Amifostine Modulates Radio-induced Apoptosis of Peripheral Blood Lymphocytes in Head and Neck Cancer Patients

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Amifostine/Radio-induced apoptosis/Radiation toxicity/Head and neck cancer/Peripheral blood lymphocytes.

Head and neck cancer is treated mainly with surgery and radiotherapy. Xerostomia and mucositis are common adverse effects of radiation therapy. One of the strategies aimed at decreasing radiation toxicity is the use of radioprotective agents, such as amifostine. We previously reported that radio induced apoptosis of peripheral blood lymphocytes was statistically associated with normal tissue toxicity in the form of severe xerostomia. The aim of the present study was to explore the effects of amifostine on the radiation-induced apoptosis of peripheral blood lymphocytes from patients suffering head and neck cancer. Eighteen consecutive patients with squamous cell carcinoma of the head and neck were included in the study. Peripheral blood lymphocytes were isolated before and after the treatment with amifostine. Then, cells were irradiated at 0, 1, 2 and 8 Gy during 24 hours. Apoptosis was measured by flow cytometry using annexin V/propidium iodide. As expected, radio-induced apoptosis values fitted to a semi logarithmic equation as follows: RIA = β ln(Gy) + α. The administration of amifostine prior to radiation therapy modulates radio-induced apoptosis of peripheral blood lymphocytes: 13.68 vs. 13.37 (P = 0.027), 19.11 vs. 17.64 (P = 0.001) and 30.70 vs. 28.84 (P = 0.001), before and after the administration of the drug for 1, 2 and 8 Gy respectively. α and β decreased significantly after the administration of the drug: 13.58 vs. 12.99 (P = 0.009) and 8.21 vs. 7.53 (P = 0.017), respectively. Our results provide new information about the biological actions of amifostine in vivo.

INTRODUCTION

Interpatient heterogeneity in normal tissue reactions due to different treatments varies considerably.1) The treatment of head and neck cancer includes surgery and, in advanced stages, radiation. Patients treated with radiotherapy (RT) will develop clinical toxicity and this may limit the success of the treatment.2) Knowledge of individual variations determining tolerance would be of great value, anyhow, the genetic and molecular mechanisms of therapeutic radiation sensitivity are still poorly understood.3,4) Radiation-induced xerostomia is highly prevalent among head and neck cancer patients treated with RT.5) Studies have quoted 60–90% rates of xerostomia syndrome.6) Survivors experience associated long-term toxicities as dry mouth, sore throat, altered taste, dental decay, changes in voice quality and impaired chewing and swallowing function.7) Treatment for radiation-induced xerostomia is difficult.8) Therefore, the identification of the most sensitive patients to RT would be of great value. Many predictive factors of tumour radiosensitivity have been described, most of them related to gene expression patterns.9) Flow cytometry evaluation of peripheral blood lymphocyte (PBLs) apoptosis has been established as a reliable method to measure radiation-induced damage since 1997.10) We have published that radiation-induced apoptosis (RIA) in PBLs increased in order to radiation dose and fitted to a semi logarithmic model defined by two constants: α (as the origin of the curve in the Y axis determining the percentage of spontaneous cell death) and β (as the slope of the curve determining the percentage of cell death induced at a determined radiation dose). β value was statistically associated with normal tissue toxicity in the form of severe xerostomia.11) Amifostine, designated WR-2721, is a selective radio-protective drug used in both radiotherapy and chemotherapy to reduce normal tissue toxicity.12) The efficacy of
this drug has been a subject of clinical studies in different cancer types.\(^{13}\) It is an effective normal tissue protector supporting its use in head and neck and gynecologic cancers.\(^{14}\) It has been reported that patients with head and neck squamous cell carcinoma treated with amifostine prior to RT had lower incidence of chronic xerostomia.\(^{15-17}\) WR-1065, the active metabolite of amifostine, protects cells from cytotoxic damage by scavenging oxygen-free radicals generated by radiation and anthracyclines and by binding to highly reactive nucleophiles and thus preventing nucleophiles from reacting with DNA.\(^{18}\) It prevents the formation of DNA crosslinks,\(^{19}\) inhibits the enzymatic activity of topoisomerase II\(^{20}\) and has been shown to activate p53 protein, to induce the expression of the cyclin-dependent kinase inhibitor p21, and to arrest cells at the G1/S transition via a p53-dependent pathway.\(^{21}\) The aim of this study was to explore the effects of amifostine on the radiation-induced apoptosis of peripheral blood lymphocytes from patients suffering head and neck cancer.

MATERIAL AND METHODS

Patients and treatment

Eighteen consecutive patients with histologically confirmed squamous cell carcinoma of the head and neck, diagnosed and treated in our institution and given informed consent, were included in the study. The study was approved by the Research and Ethics Committee of our institution. Mean age of patients was 56.39 ± 8.21 years (range 41–70, median 57). Gender of all patients was male. Clinic-pathological characteristics of patients are detailed in Table 1. All patients were treated with surgery before RT. Patients were treated with conventional RT, receiving 1.8–2 Gy per day to a total mean dose of 68.7 Gy (range 60.0–72.2). All patients were treated daily, fifteen minutes before the start of the radiation treatment, with intravenous puncture of amifostine (Ethyol\(^{®}\)) at a dose of 200 mg/m\(^2\), throughout the RT treatment period.

Sample collection

Ten ml of blood were extracted before the administration of the drug (Vacutainer, BD Biosciences, San Jose, CA). Other ten ml of blood were extracted 15 minutes after the treatment. PBLs were isolated by density gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Gibco, Life Technologies, Grand Island, NY, USA) as previously reported.\(^{22}\) The final concentration of cells was adjusted to 2 \times 10^5 cells/ml in complete RPMI, and they were separated into four 25-cm\(^2\) flasks.

Sample irradiation and preparation

Cells were irradiated at room temperature with 1, 2 and 8 Gy, 6 MV × rays (Mevatron, Siemens, Germany) at a dose rate of 50 cGy/min. After irradiation, the preparations were incubated at 37°C in 5% CO\(_2\) during 24 hours. Post incubation, four samples of 1.5 \times 10^5 cells from each flask (one negative control and three samples for triplicate study) were washed, centrifuged and incubated with 5 μl of monoclonal antibody CD45 APC-conjugated monoclonal antibody, permitting the exclusion of erythrocytes and debris, and selecting total lymphocyte population.

Apoptosis assay

The apoptosis analysis was determined by Annexin V kit (Pharmingen, Becton Dickinson) and propidium iodide (PI) as previously reported.\(^{22}\) Flow cytometric analyses were performed on a FACScalibur flow cytometer (Becton Dickinson). Each sample was analyzed using 5000 events/sample acquired in list mode by a Macintosh Quadra 650 minicomputer (Apple Computer Inc., Cupertino). Data analysis was performed via three-step procedure using the Cellquest software (Becton Dickinson). Apoptosis levels were measured at four radiation doses (0, 2, 4, and 8 Gy) in triplicate, before and after the administration of amifostine.

Statistical analyses

Statistical analyses were performed using the SPSS Statistical Package (version 15.0 for Windows). ANOVA and Kruskall-Wallis test were used to compare continuous
variables. Kolmogorov-Smirnoff analysis was made to determine the distribution of data. All tests were two sided and values of $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Radio-induced apoptosis (RIA) could be defined as the percentage of total PBLs death induced by the radiation dose minus the spontaneous cell death (control, 0 Gy: 31.97 ± 11.64 and 31.36 ± 13.12, before and after treatment with amifostine, respectively. $P = 0.815$). RIA increased significantly with radiation dose (1, 2 and 8 Gy) and data followed a normal distribution (Table 2) (Kolmogorov-Smirnoff test, $P > 0.05$).

RIA values fitted to a semi logarithmic equation as follows: $RIA = \beta \ln(\text{Gy}) + \alpha$ (Fig. 1). Alpha ($\alpha$) is defined as the origin of the curve in the Y axis and determinates the percentage of cell death at no radiation dose (spontaneous apoptosis). Beta ($\beta$) is defined as the slope of the curve and determines the percentage of cell death induced at a determined radiation dose. As previously reported, $\beta$ seems to represent an individual marker of radiosensitivity.\(^{11,22}\) $\alpha$ and $\beta$ values followed a normal distribution (Kolmogorov-Smirnoff test, $P > 0.05$). The adjustment coefficients ($R^2$) were determined and data strongly fitted to a semi logarithmic mathematical model, with correlation values of 0.999 and 0.996 before and after the administration of amifostine, respectively (Table 3).

The administration of amifostine prior to RT modulates radio-induced apoptosis of peripheral blood lymphocytes. We observed that RIA values significantly decreased after the treatment with amifostine with independence of the dose (T-test used, Table 2). The strength of this reduction increased with the dose (0.31, 1.47 and 1.86 for 1, 2 and 8 Gy, respectively), and data fitted to a semi logarithmic equation as follows: $y = 0.679 \ln(x) + 0.586$ ($R^2 = 0.795$). This data suggests that the effect of amifostine increases according to radiation dose. The amount of spontaneous apoptosis ($\alpha$) was significantly lower after the treatment with amifostine. In the same way, the percentage of cell death ($\beta$) decreased significantly after the administration of the drug (T-test used, Table 3).

Head and neck cancer is treated mainly by surgery and radiation therapy. Radiotherapy (RT) is associated with several toxicities affecting healthy tissues. Xerostomia and mucositis are common adverse effects of radiation therapy. It has been reported that patients with head and neck squamous cell carcinoma treated with amifostine prior to RT had lower incidence of chronic xerostomia.\(^{15-17}\) Anyhow, prevention of xerostomia and mucositis with amifostine is still

### Table 2. Data of Radio-induced Apoptosis (RIA) of PBLs treated with 1, 2 and 8 Gy of radiation at 24 hours. Mean ± SD were included. RIA data followed a normal distribution.

<table>
<thead>
<tr>
<th>Dose</th>
<th>RIA before Amifostine</th>
<th>RIA after Amifostine</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gy</td>
<td>13.68 ± 3.47</td>
<td>13.37 ± 2.77</td>
<td>0.027</td>
</tr>
<tr>
<td>2 Gy</td>
<td>19.11 ± 5.69</td>
<td>17.64 ± 4.03</td>
<td>0.001</td>
</tr>
<tr>
<td>8 Gy</td>
<td>30.70 ± 6.12</td>
<td>28.84 ± 5.23</td>
<td>0.001</td>
</tr>
</tbody>
</table>

### Table 3. $\alpha$ and $\beta$ constants and adjustment coefficients ($R^2$) before and after the administration of amifostine.

<table>
<thead>
<tr>
<th></th>
<th>Before Amifostine</th>
<th>After Amifostine</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>13.58 ± 3.94</td>
<td>12.99 ± 3.01</td>
<td>0.009</td>
</tr>
<tr>
<td>mean ± SD (range)</td>
<td>(6.88 – 23.09)</td>
<td>(7.67 – 21.37)</td>
<td></td>
</tr>
<tr>
<td>$\beta$</td>
<td>8.21 ± 1.62</td>
<td>7.53 ± 1.98</td>
<td>0.017</td>
</tr>
<tr>
<td>mean ± SD (range)</td>
<td>(5.18 – 11.40)</td>
<td>(4.13 – 10.98)</td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.999</td>
<td>0.996</td>
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controversial. The molecular action mechanisms of amifostine are well known, involving free-radical scavenging, DNA protection and repair acceleration, and induction of cellular hypoxia. It has been published that amifostine, indirectly through hypoxia, may upregulate the expression of a variety of proteins involved in DNA repair and inhibition of apoptosis, such as Bcl-2 and hypoxia-inducible factor-1. However, little is known about the biological effects of the drug on patients. Flow cytometry evaluation of peripheral blood lymphocyte (PBLs) apoptosis has been established as a reliable method to measure radiation-induced damage. We have recently published the capacity of PBLs to predict clinical toxicity due to RT in head and neck cancer patients. For all of this, we explored whether the drug could modify RIA due to RT in PBLs, whereas these cells as important for its ability to predict radiation toxicity. In the present study, a significant regulation of apoptosis in PBLs from patients has been observed for the first time. Amifostine reduced the level of RIA, as well as the levels of spontaneous apoptosis (α), and cell death induced at a determined radiation dose (β). Amifostine has been closely related to cell cycle regulation and apoptosis of cells. WR-1065, the active metabolite of amifostine, reduces the level of phosphorylation and inhibits the enzymatic activity of topoisomerase II. It induces genes involved in cell proliferation, including c-myc and thymidine kinase; iii) and has been shown to activate p53 protein, to induce the expression of the cyclin-dependent kinase inhibitor p21, and to arrest cells at the G1/S transition via a p53-dependent pathway. Finally, p53 protein has been identified as a mechanism of resistance to amifostine-induced apoptosis. These observations could help to understand the reduction of RIA observed in PBLs from head and neck cancer patients treated with amifostine. Independently of the clinical relevance of radiation-induced apoptosis in predicting toxicity as previously described by our group, the present results show that amifostine reduced radiation-induced death, as previously published by others. If this effect is dependent on individual characteristics should be studied in larger series of patients. Taken together, our results provide new information about the biological actions of amifostine in vivo. Thus, further experiments with larger series of patients are needed to validate these results.

ACKNOWLEDGEMENTS

This work was subsidized by FIS Grants 0855/01 and 1621/02. LAHH was supported by a grant from Instituto Canario de Investigación del Cáncer, ICIC.

REFERENCES