DNA damage-related RNA expression to assess individual sensitivity to ionizing radiation

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Predictive markers of intrinsic radiosensitivity in healthy individuals are needed in monitoring their occupational or environmental radiation exposure and may predict a patient’s response to radiotherapy. Ionizing radiation can induce a large spectrum of DNA lesions, but under optimal DNA repair conditions, the principal residual lesions of importance are misrepaired double-strand breaks. The micronucleus (MN) assay represents a useful test in measuring radiosensitivity since it reflects non-repaired DNA breaks at the time of cell division. Spontaneous and radiation-induced MN vary greatly between individuals, and little is known about the molecular mechanisms of this variability. DNA repair and apoptosis processes are involved in the cellular response to radiation-induced DNA damage, and variation in gene expression related to these cellular pathways could be linked to individual radiosensitivity. In this study we analysed by real-time quantitative RT–PCR the basal expression of 12 genes involved both in DNA repair and apoptosis in a series of 32 blood samples obtained from healthy male donors. Relationships between basal RNA expressions and MN frequency and that RAD51 gene expression is negatively correlated with radiation-induced MN frequency.

Introduction

Predictive markers of intrinsic radiosensitivity of healthy individuals may allow the monitoring of occupational or environmental radiation exposure and can also assist the stratification of patients for response to radiotherapy. Radiation can induce a large spectrum of DNA lesions and, in general, damage located on one strand is more efficiently repaired than that affecting both strands, such as double-strand breaks (DSB). It is now accepted that misrepaired DSBs are the main lesions at the origin of both chromosomal abnormalities and gene mutations (1,2). The cytokinesis-blocked micronucleus (MN) assay represents a reliable test to assess radiation-induced chromosome damage (3–5). It detects whole chromosome loss or breaks and reflects the level of non-repaired breaks at the time of cell division. Spontaneous MN in cultured lymphocytes provides an index of cumulative damage occurring during the life span of circulating lymphocytes, whereas the induction of MN following irradiation better reflects cellular radiosensitivity. Radiosensitivity assessed by the MN assay either after in vivo or in vitro irradiation of lymphocytes has shown that a significant variation exists between individuals. Little is known about the mechanisms that are responsible for this variability, but DNA repair and apoptosis processes constitute the main pathways involved in cellular response to radiation. Both mechanisms modify the radiation response by correcting or removing damaged cells. This is supported by studies in mammalian cells indicating that low DNA repair activity is correlated with a high chromosome aberration frequency. On the other hand, several studies have found either a significant (6) or non-significant (7,8) correlation between MN and cell survival. These discrepancies can be explained by either the phase of the cell cycle at the time of irradiation, the capacity of cells to divide in spite of injury and/or the intrinsic radiosensitivity of a given cellular type. Depending on the extent of damage, repair capacity and the effectiveness and duration of cell cycle blockage, cells will generate MN or undergo apoptosis. Studies have shown controversial results concerning the correlation between apoptosis and MN frequency (9,10).

Since the relationships between individual polymorphism and association with radiosensitivity are not yet understood, we investigated, by real-time quantitative RT–PCR, the basal expression of 12 genes involved both in DNA repair and apoptosis pathways in a series of 32 blood samples. The aim of this study was to determine the biological significance of these gene expressions in the measurement of radiosensitivity estimated by the MN assay after ex vivo irradiation of G0–G1 lymphocytes obtained from healthy male donors.

Materials and methods

Subjects and blood samples

Thirty-two healthy male donors regularly examined by medical staff at our institution, who presented no evidence of genetic disorders nor any prior history of exposure to known genotoxic compounds, comprised the subjects of this study. Informed consent was obtained from all donors after a thorough explanation of the nature and objectives of the study.

Whole blood samples (14 ml) were collected into heparinized tubes and transported to the laboratory. On receipt, blood samples were divided into three heparinized tubes (2 ml/tube) for subsequent irradiation (including control), 2 ml were directly used for lymphocyte isolation and RNA extraction (see below) and the remainder was used for other work.

Irradiation and MN assay

Irradiation was performed at 37°C, at doses of 0, 0.5 and 2 Gy at a dose rate of 1 Gy/min (cobalt 60 γ rays). Just after irradiation, 0.5 ml whole blood was used for MN assay according to the cytochalasin-B-blocked assay described by Fenech and Morley (11). Briefly, total blood samples were cultivated in 15 ml tubes containing 6 ml 199 Glutamax-1 culture medium (Life Technologies, Cergy-Pontoise, France), 2 ml human serum AB (Reims Bio, Reims, France), 20 IU heparin, 100 IU/ml penicillin/streptomycin (Life Technologies) and 50 μl phytohemagglutinin C (PHA-C) (Biosepra, Villeneuve la Garenne, France) at 37°C in a 95% air plus 5% CO2 incubator. After 44 h of stimulation, cells were treated for 28 h with 5 μg/ml cytochalasin-B (Sigma, Saint Quentin

Abbreviations: BN, bi-nucleated cells; DSB, double-strand breaks; MN, micronucleus.
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RNA extraction and RT–PCR

On receipt, total RNA extraction was performed using an RNAplus kit (Bioprobe, Illkirch, France) on peripheral blood lymphocytes, obtained after density centrifugation, using Ficoll-Isopaque solution (Sigma) from 2 ml fresh whole blood half-diluted with saline buffer. As previously described, for each sample, 1 µg RNA was reverse-transcribed using random hexamers (Pharmacia, Roche Diagnostics, Meylan, France) and 1/50 of the cDNA was used in each PCR reaction (12). PCR was performed using SYBR Green PCR Core Reagent (Perkin Elmer, Courtaboeuf, France) on ABI PRISM 7700 Sequence Detector apparatus and analysed with the dedicated Gene Amp software (Applied Biosystem). PCR cycles consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of annealing at 60°C for 50 s, elongation at 72°C for 20 s and denaturation at 95°C for 50 s. MgCl2 concentrations were optimized for each primer set in order to perform PCR in the linear part of the amplification together with a maximum yield of amplification. The specificity of PCR amplifications was checked by 2% agarose gel electrophoresis at least once to ensure the absence of non-specific amplification. Primer sequences are available on request. For each amplification, the Ct value, representing the cycle at which a significant fluorescent signal (as compared with the baseline i.e. no template control) is first detected, was measured. In a given sample, signals obtained for each gene were normalized to the signal obtained for the housekeeping β2 microglobulin gene, thus taking account of any variability in the initial concentration and quality of total RNA. Finally, relative quantitation of gene expression was determined by reference to a standard curve. The expression of the genes DNA LIGASE-I and -III, BCL2, BAX, WAF1, TP53, KUP80, DAD1, DNA-PK, PAPR, RAD51, RAD52 was analysed before irradiation of isolated lymphocytes.

Statistical analysis

For a given subject, MN and gene expression values represent the mean of duplicate measurements. Concerning MN before and after irradiation, standard deviation of the mean is given in Figure 1A. For gene expression, each RT–PCR experiment was duplicated; the difference between the two experiments ranged from 5 to 13% (data not shown).

Relationships between gene expression and MN frequency or distribution were analysed using the non-parametric Spearman rank test correlation. We applied the Bonferroni correction to eliminate the chance of randomly obtaining a statistically significant result that could occur with multiple comparisons. So, dealing with 12 independent variables, the threshold of significance was set at $p < 0.004$ to avoid false positives.

Comparisons between MN frequency or distribution before (MN0 or U0) and MN frequency or distribution after irradiation at both doses (MN0.5 and MN2 or U0.5 and U2) were done using the non-parametric Wilcoxon rank sum test comparing medians.

A U-test was performed to determine the degree of dispersion of MN distribution per BN as compared with a Poisson distribution (14,15). Briefly, the principle of this test is as follows: from each distribution of MN among BN cells, the total number of BN cells, $N$, the mean number of MN per cell, $Y$, and an estimate, $\sigma_Y^2$, of the population variance may be derived. A coefficient of dispersion, $d$, is defined by the equation:

$$d = \frac{(N-1)\sigma_Y^2}{Y}$$

By considering the probability of occurrence of all possible distributions of MN subject to constant values of $N$ and $Y$, it was shown that if the underlying process follows Poisson law, the mean value of $d = N - 1$, and the variance of $d$,

$$\text{var } d = 2(N - 1) \left(1 - \frac{1}{NY}\right)$$

Thus the quantity $u$ defined by the equation:

$$u = \frac{d - (N-1)}{\sqrt{\text{var } d}}$$

approaches to a unit normal deviate. A positive value of $u$ indicates over-dispersion, while a negative value indicates under-dispersion. Since $u$ approximates to a unit normal deviate, if the magnitude of $u$ is $>1.96$ then the under or over-dispersion is significant because there is only a 5% chance that the magnitude of $u$ will exceed 1.96 when the underlying distribution follows Poisson law.

Results

To classify donors according to their radiosensitivity, we analysed the number of MN per BN and the distribution of MN among the BN cell population after in vitro lymphocyte irradiation. After $\gamma$ irradiation, the MN distribution per BN
should follow a Poisson law (random distribution) if the cell population is homogeneous in terms of sensitivity to DNA breaks. In addition to the simple classification of whether or not the MN distribution follows Poisson law (−1.96 < u < 1.96), among subjects in which MN distribution absolute value does not follow Poisson law the u values indicate the extent of the deviation. Since dispersion reflects heterogeneity in cell sensitivity, the latter analysis constitutes another way to estimate individual sensitivity. For all subjects included in this study, baseline and radiation-induced MN frequencies per BN cell as well as u values (U-test) are given in Figure 1A and B, respectively. According to the limits of significance of the U-test, we found that the baseline MN distribution (MN0) was over-dispersed, as compared with a Poisson distribution, in 29 of 32 subjects. After *ex vivo* total blood irradiation, this over-dispersion decreased and was found in 24 and 21 of 32 subjects after 0.5 and 2 Gy, respectively (Figure 1B). In this series of donors, we did not observe an under-dispersion of MN distribution before or after *ex vivo* blood sample irradiation. Figure 1C shows the relationship between MN frequency and distribution per BN: no association between MN frequency and MN distribution before (controls) or after irradiation was observed.

Table I summarizes the mean, median, lowest and highest values of age, MN frequency and over-dispersion per BN cells and of mRNA expression in 32 healthy male donors.

### Table I. Mean, median, lowest and highest values of age, MN frequency and over-dispersion per BN cells and of mRNA expression in 32 healthy male donors

<table>
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<tr>
<th>Variables</th>
<th>No.</th>
<th>Mean</th>
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<th>Median</th>
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<th>Highest</th>
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<td>0.02</td>
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</table>

SD, Standard deviation of the mean.

*a* U values are only given for the over-dispersed groups (u > 1.96) because they represent the extent of dispersion of MN distribution per binucleated cell as compared with Poisson law (all other values of u < 1.96 correspond to a distribution that follows Poisson law); 0, 0.5 and 2, control, 0.5 and 2 Gy; LIG-I and -III, DNA LIGASE-I and -III; mRNA expression units corresponds to the ratio (RT–PCR signal for a given gene)/(RT–PCR signal for the housekeeping β2 microglobulin gene).

showed the same but less pronounced variations. The ratio is 3.6 and 2 (uncorrected values) or 5.9 and 2.2 (MN minus MN0) after 0.5 and 2 Gy, respectively (Table I). Depending on the dose, the MN frequency significantly increased as compared with the baseline (MN0.5 compared with MN0, MN2 compared with MN0 and MN0.5 compared with MN2, P < 0.001 for each, Wilcoxon rank sum test). As seen in Table I, the number of subjects for which MN distribution does not follow Poisson law as well as the extent of over-dispersion significantly decreased as dose increased (U0 compared with U0.5, P = 0.004; U0 compared with U2, P < 0.001; U0.5 compared with U2, P = 0.007; Wilcoxon rank test).

The mean and median values of basal (before irradiation) RNA expression of genes involved in apoptosis and DNA repair are presented in Table I. Results are given as RT–PCR signal relative to that of the control cell line for an equivalent expression of β2-microglobulin (13). For all analysed genes, we also observed an individual variability of RNA gene expression as indicated by the ratio between the highest/lowest values (Table I). It should be noted that for some subjects, the expression of DNA-PK and DADI mRNAs is below the threshold of detection, so this ratio was not calculated. Nevertheless, the range of variability appears to be of the same magnitude or higher than those found for spontaneous or radiation-induced MN frequency and distribution. So it is relevant to assess if the variability of expression is dependent on age and/or could predict radiation-induced MN frequency or distribution.

We found a significant positive correlation (r = 0.68, P < 0.001) between age and the baseline MN frequency, the increase being 0.1% per BN and per year. Age was not
significantly correlated with radiation-induced MN, with MN distribution per BN (µ values) before and after irradiation and with the basal gene expression of genes included in our study.

Since 12 genes have been analysed, significant relationships could occur only by chance. After applying the Bonferroni correction to avoid false positives, we found that RAD51 mRNA expression is significantly negatively correlated with MN induction after both doses, 0.5 and 2 Gy ($r = -0.55, P = 0.002$ and $r = -0.52, P = 0.002$).

Discussion

Interindividual variation in radiosensitivity has been well documented in normal lymphocytes and fibroblasts following in vitro irradiation and in vivo after radiotherapy. It is therefore of great interest to search for biomarkers that could help to predict individual sensitivity associated with environmental or therapeutic radiation exposures. In this regard, phenotypic predictive assays have been used, including chromosome aberrations, sister chromatid exchanges and MN (4,5,16–19), all resulting from DNA damage, but little is known about the molecular mechanisms behind this variability. Individual radiosensitivity of healthy people generally assessed by chromosome damage could result from polymorphic genes in the population (20–22) and/or variation in normal gene expression. The objective of this study was to analyse the relationship between individual radiosensitivity, assessed by measuring the induction and distribution of MN after ex vivo irradiation of human lymphocytes obtained from a series of 32 healthy male subjects, and basal mRNA expression of genes involved in the main pathways implicated in the cellular response to irradiation.

Our choice of mRNA profiling to study the molecular mechanisms of individual radiosensitivity could be debated since mRNAs constitute transient products and since it is well-known that the expression of some proteins is not regulated at the transcriptional level. On the other hand, assessment of gene expression at the protein level has obvious limitations mainly due to the constant level of expression during post-translational modifications, and their variable detection by Western blot analysis. From a technical point of view, RT–PCR is quantitatively more sensitive than Western blotting and requires minimal biological material. Moreover, it permits simultaneous analysis of numerous genes, a distinct advantage when studying biological processes involving numerous pathways.

As previously shown (23–26), a positive correlation was found between basal MN frequency and age before irradiation, whereas, after ex vivo irradiation, we did not find any correlation between age and radiation-induced MN. As observed in our experiment (Table I), there is also clear individual variability in radiation-response. Our mRNA expression results indicate that before irradiation the basal level of expression of each gene is not related to age. In a previous study, Aggarwal and Gupta (27) showed that in T lymphocytes, the expression of BAX and BCL2 increased and decreased, respectively, as a function of age. This apparent discrepancy could be due to the authors’ limits for the young (20–25 years) and ageing (65–95 years) groups, since in our series the age range was 27–58 years. It is supposed that variation in radiosensitivity could be ascribed to high or low sensitivity to DNA breaks in relation to variable efficiency of DNA repair and/or apoptosis. For example, using the Comet assay for measuring both DNA breaks and repair, it was found that, after 2 Gy irradiation, MN frequency per BN cell is not correlated with the initial degree of damage, but is correlated with the repair efficiency of irradiated human lymphocytes (28). Among the genes involved in the DNA repair process analysed in our study, we observed that RAD51 mRNA expression is negatively correlated with MN induction after both 0.5 and 2 Gy irradiation ($r = -0.55, P = 0.002$ and $r = -0.52, P = 0.002$, respectively). The rad52 epistasis group, including Rad51 protein, represents the main pathway of DSB repair by homologous recombination.

Inactivation of Rad51 renders cells sensitive to ionizing radiation and increases chromosome breaks and cell death (29). Rad51 protein is linked to DNA breaks included in MN (30). Since MN represent unrepaird DNA breaks, high basal expression of Rad51 may result in an increase in the ability of the cell to handle radiation-induced breaks and consequently may decrease MN frequency. Thus, as found by others in cell culture (31), high Rad51 expression may confer resistance to ionizing radiation. But, on the other hand, it has been shown that overexpression of RAD51 mRNA could result in an increase in spontaneous homologous recombination. Consequently, elevated basal expression may also lead to genetic instability by favouring homologous recombination between repeated sequences within the genome.

Although it has been proposed that the MN index could be influenced by the propensity of damaged cells to undergo apoptosis, the relationship between initial DNA damage, repair of DNA breaks and progress through apoptosis is not fully understood or predictable. But depending on the extent of damage and on the capacity of cells to divide in spite of breaks, it is possible that cells will generate MN or will go to apoptosis. Therefore, post-irradiation the frequency of MN might be negatively correlated with apoptosis. In mammals, many genes have been identified whose products are necessary to prevent entry into the apoptotic process. None of the apoptosis-related gene expression analysed, even if we considered the BAX/BCL2 ratio (data not shown), seems to predict alone individual radiosensitivity.

Before and after irradiation the MN distribution per BN showed marked variations between individuals and for some subjects this distribution differed from the Poisson distribution (Table I and Figure 1B and C). If damage is expressed randomly throughout the cell population, the distribution of MN per BN should follow Poisson law. If not, it indicates that variability in susceptibility to damage exists among the cell population and could be caused by the presence of different subtypes. In our experiments, MN were analysed on T-lymphocytes (PHA stimulation) which survive one nuclear division (cytochalasin-B treatment) that correspond to one cellular subtype at least phenotypically. Consequently, under our conditions a non-Poissonian distribution is not linked to the presence of different subtypes in the cell population but rather reflects a real polymorphism of cellular radiosensitivity. Although this polymorphism of radiosensitivity may be ascribed to different repair capacities among the cells, none of the genes analysed seem related alone to the MN distribution.

In conclusion, our results indicate that basal RAD51 gene expression could partly explain the individual sensitivity to ionizing radiation assessed by radiation-induced MN. Since several of these genes are regulated by radiation, we are currently analysing their expression after irradiation to define the predictive value of this dynamic measure for measurement of individual radiosensitivity.
These first results are promising but should be complemented since there are probably several hundred genes that govern the cellular response to ionizing radiation. Moreover, individual radiosensitivity represents a composite integration of the level of expression of multiple genes and the contribution of partially dysfunctional variants that may be numerous in the population. So, gene profiling by RT–PCR, and soon using DNA chips, could help to define the gene expression ‘imprint’ conferring sensitivity or resistance to ionizing radiation, give indications for identifying new phenotypic or genotypic polymorphisms, and, lastly, help to conduct mechanistic approaches, even though the protein levels are also critical and not necessarily directly related to transcript levels.

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