Individualisation of radiotherapy in breast cancer patients: possible usefulness of a DNA damage assay to measure normal cell radiosensitivity

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Abstract

Purpose: The purpose of this study was to determine whether the distribution of sensitivities in breast cancer patients, measured using a DNA damage assay on lymphocytes, is likely to provide sufficient discrimination to enable the reliable identification of patients with abnormal sensitivities.

Material and methods: Radiosensitivity (x) was assessed in 226 samples of lymphocytes from unselected women with breast cancer and was quantified as the initial number of DNA double-strand breaks (dsb) induced per Gy and per DNA unit (200 Mbp).

Results: The existence of an inter-individual variation in the parameter (x) is described through the range (0.40–4.72 dsb/Gy/DNA unit) of values found, which have been fitted to the mathematical model defined by the log–normal distribution (μ = 0.42 ± 0.03; σ = 0.52 ± 0.03; R² = 0.9475). A total of 189 patients received radiotherapy after surgical treatment. Among them, we have detected 15 patients who developed severe skin reactions and we have compared their radiosensitivity values with the rest of patients treated.

Conclusions: Our results suggest that DNA initial damage measured on lymphocytes offers an approach to predict the acute response of human normal tissues prior to radiotherapy. Values of x higher than 3.20 dsb/Gy/DNA unit theoretically should correspond to the highly radio-sensitive patients. Using the experimental results, we have calculated the strength of the test by means of the area under the receiver operator characteristic curves (AZ) to determine whether the radiosensitivity assay can discriminate between patients according to their radiation response. The value found (AZ = 0.675 ± 0.072) is indicative of a fair–poor discriminating capacity of the test to identify the patients with higher risk of developing a severe acute reaction during the radiotherapy treatment. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Breast cancer; Radiotherapy; Cellular radiosensitivity; Normal tissue damage; Predictive assay

1. Introduction

Following radiotherapy, patients show a wide variation in response of both tumour and normal tissues [24,26]. Although a significant proportion of this variation can be attributed to treatment-related factors [25] such as dose inhomogeneity, there is increasing evidence showing that the major factors determining these differences are related to intrinsic biological factors [1].

The ability to predict the determinants of these differences in radiation sensitivity would have important implications with regard to cancer treatment. For example, a strategy based on testing human normal-tissue radiosensitivity to identify the patients with a high risk of developing unacceptable severe reactions after radiotherapy might also permit the individualisation of treatment [5] by dose-escalation in resistant patients without increasing normal-tissue complications [3].

Over the last few years, a number of publications have suggested that this objective may be achieved by assays based on cell or tissue samples withdrawn from the patients prior to radiotherapy treatment [4,8,11]. These recent observations mirror the earlier studies relating tumour cell radiosensitivity to in vivo response [6,7].

However, even if this approach was validated, clinical implementation would be difficult because of the lack of a practical rapid assay of sensitivity. We have previously reported the potential of assays of DNA damage in lympho-
cytes for this purpose [15]. In this paper, we have set out to determine whether the distribution of sensitivities in breast cancer patients measured using this technique is likely to provide sufficient discrimination to enable the reliable identification of patients with abnormal sensitivities.

The three objectives of this study are:

(i) to prove if there is a real and significant variation in the levels of initial damage among lymphocytes taken from different patients and to check the reproducibility of this assay;
(ii) to study the statistical distribution of the values found; and
(iii) to advance the possible applications of the test (ex vivo DNA assay) in the prediction of the normal tissue variations in the response to radiotherapy.

2. Material and methods

2.1. White mononuclear cells from patients and healthy donors

To assess normal lymphocyte cell sensitivity to radiation, between March 1996 and October 1999, we have performed the radiosensitivity assay on a total of 226 samples from the same number of women with breast cancer. A total of 189 of these patients received radiotherapy after surgical treatment. In this subgroup, as well as lymphocyte radiosensitivity, we have also evaluated, using the method proposed by Burnet [5] the early response of skin just at the end of radiation treatment. Ethical committee approval was obtained before starting this work. Blood samples were taken after patients’ informed consent. The samples (5 ml) were obtained by venipuncture, using sodium heparin (10 units per ml) as an anticoagulant and stored at room temperature until use. In no case was the storage period longer than 18 h.

2.2. Radiosensitivity assay

Mononuclear cells were isolated from red blood cells and neutrophils by centrifugation on a Ficoll–Hypaque density gradient (Sigma, USA). The mononuclear cell layer was removed and rinsed twice with Dulbecco’s modified Eagle medium (DMEM), counted under microscope and resuspended in cold DMEM at 4°C. Cell irradiation and radiosensitivity assay was performed as previously described [15].

To assess the reproducibility of the DNA damage assay used in this study, the test has been carried out on eight healthy donors. In this group of persons, we have performed repeated venipuncture (three samples, 5 ml of blood, obtained on 3 different days). Each sample was processed independently in all steps of the experimental procedure.

2.3. Data analysis

The ex vivo normal tissue radiosensitivity measured on lymphocytes is a variable \(x\), whose specific characteristics, are as follows:

1. The value of \(x\) (dsb/Gy/200 Mbp) is a measure of the average of the initial DNA damage expressed as the number of DNA double strand breaks (dsb) induced on the cells per Gy of low LET ionizing radiation and per DNA unit (200 Mbp) [20]. The distribution of values found here is asymmetric (Fig. 1) and the random variable, \(x\), is continuous for the whole range studied.
2. Cells with different sensitivities show different amounts of DNA lesions [14,21].
3. The results obtained for \(x\) are always positive. Negative values lack biological meaning. As \(x\) is the value of DNA dsb induced on each sample, within a particular data set, there will be two limits, a maximum (\(x_{\text{max}}\)) and a minimum (\(x_{\text{min}}\)), represented by the amount of DNA damage produced by the unit of dose in the most sensitive and in the most radioresistant cells, respectively.

We have used the mathematical log–normal model [12] to transform the variable \(x\) to a statistical distribution easier to study. The equation is as follows:

\[
P_X(x) = \frac{1}{x\sigma\sqrt{2\pi}} \exp\left[-\left(\ln x - \mu\right)^2/2\sigma^2\right] \quad x > 0
\]

2.4. Assay reproducibility

The inter-patient variability of the test has been estimated by the coefficient of variation (CV) resulting from the statistical analysis of the measures of DNA damage. This para-

![Fig. 1. Histogram of the experimental distribution of initial number of DNA double strand breaks produced by Gy of low-LET radiation dose and per DNA unit in lymphocytes taken from women with breast cancer. The line included in the graph shows the fit of experimental data to the log–normal distribution used.](image-url)
mometer has been calculated as the ratio between the standard deviation and the mean value of the samples. The intra-individual variability has been estimated from the replicated measurements from the same patient. For this, keeping the experimental conditions constant, we have performed the radiosensitivity test three different times, on different days, on each one of the lymphocyte samples taken from eight voluntary donors. Both inter- and intra-variation coefficients allow us an approach to validate the test statistically.

2.5. Comparison between groups of patients

The collateral acute side-effects of radiotherapy were clinically assessed according to Burnet’s system [5]. Among the breast cancer women included in this work, we have identified 15 patients who developed severe acute skin reactions; we have named that group as highly radio-sensitive patients (skin severe effects: SSE) to distinguish them from the rest of patients treated, named here as normal radio-sensitive (skin normal effects: SNE). According to Burnet’s system, the concept of ‘normal’ range used to describe patients’ normal tissue reactions represents the variation in normal tissue reactions seen in typical radio-therapeutic practice, treating large number of patients. The variation in severity is within clinically acceptable limits, given and doses used, and de variation in normal tissue reactions seen in typical radio-sensitive patients (skin normal effects: SNE). According to them from the rest of patients treated, named here as normal sensitive patients (skin severe effects: SSE) to distinguish skin reactions; we have named that group as highly radio-sensitive patients (skin severe effects: SSE). According to Burnet’s system, the concept of ‘normal’ range used to describe patients’ normal tissue reactions represents the variation in normal tissue reactions seen in typical radio-therapeutic practice, treating large number of patients. The variation in severity is within clinically acceptable limits, given and doses used, and defines the ‘tolerance dose’ of the relevant normal tissues. There are patients in whom reactions are much more severe. Differences in normal cellular and tissue radiosensitivity are of major importance in determining the severity of the normal tissue response, although other factors also have to be considered. To assess whether the results of our test are indicative of the skin response, the intrinsic radiosensitivity measured on lymphocytes from patients included in both groups, SNE and SSE, was compared using the statistical Kruskall–Wallis test.

It is clear that the distributions of the ex-vivo radiosensitivity values corresponding to the normal and the highly radio-sensitivity patients’ populations are, at least in part, overlapped. As a consequence, the number of correct predictions of the normal tissue response – true positives cases: TP – identified by means of the test now depends on the threshold radiosensitivity level chosen; if a very lax criterion (cut-off level very low) is adopted, the true positives could be near 100%, but there is also a high percentage of false positives, FP. If the threshold level adopts a more stringent criterion, the results are quite different with few false positives but many true positives missing.

The receiver operator characteristic curves (ROC) overcome this problem. Using different thresholds, it is possible to obtain successive pairs of false positive and true positive values that can be plotted as a curve. An useful numerical parameter that arise from this graph is the proportion of the ROC space that lies below the ROC curve, $A_Z$. The larger the value of $A_Z$ the greater the separation of the distributions whatever be the experimental situation. This coefficient $A_Z$ has been used to estimate the discriminative power of our radiosensitivity assay.

3. Results

3.1. Initial DNA damage levels in peripheral lymphocytes

We have performed the radiation-induced DNA damage experiments on a total of 226 lymphocyte samples taken from 226 women with breast cancer. For each patient sample, initial dsb number increased linearly with dose with significant differences in the slope ($\xi$) of dose–response straight lines as has been previously shown [15]. Table 1 summarises the results obtained.

The histogram of values found is shown in Fig. 1. Testing for a Gaussian distribution, using the Kolmogorov–Smirnov (KS) distance and the corresponding $P$-value, we can say that the data are inconsistent with a normal distribution. The line on Fig. 1 draws the approach of the experimental values to the theoretical log–normal distribution chosen. We have fitted the empirical histogram to other different distribution functions, i.e. gamma and Weibull distributions, which also fit closely with the experimental values found (data not shown).

Fig. 1 shows the concordance between experimental and the theoretical values. The values of the parameters included in the log–normal equation have been calculated and are: $\mu = 0.42 \pm 0.03; \sigma = 0.52 \pm 0.02$; mean value $= 1.74 \pm 0.06; R^2 = 0.9475$. The uncertainty, when included, is referred to one standard deviation.

Trying to separate normal and severe reactions, we have defined the value $x_m = 3.20$ dsb/Gy/DNA unit as cut-off for the ex-vivo lymphocyte radiosensitivity. This point divides the area under the distribution curve into two portions of different weights on behalf of what the first one contains normal patients and the second, mainly the highly radio-sensitive; what certainly could be arbitrary but reasonable. A quick qualitative impression of the predictive value of the test can be gained from the scatter plot of the quantified observed reaction (normal radio-sensitive and highly

<table>
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<td>Radiosensitivity measured on lymphocytes from breast cancer patients (statistic descriptors)</td>
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<td>Size sample (patients)</td>
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<td>Minimum value</td>
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<td>Median</td>
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<td>Passed normality test ($P &lt; 0.05$)</td>
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<td>Coefficient of variation (%)</td>
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* Radiosensitivity units are: double strand breaks by dose unit and by DNA unit (dsb/Gy/200 Mbp).
radio-sensitive patients) presented in Fig. 2. As result of a perfect test of radiosensitivity, we would expect to be able to separate the people who are developing excessive or unacceptable early reactions after treatment as positive (reaction of sensitivity) and those without collateral effects as negative (reaction of specificity). Results from the cut-off level chosen are shown in Table 2. The positive predictive value is the likelihood that a patient whose result is positive on the test develops an acute severe skin reaction during the radiotherapy course.

We have also compared the radiosensitivity values found in both groups of patients highly and normal radio-sensitive tissues response after radiotherapy, SNE and SSE, using the two-sided Fisher exact test. The result of this comparison gives a $P$ value of 0.0055, the relative risk being 5.059 (95% confidence interval from 1.99 to 22.94). A more detailed information could be extracted from the contingency table analysis performed (Table 2).

To determine whether the radiosensitivity assay can discriminate between the patients according to their radiation response, we have calculated the area under the ROC curve and obtained a value of 0.675 ± 0.072 with 95% confidence interval from 0.534 to 0.817 (Fig. 3). These results are indicative of a fair–poor discriminating capacity of the test to identify the patients with higher risk of developing a severe acute reaction during the radiotherapy treatment.

3.2. Reproducibility of the assay

Table 1 summarises the results obtained measuring the radiosensitivity of lymphocytes from patients. The CV reflects the variability of inter-patient observed (226 patients). The results obtained from the assay performed three times, on different days, on the cells taken from eight volunteers, provides a way to assess the magnitude of errors within the experimental procedure (intra-experimental error). The essential feature of any test aimed at identifying the most highly sensitive patients to radiation treatment is that differences found among individuals must be significantly greater than variation due to technical or sampling errors. The inter-assay coefficients of variation ranged from 2.9 to 23.4%. In Fig. 4, we have included the CV values found for the replicated experiments performed.
with lymphocyte cells. As a comparison, we have also included the inter-patient coefficient of variation (CV = 53%), which is clearly higher than variation among replicates of the same sample measured on different days. To estimate the intra- and inter-individual variability of the assay, we have used the one-way analysis of variance (ANOVA) applied to the results obtained measuring the lymphocyte radiosensitivity three times in each one of the eight healthy donors included in the control group. So, after calculating the intra-assay variation coefficient, we have used the one-way analysis of variance to estimate the intra- and inter-individual variability of the replicates of the same sample measured on different days.

4. Discussion

A key question, when considering the possible clinical relevance of radiosensitivity measurements, is whether differences between replicates from the same patient are due to a real variation in the intrinsic cell sensitivity or to experimental errors. We have discarded the intra-assay variability as a confounding factor and we have accepted the usefulness of the assay. Other authors [8,16,22] also found that the variation due to technical or sampling errors was significantly lower than variation between individuals.

Using lymphocytes as cellular model, we have found a wide variation in the level of initial radiation-induced DNA damage (range from ~0.4 to ~5). These results support the suggestion that variation in cell radiosensitivity can be detected in vitro using radiosensitivity assays on lymphocytes derived from normal tissues of cancer patients prior to radiotherapy. We know that patients with radiosensitive lymphocytes had a significantly increased risk of developing late complications [27]. To assess the predictive value of the DNA radiation-induced damage assay to identify the most sensitive patients, it is necessary to define the normal range of the values. However, the exact proportion of cases in which we could suspect the possibility of an excessive reaction is highly dependent on the level of the ‘cut-off’ used. We can initially choose the limit as a figure that allows us to ensure that about 95% of results belong to a normal range. We have initially considered that, for our series, the range of normality for lymphocyte radiosensitivity might be below 3.20 dsb/Gy/200 Mbp. Higher values than these ones should correspond theoretically to the most sensitive patients in our series.

To assess the discriminatory power of the radiosensitivity test, we have used the Kruskall–Wallis test to do the comparison between the 15 patients displaying severe normal tissue reactions after radiotherapy and the rest of patients (211) included in our series. The mean value and the standard radiosensitivity for result obtained in each group are, $M_1 = 2.435 \pm 0.325$ and $M_2 = 1.648 \pm 0.057$ dsb/Gy/200 Mbp, respectively. The statistical comparison between both groups reveals ($P$-value = 0.0223) that patients who develop acute severe reactions have higher mean value of ex-vivo lymphocyte radiosensitivity.

The main interest of a radiobiological test is to find out the possible correlation between the amount of initial radiation-induced damage on DNA, measured ex vivo, and the tissue (or tumour) response after radiotherapy. If there is a common genetic basis for determining radiosensitivity in an individual, then it could be that the radiosensitivity between different normal cells derived from the same patient would be well correlated [27]. A similar result has been obtained studying CD4 and CD8 lymphocyte subpopulations from the same individuals [17]. The existence of a reasonable correlation between the radiosensitivity of two different normal cells derived from the same patients has been demonstrated in a paired comparison [19]. This has also been suggested by others [10]. However, this finding has not been described universally; some authors have shown that there is no significant correlation between the radiosensitivity of different normal cells derived from the same donors. Kushiro et al. [13] found no correlation between matched samples of peripheral T-lymphocytes and fibroblasts from a total of 22 patients. This absence of

Fig. 4. Inter-assay variation coefficient calculated for three different samples from eight volunteers (L1–L8). The bar named P show the inter-patient variation coefficient. Inter-patient variations are significantly different, $P = 0.0002$. 

### References

correlation most probably derives from the fact that the apparent inter-individual variability of dose–survival curves is caused primarily by random experimental fluctuations at least in the case of lymphocytes. Green et al. [9] found significant differences in radiosensitivity for fibroblasts but not for T-lymphocytes, suggesting limitations in the predictive value of conventional measurement of cell survival. Geera et al. [8] observed no correlation between the radiosensitivity of lymphocyte and fibroblast cultures derived from the same donors perhaps due to tissue-specific characteristics, such as differentiation status, which may variably modulate radiosensitivity. Besides, no correlation has been found between survival fraction at 2 Gy for fibroblast and tumour cells obtained from the same biopsy in carcinoma of the head and neck [23].

It is possible that changes in gene expression, occurring during the time required to grow the cells and to perform clonogenic assays, could be responsible for the lack of correlation found by some authors. We have used a potential predictive assay of radiosensitivity where, in contrast to the process used in clonogenic assays, the cells do not need to be cultured. Lymphocytes are also very attractive for the radiosensitivity test since large number of cells can be readily obtained without causing any pain to the patients. From our point of view, this is one of the most important advantages of this assay. The relationship between lymphocytes and epidermal skin cells taken from the same patient has been previously shown by our group [15]. Finally, two recent manuscripts address the problem of predicting a patient’s risk for severe normal tissue complications following radiotherapy and have described mixed results [2,18].

We have demonstrated an important variation in the susceptibility ex vivo of normal cells against ionising radiation. We can theoretically suggest that the initial radiation-induced damage measured on lymphocytes could be proportional to the acute damage evaluated on the skin of patients treated. Actually, the risk coefficient 5.059 indicates that the probability of developing more severe reactions is higher for the patients with larger values of the radiosensitivity parameter; however, the predictive positive value (0.29) indicates a weak predictive power. The current concept of predictive testing of normal tissue response to individualise radiotherapy prescriptions is founded on the hypothesis that a relationship exists between cellular and normal tissue radiosensitivity. However, the uncertainty in the measure of the radiosensitivity parameter and overall, the lack of a clinical trial on the usefulness of predictive test of normal tissue response force us to be cautious.

On the other hand, taking into account the differences in the lymphocyte radiosensitivity measured (slope of the relationship between DNA dsb induced and dose), it would be possible to get double information: first, steeper slopes are indicative that the threshold will be reached at low dose values and secondly, slighter slopes meaning that a higher dose is necessary to reach the level of severe tissue adverse effects. Moreover, we could suggest that the severe normal tissue reactions appear when initial DNA damage reaches a determined threshold level related to the amount first in the most sensitive patients because their cells suffer most DNA molecular damage per Gy.

If we would know from a radiosensitivity test what is the normal tissue damage threshold level, we could increase the dose in the most resistant patients without reaching the tolerance level and improving the probability of tumour control.

In fact, between the parameters of radiosensitivity, measured in normal and tumour cells belonging to the same patients, a mixed balance can be drawn from some published reports [23,27]. Firstly, the probability of tumour control is mainly dependent on dose administered [14]. Secondly, the probability of severe complications is dependent on both dose and normal tissue radiosensitivity. Finally, the increment of dose in the selected patients must have a positive effect on tumour control probability. If cellular or molecular sensitivity could be measured prospectively, it should be possible to individualise radiotherapeutic treatment according to the predicted sensitivity of the relevant normal tissues. Besides, modifications in the treatment of patients with high risk of complications could be linked to a decrease in the incidence of radiotherapy morbidity, which is another important consequence of the program.

The close relation between chromosome fragment production and killing in many cell systems has been important in linking DNA dsb to death because it is a natural step to relate DNA strand breakage to chromosome breakage. However, the recognition that apoptosis may be an important mode of radiation-induced death in some cell types raise the possibility that other types of damage may induce apoptosis; so we need to consider that although dsb has received the most attention among the various types of radiation-induced DNA damage, other lesions or the repair process may play an important role. It is therefore possible that while dsb are clearly important – and they may be the most important lesion in some tissues – other damage types may significantly trigger the cell death pathways after ionizing radiation treatment [28]. Whether DNA initial radiation-induced damage is related or not to the severity of normal tissue effects after radiation treatment might be better clarified with further research that is currently underway in several centres including ours. Only in this way, it would be possible to prove the benefits described above.

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