Radiosensitization of cervical cancer cells with epigenetic drugs hydralazine and valproate

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Summary

Purpose: To evaluate the radiosensitizing effects of the DNA methylation inhibitor hydralazine in combination with valproic acid, a histone deacetylase inhibitor in cervical cancer cells. *Materials and Methods:* Cell viability assays were performed in the SiHa cervical cancer cell line treated with hydralazine and valproic acid for five days with and without cisplatin. Cell irradiation was performed using teletherapy (1.25 MV). *Results:* Neither hydralazine, valproic acid or cisplatin as single agents increased the cytotoxicity from radiation, however, the combination of hydralazine with valproic acid at ten μM and one mM, respectively, did induce radiosensitization ($p = 0.046$). Interestingly, this effect was further increased with the triple combination of hydralazine, valproic acid, and cisplatin ($p = 0.041$), where cell viability decreased more than 50% as compared to radiation alone. *Conclusions:* The present results demonstrate that epigenetic drugs increase the efficacy of cisplatin chemoradiation in cervical cancer cells.

Key words: Cervical cancer cells; Radiosensitization; Epigenetic drugs; Hydralazine; Valproate.

Introduction

Cervical cancer is the third most commonly diagnosed cancer worldwide and the fourth leading cause of cancer death in females, accounting in 2008 for 9% (529,800) of the total new cancer cases, and 8% (275,100) of the total cancer deaths among females [1].

Pelvic external beam radiation therapy and intracavitary brachytherapy (BCT) continue to be the cornerstone in the primary treatment of locally advanced cervical cancer (FIGO Stages IB2-IVA). In the last decade, the results of radiation treatment were significantly improved with the addition of cisplatin-based chemotherapy concurrent to radiation. This regimen became the standard of care for IB2-IVA patients and was rapidly adopted in clinical practice [2, 3].

Most recently, the addition of gemcitabine to cisplatin-chemoradiation plus two adjuvant courses of cisplatin-gemcitabine increased 9% further the three-year absolute survival [4], however, it appears that toxicity of this regimen may limit further improvements using combinations of classical cytotoxics as radiosensitizers, thereby the need to investigate molecular-targeted agents to be used in combination with radiation, as well as, in the adjuvant setting.

Among molecular-targeted approaches, epigenetic drugs are promising in cervical cancer due to the fact that as many other tumor types, this malignancy has a vast number of epigenetic alterations including the known interaction between human papilloma virus (HPV) oncoproteins with epigenetic machinery players such as DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) [5]. Interestingly, DNMTs and HDACs inhibitors are known to have radiosensitizing properties in head and neck cancer cell lines [6], however, these effects remain to be evaluated in cervical cancer.

The combination of hydralazine, an antihypertensive agent repositioned as DNA methylation inhibitor [7], with valproic acid, a HDAC inhibitor shows inhibitory growth effect *in vitro* and *in vivo*, chemosensitization, synergistic effect on global gene expression [8, 9], up-regulation of class-I human leukocyte antigen expression, and antigen specific cytotoxicity by T-lymphocytes in cervical cancer cells [10]. These data lead the present authors to evaluate whether the combination of hydralazine with valproic acid could show radiosensitizing activity in cervical cancer.

Materials and Methods

Cervical cancer cell line SiHa, was cultured at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10% (v/v) fetal calf serum.

Cell irradiation was performed using teletherapy, in a 15 x 15 cm^2 field size at 80 cm source-to-surface distance (SSD) for viability assays. The absorbed doses evaluated were one, three, and five Gy.

To assess cell viability, cells were seeded into 24-well microtiter plates at a cell density of 2.5 x 10^4 and cultured in complete medium. The next day, cells were treated for five days with hydralazine, valproic acid, or both. Cisplatin was added at day 5 only for 24 hours. Medium with drugs was changed every other day. At day 6 cell viability was measured by conventional crystal violet assay. Briefly, after aspiration of culture medium, surviving cells were fixed and stained with 0.5% crystal violet in 95% ethanol for five minutes and washed with tap water several times. Then one percent sodium dodecyl sulfate (SDS) solution was added to each well to elute the blue dye, and the absorbance of the eluted samples was measured at 595 nm spectrophotometrically, for quantitative evaluation. Assays were performed twice in tripli-

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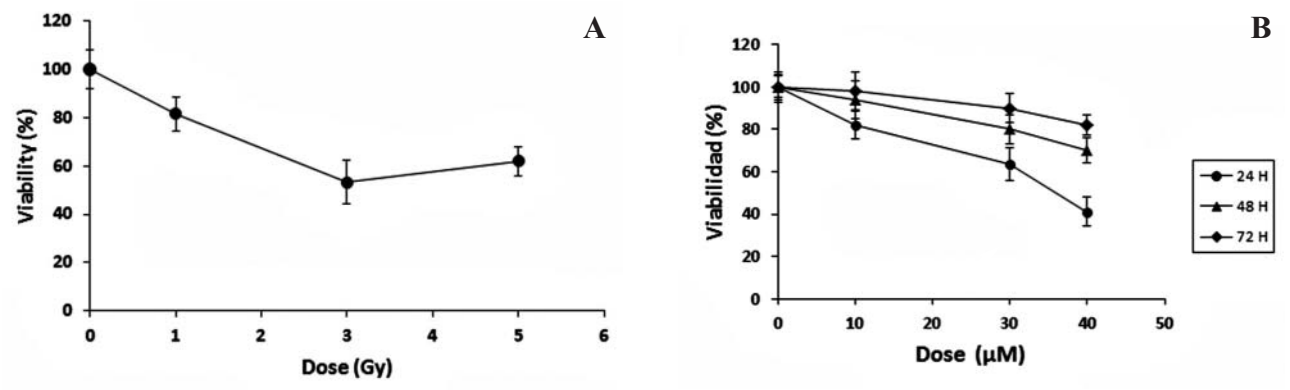

Figure 1. — Dose-effect curve of radiation and cisplatin. A) At 24 hours cell viability decreased 20% at 24 hours after radiation. B) Viability after treatment with cisplatin. The IC10 for SiHa was 5.98 µM at 24 hours.

cate. The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to untreated control cells.

Results

To determine the experimental conditions to test the radiosensitizing properties of hydralazine and valproic acid upon cervical cancer cells, untreated SiHa cells were exposed to one, three, and five Gy of radiation dose. After 24 hours of the radiation exposition, the percentage of cell viability was determined. Figure 1A shows the viability dose curve to radiation. It can be seen that 24 hours after the exposition to one Gy there is an approximate 20% decrease in cell viability, hence this dose was set for further assays.

Likewise, a cisplatin viability dose curve was built to assess the cytotoxic effect of cisplatin. Figure 1B, shows that viability decreased as a function of the cisplatin dose evaluated at ten, 20, and 30 µM at 24, 48, and 72 hours. The IC_{10} for cisplatin for SiHa was 5.98 µM.

To determine whether there was a synergistic effect of epigenetic agents with radiation, and radiation plus cisplatin, SiHa cells were treated with hydralazine (at two different doses), valproic acid and the combination of these two. As shown in Figure 2, hydralazine at any dose, valproic acid or cisplatin did not increase cytotoxicity from radiation. On the other hand, the combination of hydralazine with valproic acid (at ten µM and one mM, respectively) did induce radiosensitization ($p = 0.046$). Interestingly, this effect was further increased with the triple combination of hydralazine, valproic acid, and cisplatin ($p = 0.041$), where cell viability decreases more than 50% as compared to radiation alone.

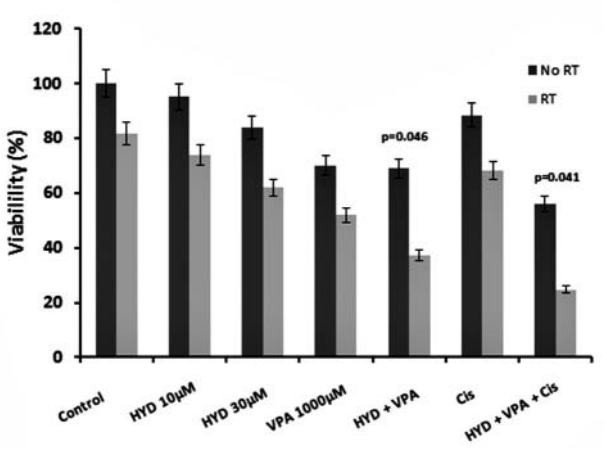

Figure 2. — Radiosensitation with hydralazine and valproate. As single agent neither hydralazine (at both doses), valproic acid nor cisplatin showed increased cytotoxicity as compared to untreated cells. The combination of hydralazine and valproate showed a clear radiosenzitation effect ($p = 0.046$) and it increased further, more than 50% decreased viability when the combination was added to cisplatin ($p = 0.046$).

Discussion

The results of this study show that in SiHa cells, the combination of both epigenetic agents hydralazine and valproic acid increased radiation sensitivity, as shown by the increased cytotoxic effect in comparison with radiation alone. This radiosensitizing effect was further increased with the triple combination of hydralazine, valproic acid, and cisplatin, however, none of these agents showed statistically significant radiosensitization by their own.

Despite the radiosensitizing effects of epigenetic agents, mostly HDACs inhibitors are well documented in literature [11, 12], their clinical efficacy is yet to be evaluated. The authors recently reported the results of a small exploratory study in FIGO Stage IIIB cervical cancer patients who received hydralazine and valproic acid added to standard chemoradiation using weekly cisplatin. An interesting increase in clinical response rate was observed suggesting that epigenetic therapy indeed may increase the efficacy of chemoradiation [13]. Nonetheless, the radiosensitizing effects of this combination had not been tested in cell culture.

Valproic acid on its own has shown radiosensitization properties in a number of cancer cell lines and these effects corre-

late with its ability to increase histone acetylation and the inhibition of DNA double-strand break repair [14-16]. It has also been demonstrated that valproic acid may also induce radiosensitization independent of its transcriptional nuclear effects via acetylation of p53 protein [17-19]. In this regard, it has been demonstrated that valproic acid induces p53 acetylation not only *in vitro* but also in the primary tumors of patients receiving the combination of hydralazine and valproate [9].

DNA methylation inhibitors, on the other hand, have been less tested as radiosensitizers. One of these studies shows that zebularine can enhance tumor cell radiosensitivity *in vitro* and *in vivo* and suggests that this effect may involve an inhibition of DNA repair [20]. 5-Aza-2'-deoxycytidine has also radiosensitization effect in colon, breast [21] and head neck cancer cell lines [6]. In concordance with the findings of this study, the combination of a DNA demethylating agent and a HDAC inhibitor either 5-Aza-2'-deoxycytidine plus TSA LBH589, or MGCD0103 [6] or 5-Aza-2'-deoxycytidine plus butyrate [21] are more effective than that of single agent treatment.

The present results are limited by the fact that only one cervical cancer cell line was tested and that no molecular analyses were performed; however, the combination of hydralazine and valproate has shown in previous clinical trials to induce DNA demethylation and histone hyperacetylation, as well as, to synergize gene expression [8, 9, 22, 23].

In conclusion, the results of this study and published data on hydralazine and valproic acid in cervical cancer support the continuing testing of this epigenetic combination for the treatment of locally advanced cervical cancer.

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