Prediction of clinical toxicity in localized cervical carcinoma by radio-induced apoptosis study in peripheral blood lymphocytes (PBLs)

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Abstract

Background: Cervical cancer is treated mainly by surgery and radiotherapy. Toxicity due to radiation is a limiting factor for treatment success. Determination of lymphocyte radiosensitivity by radio-induced apoptosis arises as a possible method for predictive test development. The aim of this study was to analyze radio-induced apoptosis of peripheral blood lymphocytes.

Methods: Ninety four consecutive patients suffering from cervical carcinoma, diagnosed and treated in our institution, and four healthy controls were included in the study. Toxicity was evaluated using the Lent-Soma scale. Peripheral blood lymphocytes were isolated and irradiated at 0, 1, 2 and 8 Gy during 24, 48 and 72 hours. Apoptosis was measured by flow cytometry using annexin V/propidium iodide to determine early and late apoptosis. Lymphocytes were marked with CD45 APC-conjugated monoclonal antibody.

Results: Radiation-induced apoptosis (RIA) increased with radiation dose and time of incubation. Data strongly fitted to a semi logarithmic model as follows: \( RIA = \beta \ln(Gy) + \alpha \). This mathematical model was defined by two constants: \( \alpha \), is the origin of the curve in the Y axis and determines the percentage of spontaneous cell death and \( \beta \), is the slope of the curve and determines the percentage of cell death induced at a determined radiation dose (\( \beta = \Delta RIA/\Delta \ln(Gy) \)). Higher \( \beta \) values (increased rate of RIA at given radiation doses) were observed in patients with low sexual toxicity (Exp(B) = 0.83, C.I. 95% (0.73-0.95), p = 0.007; Exp(B) = 0.88, C.I. 95% (0.82-0.94), p = 0.001; Exp(B) = 0.93, C.I. 95% (0.88-0.99), p = 0.026 for 24, 48 and 72 hours respectively). This relation was also found with rectal (Exp(B) = 0.89, C.I. 95% (0.81-0.98), p = 0.026; Exp(B) = 0.95, C.I. 95% (0.91-0.98), p = 0.013 for 48 and 72 hours respectively) and urinary (Exp(B) = 0.83, C.I. 95% (0.71-0.97), p = 0.021 for 24 hours) toxicity.

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Conclusion: Radiation induced apoptosis at different time points and radiation doses fitted to a semi logarithmic model defined by a mathematical equation that gives an individual value of radiosensitivity and could predict late toxicity due to radiotherapy. Other prospective studies with higher number of patients are needed to validate these results.

Background
Interpatient heterogeneity in normal tissue reactions varies considerably, yet the genetic determinants and the molecular mechanisms of therapeutic radiation sensitivity remain poorly understood [1]. Patients treated with radiotherapy (RT) will develop clinical toxicity and this may limit the efficacy of the treatment [2]. Even with rigid dose tolerance limits, patients respond with different levels of toxicity to a given RT schedule [3]. The treatment of cervical carcinoma includes surgery and/or irradiation. The prediction of the toxicity induced by RT could help to select the most appropriate treatment for each patient. Many predictive factors of tumour radiosensitivity have been described, most of them related to gene expression patterns [4,5]. Intrinsic radiosensitivity is correlated to the ability of the cell to detect and repair DNA damages [6]. Flow cytometry evaluation of lymphocyte apoptosis has been established as a reliable method to measure radiation-induced damage [7]. Quantification of radiation-induced apoptosis (RIA) in peripheral blood lymphocytes (PBLs) has been proposed as a possible screening test for cancer-prone individuals and also for the prediction of normal tissue responses after RT [8]. Previous reports have suggested that PBLs apoptosis could be used to identify radiosensitive patients based on the apoptotic response of T lymphocytes to large in vitro doses [9]. In this way, radiation-induced T-lymphocyte apoptosis can significantly predict differences in late toxicity between individuals [10]. A correlation existed between low levels of RIA in lymphocytes and increased late toxicity after radiation therapy. The radiation-induced apoptotic responses of the CD4 and the CD8 T-lymphocytes from both groups of hypersensitive patients are significantly lower than the responses of the CD4 and the CD8 T-lymphocytes from normal individuals [7]. Moreover, apoptosis in subpopulations of T lymphocytes (CD4+ and CD8+) could be used to identify radiation-sensitive patients before therapy [10]. Development of predictive assays for clinical prediction requires that the diagnostic test employed display both high reproducibility and low variation [11]. The ideal pre-RT predictive assay must be cheap, fast, with low false positives or negatives results and accessible for clinical implementation. Intrinsic radiosensitivity is genetically determined and varies in dependence of the patient and the tumour type. For this, the aim of the present study was to define a radiosensitivity value for each individual patient and to test if such marker of apoptosis will predict the risk of late toxicity in cervical cancer patients treated with radiotherapy in a prospective assay.

Methods
Patients
Ninety four consecutive patients with histological confirmed localized carcinoma of the uterine cervix, diagnosed and treated in our institution between February 1998 and October 2003, and given informed consent, were...

Table 1: Characteristics of the patients in study (n = 94)

| Stage | Cases | Percentages |
|-------|-------|-------------|
| I     | 51    | 52.6        |
| II    | 40    | 41.2        |
| III   | 5     | 15.2        |
| IVA   | 1     | 1.0         |

| Histology | Cases | Percentages |
|-----------|-------|-------------|
| Squamous  | 76    | 76.8        |
| Adenocarcinoma | 23  | 23.2        |

| Grade | Cases | Percentages |
|-------|-------|-------------|
| I     | 11    | 13.1        |
| II    | 41    | 48.8        |
| III   | 32    | 38.1        |

| Treatment | Cases | Percentages |
|-----------|-------|-------------|
| Surgery + RT (posterior) | 22    | 22.2        |
| Surgery + RT (posterior) + Chemotherapy | 7     | 7.1         |
| Radical RT | 19    | 19.2        |
| Radical RT + Chemotherapy | 51    | 51.5        |
recruited prospectively for the study. Apoptosis was determined between April 2003 and March 2004. The study was approved by the Research and Ethics Committee of our institution. Mean age of patients was $51.12 \pm 13.18$ years (range 26-89). Evaluation of clinical toxicity was made, and the mean follow-up was $26.14 \pm 7.61$ months (range 3-73). The majority of patients had squamous cervical carcinoma (76 cases; 76.8%) in early stage of the disease (I (52.68%)-II (40.20%)). Characteristics of patients are detailed in Table 1. A total dose of 45-50 Gy was administered in a 1.8-2 Gy/day schedule followed by brachytherapy treatment at a dose of 20 Gy to a total dose of $69.39 \pm 15.28$ Gy. Sexual, bowel, rectal and urinary toxicity were evaluated according to the LENT SOMA system for recording late effects [12], stratifying toxicity reaction from 0 (no toxicity) to 4 (serious toxicity). Clinical toxicity of patients is detailed in Table 2. Four healthy donors were included as controls. All samples were processed anonymously.

**Sample collection**

A total of 10 ml of blood was collected into lithium heparin venous blood collection tubes (Vacutainer, BD Biosciences, San Jose, CA). PBLs were isolated by density gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Cybco, Life Technologies, Grand Island, NY, USA). Cells were suspended in RPMI 1640 medium (0.05% L-glutamine, 20 nM HEPES, 50 IU/ml penicillin, 50 ug/ml streptomycin and 10% heat inactivated fetal calf serum (FCS)). The final concentration of cells was adjusted to $2 \times 10^5$ cells/ml in complete RPMI, and they were separated into four 25-cm² flasks (three flasks for irradiation and one control flask) with 5 ml of medium.

**Sample irradiation and preparation**

Cells were irradiated at room temperature with 1, 2 and 8 Gy, 6 mV X rays (Mevatron, Siemens, Germany) at a dose rate of 50 cGy/min. After irradiation, the preparations were incubated at 37°C in 5% CO₂ during 24, 48 or 72 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 placed into 5-ml centrifuge tubes and washed once with 3 ml of PBS free of Ca²⁺ and Mg²⁺. The tubes were centrifuged at 500 g for 10 min and the supernatant was removed. Cells were incubated with 5 μl of monoclonal antibody CD45 APC-conjugated monoclonal antibody, permitting the exclusion of erythrocytes, debris, and leukocytes (clone HI30, Pharmingen, Becton Dickinson, San José, CA, USA).

**Apoptosis assay**

The apoptosis analysis was determined by Annexin V kit (Pharmingen, Becton Dickinson, San José, CA, USA) according to manufacturer instructions. The supernatant was decanted and the pellet was resuspended in 100 μl of 1× annexin V binding buffer. Cells were incubated with 4 μl of annexin V-FITC and 10 μl of propidium iodide (PI) for 15 minutes at room temperature in the dark. Finally, 400 μl of 1× annexin V Binding Buffer was added.

**Flow cytometry**

Flow cytometric analyses were performed on a FACScalibur flow cytometer (BD, San Jose, CA) equipped with an argon-ion laser. Each sample was analyzed using 5000 events/sample acquired in list mode by a Macintosh Quadra 650 minicomputer (Apple computer Inc., Cupertino, C). Data analysis was performed via three-step procedure using the Cellquest software (BD, San Jose, CA). Apoptosis levels were measured at four radiation doses (0, 2, 4, and 8 Gy) in triplicate. Detection of phosphatidyl serine exposure on the external leaflet of the plasma membrane employing annexin V was assayed. This phenomenon is one of the early events in the cascade of events characterizing apoptosis.

**Statistical analyses**

Statistical analyses were performed using the SPSS Statistical Package (version 15.0 for Windows). χ² test was used to compare discrete variables. ANOVA and Kruskall-Wallis test were used to compare continuous variables. Cox regression was used for multivariate analysis. 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**

A mathematical model predicts toxicity due to radiotherapy

After irradiation of lymphocytes, three different populations of PBLs were observed: i) early apoptotic cells identified with annexin V, ii) late apoptotic cells stained with propidium iodide (PI) and iii) non-apoptotic cells (Figure 1). Radio-induced apoptosis (RIA) could be defined as the percentage of total PBLs death induced by the radiation

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**Table 2: Toxicity of 94 cervical cancer patients**

| Toxicity | Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|----------|---------|---------|---------|---------|---------|
| Sexual   | 15 (16.0%) | 31 (33.0%) | 32 (34.0%) | 12 (14.9%) | 2 (2.1%) |
| Bowel    | 67 (71.3%) | 21 (22.3%) | 3 (3.2%) | 2 (2.1%) | 1 (1.1%) |
| Rectal   | 70 (74.5%) | 11 (11.7%) | 9 (9.6%) | 3 (3.2%) | 1 (1.1%) |
| Urinary  | 67 (71.3%) | 16 (17.0%) | 8 (8.5%) | 2 (2.1%) | 1 (1.1%) |
dose minus the spontaneous cell death (control, 0 Gy).
RIA data followed a normal distribution (Table 3). RIA values increased with radiation dose (0, 1, 2 and 8 Gy), and fitted to a semi logarithmic equation as follow: RIA = \( \beta \ln(\text{Gy}) + \alpha \) (Figure 2). Alpha (\( \alpha \)) is defined as the origin of the curve in the Y axis and determines 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 (\( \beta = \Delta \text{RIA}/\Delta \ln(\text{Gy}) \)). \( \beta \) seems to represent an individual marker of radiosensitivity. RIA increased significantly with radiation dose and incubation time. The \( \alpha \) value raised with the incubation time (\( p < 0.001 \)) while the \( \beta \) value did not (Table 4). The adjustment coefficients (R) were determined and data strongly fitted to a semi logarithmic mathematical model, with correlation values of 0.99 for 24 and 48 hours and 0.98 for 72 hours (Table 4). \( \beta \) values of 24 h (11.19 ± 4.18), 48 h (9.48 ± 4.40) and 72 h (10.22 ± 5.39) were closely statistically correlated with Pearson test (24 vs. 48 h, \( p < 0.014 \); 48 vs. 72 h, \( p < 0.001 \)). Intraindividual variation for \( \beta \) value for healthy donors was lower than interindividual variation for patients (14.30% vs. 45.49%).

**RIA predicts radio-induced toxicity. Role of \( \alpha \) and \( \beta \) constants**

Sexual, bowel, rectal and urinary toxicity were evaluated according to the Lent-Soma system for recording late effects (Table 2). The majority of patients did not suffer toxicity or suffered low grade of toxicity, especially for gastrointestinal (71.3%), rectal (74.5%) and urinary damage (71.3%). Half of the population in study (48 cases; 51.1%) maintained sexual relations when the analysis were performed. A simple Cox regression analysis or proportional hazards model was performed to evaluate the relationship between \( \beta \) and the different normal tissue toxicity reactions observed. The hazard ratio was also estimated (Table 5). Rectal, urinary and sexual toxicities were significantly predicted by the \( \beta \) constant at different time points. PBLs apoptosis, measured as an integrated value of radiosensitivity (from 1 to 8 Gy), seems to have the potential to predict which patients will be spared late toxicity after radiation therapy. Patients with late toxicity after radiotherapy showed lower lymphocyte apoptotic responses than patients who had not developed late toxicity.

**Discussion**

Normal tissue toxicity due to radiotherapy (RT) limits the efficacy of the treatment. The development of predictive toxicity assays would improve RT results. Analysis of radiation induced apoptosis (RIA) in peripheral blood lymphocytes (PBLs) by flow cytometry seems to be a useful approach to determine individual variability to RT. The present study suggests a new approach to the evaluation of individual radiosensitivity scored by flow cytometry. A mathematical model where RIA and late toxicity were related at different radiation doses and time points was developed. We observed that radiation-induced apoptosis of lymphocytes (constant \( \beta \)) varied between patients, representing an intrinsic condition of each individual. \( \beta \) value predicted all radiation toxicities evaluated but bowel morbidity. This fact could be explained by the

| Dose (Gy) | 24 h | 48 h | 72 h |
|-----------|------|------|------|
| 0         | 25.32 ± 21.31 | 28.33 ± 21.30 | 24.03 ± 19.13 |
| 1         | 31.88 ± 20.24 | 40.29 ± 20.34 | 41.58 ± 21.20 |
| 2         | 35.52 ± 20.78 | 46.68 ± 20.74 | 49.18 ± 21.78 |
| 8         | 46.02 ± 21.11 | 62.63 ± 20.44 | 69.09 ± 19.06 |

| Table 3: Data of apoptosis and radio-induced apoptosis (RIA) of PBLs treated with 0, 1, 2 and 8 Gy of radiation at 24, 48 and 72 hours. Cells isolated from 94 cervical cancer patients. Mean ± SD was included. RIA data followed a normal distribution (Kolmogorov-Smirnoff test, \( p = NS \)) and strongly fitted to a semi logarithmic model |

| Time (hours) | \( \alpha \) (mean ± SD, median, range) | \( \beta \) (mean ± SD, median, range) | R (mean ± SD, median, range) |
|--------------|-------------------------------------|-------------------------------------|-----------------------------|
| 24           | 14.50 ± 9.69, 15.42 (-29-46.7)       | 11.19 ± 4.18, 11.19 (-5.42-23.70)   | 0.94 ± 0.11; 0.99 (0.29-1)   |
| 48           | 25.84 ± 9.99, 26.22 (-5.07-44.44)    | 9.48 ± 4.40, 9.34 (-8.00-19.83)    | 0.96 ± 0.068, 0.99 (0.64-1)  |
| 72           | 28.46 ± 10.76, 30.31 (-0.46-51.32)   | 10.22 ± 5.39, 10.75 (0.24-32.83)   | 0.93 ± 0.15, 0.98 (0.11-1)   |

| Table 4: \( \alpha \) and \( \beta \) constants and adjustment coefficients (R) at 24, 48 and 72 hours |
lower dose of radiation given in the bowel compared with rectum and bladder [13]. This higher dose received in these organs caused higher number of patients with toxicity that can be evaluated (Table 2). Bowel toxicity is infrequent and mild compared with other toxicities [14]. Sexual difficulties after treatment for gynaecological malignancy have received increased attention in recent years. Women receiving primary or adjuvant pelvic radiotherapy experienced greater and more prolonged disruption to their sexual well-being [15]. In fact, sexual toxicity was predicted by $\beta$ values at 24, 48 and 72 hours. Higher levels of $\beta$ values were significantly associated to lower levels of late toxicity. This finding agree with previous studies [7,16] where RIA presented higher levels in healthy patients compared with radiosensitive patients and patients who suffered ataxia-telangiectasia (AT) [9]. This profile was observed in different lymphocyte subpopulations [10,17]. Different molecular and genetic alterations could help to explain those findings; however the mechanism behind the relationship between increased radiation toxicity and reduced apoptotic response in PBLs is still unclear. Lymphocytes from patients who suffered AT, Bloom syndrome, Fanconi anaemia and other syndromes related with radiosensitivity, showed absence of induction of p53 [18,19] and

**Figure 1**
**Determination of apoptosis.** Three different cell populations were detected after the Annexin V/PI staining of isolated PBLs: Alive cells grouped in the lower left part of the panel, early apoptotic cells grouped in the lower right part of the panel and late apoptotic cells grouped in the higher right part of the panel.

**Figure 2**
**Radio-induced apoptosis (RIA) of lymphocytes after 24, 48 and 72 hours.** RIA values at 1, 2 and 8 Gy were adjusted perfectly to a semi logarithmic model where two constants were defined: $\alpha$ is the origin of the curve in the Y axis and determines the percentage of spontaneous cell death and $\beta$ is the slope of the curve and determines the percentage of cell death induced at a determined radiation dose ($\beta = \Delta RIA/\Delta\ln(Gy)$).
list of abbreviations
AT: Ataxia-Telangiectasia; PBLs: Peripheral Blood Lymphocytes; PI: Propidium Iodide; RIA: Radio-induced Apoptosis; RT: Radiotherapy.

competing interests
The authors declare that they have no competing interests.

authors' contributions
EB has written the first draft of the manuscript, has been involved in conception and design of the project and has made all the cell experiments with lymphocytes, irradiation of cells, flow cytometry experiments, data acquisition and statistical analysis. LAHH has written the manuscript, made all the cell experiments with lymphocytes, flow cytometry experiments, data acquisition and statistical analysis. CRG has been involved in flow cytometry experiments as well as in RIA measurements. All authors read and approved the final manuscript.

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