as 3, and 81-100% as 4. Staining intensity was rated on a scale of 0 to 3, with 0-5 negative; 1-5 weak; 2-5 moderate, and 3-5 strong. The raw data were converted to the immunocytochemical evaluation by multiplying the quantity and staining intensity of scores. Theoretically, the scores could range from 0 to 12. An immunocytochemical evaluation score of 9-12 was considered strong immunoreactivity, 5-8 was considered moderate, 1-4 was considered weak, and 0 was scored as negative.

Statistical analysis

Experimental data are expressed as mean ± SD and analyzed by the *t*-test. Analysis of variance was used to compare the two groups. SPSS 13.0 statistical software was used and the test level was 0.05.

Results

Effects of TSA on cell growth

TSA significantly inhibited the proliferation of Hela cells in a dose-and time-dependent manner under normoxic and hypoxic conditions as shown in Figure 1. The 50% inhibition concentration and 10% inhibition concentration under normoxic conditions were 0.031 μmol/l, 0.587 μmol/l; the 50% inhibition concentration (IC50) and 10% inhibition concentration (IC10) under hypoxic conditions were 0.076 μmol/l and 0.947 μmol/l. Multivariate analysis of variance of cells under normoxic conditions showed that for different concentrations of the experimental group inhibition rate there was a significant difference ($F = 37.930$, $p = 0.000$), and the different times of experimental groups had a significantly statistical difference ($F = 48.420$, $p = 0.000$); multivariate analysis of variance of cells under hypoxic conditions showed that for different concentrations of the experimental group inhibition rate there was a significant difference ($F = 54.429$, $p = 0.000$), and the different times of the experimental groups had significant statistical difference ($F = 59.398$, $p = 0.000$). According to the experimental requirements, selection of the IC10 of TSA under hypoxic conditions was used as the drug concentration in the follow-up experiments. Futhermore, IC50 of Hela cells under hypoxic conditions were higher than under normoxic conditions, and resulting in chemo-resistance.

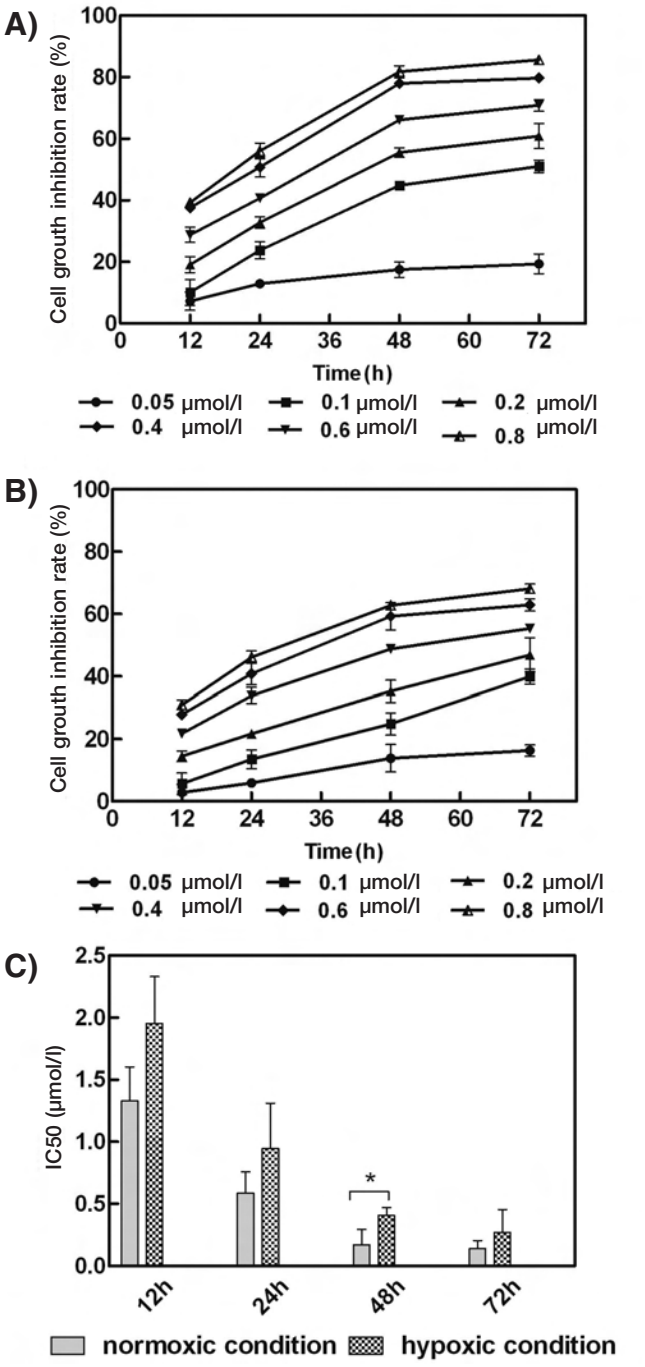
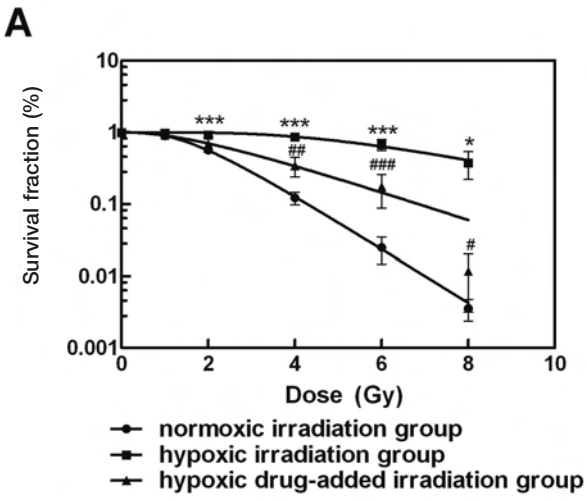


Figure 1. — A) Cell growth inhibition rate of Hela cells under normoxic conditions by different concentrations of TSA. Cells were exposed to TSA for 12, 24, 48 or 72 hours; cytotoxic effects were assessed by methylthiazolyl tetrazolium assay. Means ± SD of three experiments in quadruplicates are shown. B) Cell growth inhibition rate of Hela cells under hypoxic conditions by different concentrations of TSA. Cells were preconditioned in hypoxic conditions, then exposed to TSA for 12, 24, 48 or 72 hours; cytotoxic effects were assessed by methylthiazolyl tetrazolium assay. Means ± SD of three experiments in quadruplicates are shown. C) IC50 of Hela cells under normoxic and hypoxic conditions by TSA for 12, 24, 48 or 72 hours; cytotoxic effects were assessed by methylthiazolyl tetrazolium assay. Means ± SD of three experiments in quadruplicates are shown (hypoxic condition vs normoxic conditions: $*p < 0.05$).



B

	D_0	D_q	SER_{D0}	SER_{Dq}	SER_{SF2}
normoxic irradiation group	1.16	1.57	—	—	—
hypoxic irradiation group	3.24	2.95	—	—	—
hypoxic drug-added irradiation group	2.19	1.64	1.48	1.80	1.34

Figure 2. — A) Surivival fractions of Hela cells under different conditions. Cells of the normoxic irradiation group were directly exposed to X-ray, incubated for 14 days, and survival fraction was calculated by the number of colonies; cell survival curve was fitted by a multitarget-single hitting model. Means ± SD of three experiments in quadruplicates are shown. Cells of the hypoxic irradiation group were preconditioned in hypoxic conditions, and exposed to X-ray, and then incubated for 14 days; surgical fraction was calculated by the number of colonies; cell survival curve was fitted by multitarget-single hitting model. Means ± SD of three experiments in quadruplicates are shown (hypoxic irradiation group vs normoxic irradiation group: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Cells of the hypoxic drug-added irradiation group were preconditioned in hypoxic conditions and given IC10 TSA for 24 hours, then exposed to X-ray, and incubated for 14 days; survival fraction was calculated by the number of colonies; cell survival curve was fitted by a multitarget-single hitting model. Means ± SD of three experiments in quadruplicates are shown (hypoxic drug-added irradiation group vs hypoxic irradiation group: ($\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$). (B) Main parameters of Hela cells after radiation survival curve.

Effect of pretreatment with TSA on radiation sensitivity

The Hela cell line had a D_o value of 1.16 Gy and a D_q value of 1.57 Gy in the normoxic irradiation group, a D_o value of 3.24 Gy and a D_q value of 2.95 Gy in the hypoxic irradiation group, and a D_o value of 2.19 Gy and a D_q value of 1.64 Gy in the hypoxic drug-added irradiation group. Figure 2 shows the radiation survival curves and parameters of Hela in the normoxic group, hypoxic group and hypoxic drug-added group, respectively. Hela cells had a significantly lower radiosensitivity after treat-

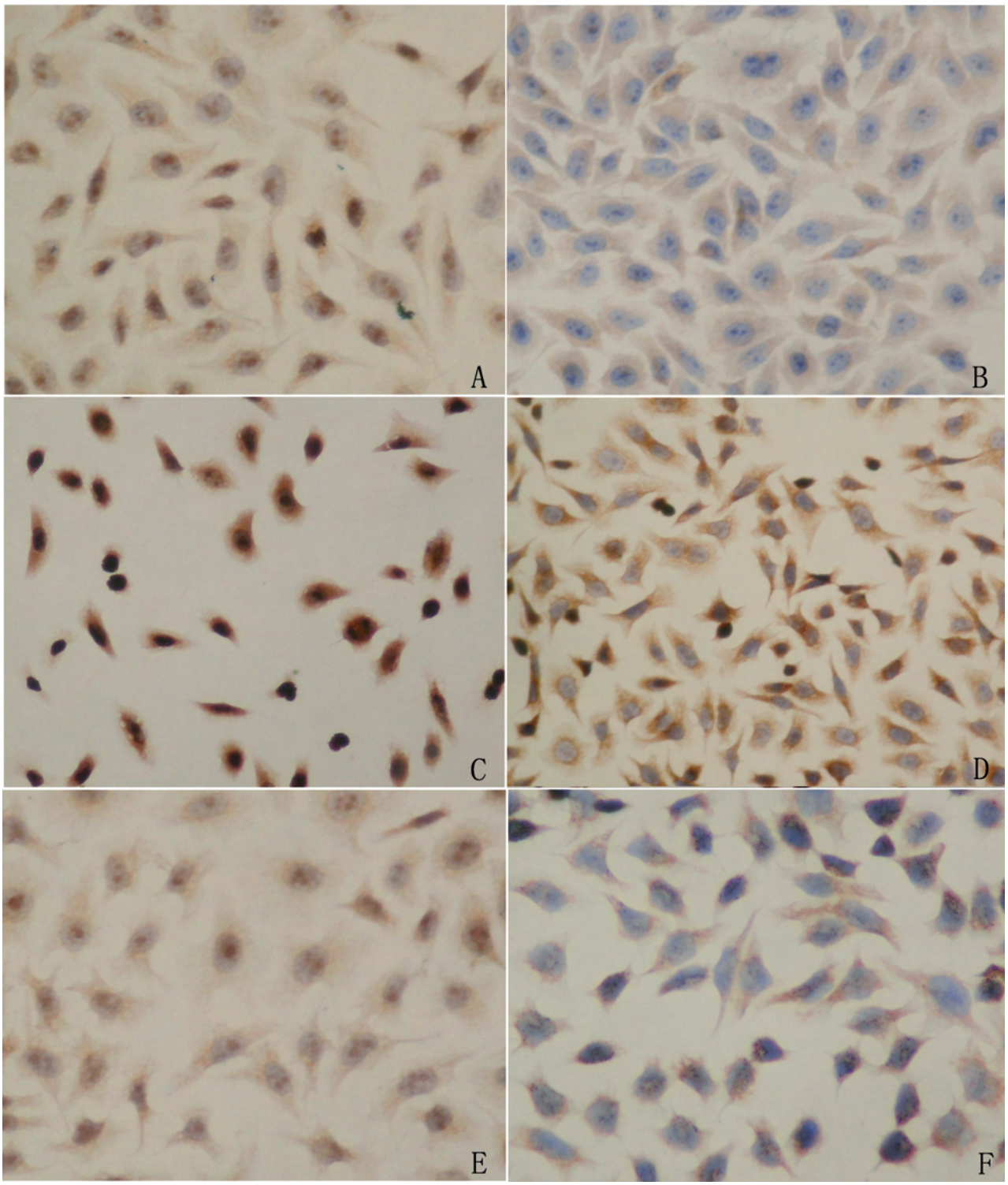


Figure 3. — Expression of HIF-1α and VEGF by an immunocytochemical method (DAB × 400). Cells of the normoxic condition group were collected in the cell exponential growth phase and inoculated into sterile coverslips; when the confluence reached approximately 70%, the expressions of HIF-1 and VEGF proteins were detected by immunocytochemistry (A, B). Cells of the hypoxic condition group were collected in the cell exponential growth phase and inoculated into sterile coverslips; when the confluence reached approximately 70%, the expressions of HIF-1 and VEGF proteins were detected by immunocytochemistry (C, D). Cells of TSA combined with the hypoxic condition group were collected in the cell exponential growth phase and inoculated into sterile coverslips; when the confluence reached approximately 70%, cells were put into a hypoxic condition and treated with IC10 TSA for 24 hours. The expressions of HIF-1 and VEGF proteins were detected by immunocytochemistry (E, F). The location expression of HIF-1 was cytoplasm and (or) nucleus, and VEGF was cytoplasm.

ment by hypoxia ($p < 0.05$), while the radiosensitivity significantly increased after TSA treatment ($p < 0.05$). Compared with the hypoxic irradiation group, the hypoxic drug-added irradiation group SER_{D_0}, SER_{D_q} and SER_{SF2} were 1.48, 1.80 and 1.3, respectively.

Expression of VEGF and HIF-1α proteins

As shown in Figure 3, in the cervical carcinoma cell line Hela under normoxic conditions, expression of HIF-1α in the nucleus was negative (–), in the cytoplasm it was weakly positive (+) (A), and the expression of VEGF in the cytoplasm was weakly positive (+) (B); after preconditioning in the hypoxic condition for two hours, the expression of HIF-1 was strongly positive in the nucleus and cytoplasm (+++) (C), the expression of VEGF was strongly positive in the cytoplasm (+++) (D), the cells were deformed into polygonal shapes and the expression of nuclear particles was increased. As for the role of the hypoxic drug-added group, the cells further deformated, emerged into a membrane dissolution phenomenon, the expression of HIF-1α in the cytoplasm and nucleus was weakly positive (+) (E), and the expression of VEGF in the cytoplasm was weakly positive (+) (F), similar to the normoxic group.

Discussion

HDAC inhibitors including TSA have shown potential as antineoplastic agents for the treatment of many solid and hematological malignancies, and currently there is an intense research focus aimed at developing this new class of targeted anticancer agents [21, 22]. They predominantly act by inducing differentiation, apoptosis, cell-cycle arrest, anti-angiogenic, anti-invasive and immunomodulatory activities related to transcriptional changes with a preferential cytotoxicity for tumor cells.

In this study the results showed that under normoxic or hypoxic conditions the concentration of TSA from 0.05 μmol/l to 0.8 μmol/l induced a dose-and time-dependent inhibition on the human cervical carcinoma cell line Hela. Hela cells under hypoxic conditions performed drug resistance to TSA, while the IC50 of Hela cells under normoxic conditions and hypoxic conditions rose from 0.587 μmol/l to 0.947 μmol/l. Many experiments have produced similar results [23-25]. Hypoxia enhances chemoresistance of cancer cells. First, the delivery of drugs in a hypoxic area and cellular uptake are affected by hypoxia or associated acidity. Second, some chemotherapeutic drugs require oxygen to generate free radicals that contribute to cytotoxicity. Last, hypoxia induces cellular adaptations that compromise the effectiveness of chemotherapy. In response to nutrient deprivation due to hypoxia, the rate of proliferation of cancer cells decreases and chemotherapeutic drugs are more effective against proliferating cells. On the other hand, hypoxia induces adaptation by post-translational and transcriptional changes that promote cell survival and resistance to chemotherapy.

In addition, there is emerging interest in the use of HDAC inhibitors to modulate the effects of irradiation [26-29]. Preclinical studies have also shown that HDAC inhibitors exhibit radiosensitizing effects in a variety of malignancies, such as glioblastoma, non-small cell lung cancer, colorectal cancer, prostate cancer, and metastatic breast cancer. While the mechanism of radiosensitization is not well understood, accumulating evidence suggests that it is in part due to inhibition of DNA DSB repair as evidenced by prolonged expression of γH2AX [30]. Besides influencing repair pathways, HDAC inhibitors can also change the acetylation status of other proteins such as Hsp90. Some authors have attributed the observed radiosensitization to dissociation of EGFR from the Hsp90 complex, resulting in receptor degradation [31]. Furthermore, in preclinical studies HDAC inhibitors appeared to radiosensitize tumor cells without an increase in radiosensitization of normal cells, potentially improving the therapeutic effect. Interestingly, HDAC inhibitors not only act as tumor-selective radiosensitizers, but also as protectors of radiation-induced normal tissue damage. Topical applications of TSA, VPA and NaB were shown to enhance protection from radiation-induced injuries in an animal model of skin radiation syndrome. Although the mechanisms behind this radioprotection remain to be clarified, HDAC inhibitors mediated decrease in the inflammatory cytokine tumor necrosis factor alpha (TNF-β) and transforming growth factors (TGF)-β1 and TGF-β2 could be partly responsible [32].

In this study, we have found that TSA is a potent radiation sensitizer in Hela cells under hypoxic conditions. Compared to the normoxic irradiation group, the D0 value of the hypoxic irradiation group rose from 1.16 to 3.24, and the D_q value rose from 1.57 to 2.95. Compared to the hypoxic irradiation group, the D_o value of the hypoxic drug-added irradiation group decreased from 3.24 to 2.19, and the D_q value decreased from 2.95 to 1.64. In short, data from the clonogenic survival assays indicated that under hypoxic conditions the radiosensitivity of Hela cells was reduced, and exposure of Hela cells under hypoxic conditions to TSA prior to irradiation results in a radiosensitization effect. Previous studies have demonstrated that low doses of TSA as a result of increasing human leukemia cell levels of histone acetylation, enhanced the radiosensitivity to the γ-ray [33].

In an attempt to elucidate the mechanisms by which TSA potentiates the biological effects of irradiation, experiments were performed with immunocytochemistry. Furthermore, the results demonstrated that Hela cells under hypoxic conditions resulted in increased levels of the protein expressions of HIF-1α and VEGF, and pretreatment of Hela cells under hypoxic conditions with TSA resulted in decreased levels of protein expressions of HIF-1α and VEGF. An experiment done on liver cancer cells showed that [34] TSA could activate the expressions of tumor suppressor gene p53 and von Hippel Lindau (VHL) and via the ubiquitin-proteasome pathway reduced hypoxia-induced over-expression of HIF-1α. The low expression of HIF-1α decreased the DNA binding activity of the hypoxia response element (HRE). VEGF as a downstream signaling regulator of HIF-1α reduces expression of HIF-1α affecting expression of VEGF, and thus has targeted anti-tumor activity

and a radiosensitizing effect. Some other studies have demonstrated TSA to be a potential anti-angiogenic agents [35]. TSA reduced the VEGF stimulated human vein endothelial cell invasion of collagen type I-like structures and capillary formation by decreasing the expression of human endothelial growth factor receptor (VEGFR) VEGFR1, VEGFR2, and neuropilin-1.

In summary, we demonstrated that HDAC inhibitor TSA enhanced the radiosensitization of Hela cells under hypoxic conditions via down-regulated expression of HIF-1 and VEGF. In conclusion, the current data provide new evidence for the potential application of combining TSA and X-ray irradiation as a valuable anticancer strategy for cervical carcinoma treatment.

Acknowledgements

Grant support: Scientific and Technological Project of Luoyang (No. 0801059A).

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