Application of the single cell gel electrophoresis (SCGE) assay to the detection of DNA damage induced by ^{131}I treatment in hyperthyroidism patients

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To provide further data on the possible applications of the single cell gel electrophoresis (SCGE) or Comet assay in human biomonitoring studies, we have evaluated the eventual genetic damage induced by therapeutic exposure to ^{131}I, by measuring the tail length of the comet and the amount of DNA damage in peripheral blood cells from 16 hyperthyroidism patients treated with [^{131}I]sodium iodide by oral administration. Blood samples were taken just before the treatment and 1 week and 1 month after. The results show a slight but significant increase in the mean tail length in the sample obtained 1 month after treatment. When the cells were classified according to the grade of damage the two post-treatment samples showed a clear increase in the proportion of damaged cells. The results of this study indicate that the DNA damage caused by ^{131}I can be detected with the Comet assay, but when comparing the data reported here with our previous results obtained from the same patients and sampling times with the sensitive and well-established micronucleus test, the response in the Comet assay was less clear.

Introduction

The single cell gel electrophoresis (SCGE) or Comet assay seems to be a promising tool for estimating DNA damage at the individual cell level in both *in vitro* and *in vivo* studies. When the assay is performed under alkaline conditions a suspension of single eukaryotic cells is embedded in agarose, placed on microscope slides and taken for lysis and unwinding of DNA followed by electrophoresis at high pH, neutralization and staining with a fluorescent DNA binding dye (McKelvey-Martin *et al.*, 1993; Fairbairn *et al.*, 1995). Cells with increased DNA damage display an increased migration of the DNA from the nucleus (comet head) towards the anode (tail) (Singh *et al.*, 1988). This migration could result from DNA single-strand breaks, damage at alkali-sensitive sites and incomplete excision repair sites present at the time of lysis (Tice and Strauss, 1995).

This assay has been shown to be very sensitive in the detection of genetic damage induced *in vitro* by radiation (Olive *et al.*, 1990; Vijayalaxmi *et al.*, 1992; Alapetite *et al.*, 1996) and chemicals (Hartmann and Speit, 1994; Ribas *et al.*, 1995), as well as to detect and measure DNA repair (Nocentini, 1995). The high sensitivity of the Comet assay has moved different authors to propose its use in human biomonitoring studies.

Radionuclides emitting γ-rays and energetic β-particles are widely used as radiopharmaceuticals in nuclear medicine

(Prasad, 1995) and are an important source of therapeutic or occupational exposure to ionizing radiation. Thus radioactive iodine (^{131}I) therapy is commonly used in the treatment of hyperthyroidism and thyroid cancer.

Recent studies indicate that radioiodine therapy induces significant increases in the frequency of chromosome aberrations (Gundy *et al.*, 1996; M'Kacher *et al.*, 1996) and micronuclei in peripheral blood lymphocytes of exposed people (Catena *et al.*, 1994; Wuttke *et al.*, 1996; Gutiérrez *et al.*, 1997). In this context, the goal of this work was to study the suitability of the Comet assay to assess the presence and persistence of the eventual genetic damage induced in hyperthyroidism patients by therapeutic exposure to [131]I.

Materials and methods

Patients

The study was performed with 16 hyperthyroidism patients (11 women and five men) treated at the Nuclear Medicine Service of the University Hospital of Vall d'Hebron in Barcelona who received [^{131}I]sodium iodide by oral administration.

Before proceeding with the study we obtained clearance from the ethical committee of our Institutions. All patients gave informed consent and blood samples were collected and further manipulated in accordance with ethical standards.

Three different blood samples were taken from each individual in a follow-up study: the first was obtained before the treatment and the following were taken 1 week and 1 month after respectively.

Comet assay

Before slide preparation, 0.2 ml whole blood were centrifuged and the supernatant was removed. After that the cell pellet was resuspended in 1.4 ml chromosome medium (RPMI 1640; Gibco BRL) with L-glutamine.

The Comet assay was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). The cell pellet obtained from 60 μl RPMI-diluted blood was mixed with 85 μl 0.7% low melting point agarose (LMA) and then placed on fully frosted roughened slides, previously coated with 110 μl 1% normal melting point agarose (NMA). When this layer had solidified, a third layer of 85 μl LMA was applied. The slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris–HCl, 1% Na sarcosinate, pH 10) with 1% Triton X-100 and 10% DMSO added fresh to lyse the cells and to allow DNA unfolding. The slides were then placed on a horizontal gel electrophoresis tank, facing the anode. The unit was filled with fresh electrophoretic buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH 13) and the slides were allowed to set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (300 mA). After that, to remove alkali and detergents, a neutralization buffer (0.4 M Tris–HCl, pH 7.5) was added dropwise to the slides and allowed to sit for 5 min, then the DNA was stained with 4,6-diamidino-2-phenylindol (DAPI) (5 μg/ml). Slides were examined by eye at 400× magnification using a fluorescence microscope. The tail length was measured according to Singh *et al.* (1988). The width of the nucleus and the extent of migration of DNA fragments of 50 randomly selected cells per slide were determined. Two parallel replicates were performed per sample and the mean tail length was calculated. Moreover, cells were graded by eye into five categories (A–E) according to the amount of DNA in the tail (Anderson *et al.*, 1994), where A are undamaged cells and E highly damaged cells. To quantify the damage in this scoring, a rank number ranging from 0 (A) to 400 (E) was assigned to each of the categories, in order to calculate a mean of DNA damage grade for all samples.

Statistical analysis

The distributions of mean tail length of the comet and mean of the grade of DNA damage obtained before and after treatment were compared with the

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Table I. Characteristics of the hyperthyroidism patients and Comet parameters (average tail length and coefficient of variation) before and after ^{131}I treatment

Patient code	Dose (MBq)	Age (years)	Sex	Pre-treatment		1 week after		1 month after	
				Tail length[a]	CV	Tail length[a]	CV	Tail length[a]	CV
302	148	48	F	34.50	18.51	38.20	25.60	43.22	24.42
319	281	30	F	35.75	17.99	42.77	29.80	37.70	30.18
333	289	20	F	41.61	35.56	36.89	27.60	48.28	34.50
290	296	32	F	35.03	24.65	40.18	26.72	53.78	30.01
261	300	30	F	29.31	22.73	32.78	24.06	35.80	25.09
284	303	68	F	37.46	34.47	32.21	22.99	62.08	32.50
262	315	44	F	35.05	21.60	31.71	19.16	45.38	28.29
282	444	72	M	36.86	20.71	34.87	24.51	38.11	25.26
348	555	33	F	32.10	23.79	45.36	31.22	42.09	33.39
314	555	36	M	52.05	22.07	33.14	27.47	33.25	26.74
283	555	48	F	36.66	26.72	31.48	17.44	47.26	31.16
289	555	60	M	34.07	29.95	37.27	26.81	31.35	26.75
291	555	67	M	35.23	28.26	42.40	37.99	36.93	19.59
254	555	69	F	46.01	30.25	30.65	27.47	38.82	25.31
359	555	75	F	34.78	26.04	56.57	29.68	40.16	24.99
265	762	48	M	32.03	23.21	29.19	25.35	41.70	39.40
Mean	438.91	48.75		36.78	25.38	37.23	26.49	42.24*	28.60
SE	41.06	4.43		1.40	1.30	1.76	1.19	1.96	1.21

[a]Data based on 100 cells/sample and expressed in μm.
CV, coefficient of variation.
*P < 0.05 (t-test for dependent samples), comparison with the pre-treatment value.

normal distribution by means of the Kolmogorov–Smirnov test of goodness of fit. Neither of them departed significantly from normality and therefore parametric tests were adequate for statistical analysis.

The t-test for dependent samples was used to compare means of average tail length and grade of DNA damage, between the pre- and two post-treatment samples. Given the existence of great variability in the comet length, within and between individuals, we have calculated the coefficient of variation in order to obtain an estimate of the relative amount of variability. This coefficient derives from expression of the standard deviation as a percentage of the mean. To determine any relationship between the DNA migration length and activity of ^{131}I administered to the patients, a linear regression coefficient was calculated. A possible age effect on mean tail length was evaluated using linear regression analysis. To determine the relationship between the results for comet length and grade of DNA damage the Pearson correlation analysis was applied.

The distribution of the degrees of DNA damage after treatment was compared with the distribution obtained before it by means of the χ^2 test.

Results

Table I shows comet tail length values obtained before treatment and 1 week and 1 month after. These data indicate a slight but significant increase in mean tail length of the comet in the sample taken 1 month after ^{131}I therapy. It is noteworthy that before as well as after treatment the tail length exhibits high intra- and inter-individual variation. When the coefficient of variation was computed a significant heterogeneity within and between individuals was observed. As can be seen in Table I, concerning variability within individuals the coefficient of variation before the treatment ranged from 17.99 to 35.56, with an average of 25.38. In the sample taken 1 week later it ranged from 17.44 to 37.99, with an average of 26.49, while after 1 month the value ranged from 19.59 to 39.40, with an average of 28.60. In addition, when the variability between individuals is considered the corresponding values of the coefficient of variation are 30.20 before therapy and 33.67 and 35.04 1 week and 1 month later respectively. From these data it appears that comet length variability increases after treatment. Table I also indicates the dose administered to each patient together with their age and sex. The mean activity was 438.91 ± 41.06 MBq (range 148–762), the mean age of the group

being 48.75 ± 4.43 years (range 20–75). The regression coefficient between comet tail length and activity of the treatment in the two post-treatment samples studied does not indicate any significant relationship. Similar results were obtained when tail length and age were compared.

Table II shows the results obtained when the cells were classified into different categories according to the amount of DNA in the tail of the comet, indicating that treatment significantly increased the proportion of cells with damaged DNA. Thus it is observed that the proportion of damaged cells rises from 41% before treatment to 57% in the sample taken 1 month after ^{131}I treatment. Another way to determine this increase is by calculating the ratio of damaged to non-damaged cells. When this ratio was computed for the three samples the values 0.70, 0.88 and 1.31 were obtained, showing a nearly 2-fold increase in the sample taken 1 month after ^{131}I treatment. However, when the analysis was carried out using the mean of the arbitrary values assigned to each category the increase obtained in this mean in the two post-treatment samples did not reach statistical significance (t-test). Finally, a clear linear relationship between the values for DNA damage grade and tail length was obtained, the r values being 0.80 (P < 0.001), 0.95 (P < 0.001) and 0.70 (P < 0.003) for the pre- and two post-treatment samples respectively.

Discussion

The Comet assay allows detection of DNA damage and repair at the level of individual cells. The use of this technique is increasing and it has been employed in different *in vitro* studies conducted to detect the genotoxic effects of ionizing radiation, as well as the repair kinetics of such damage in human blood cells (Vijayalaxmi *et al.*, 1993; Tice and Strauss, 1995; Lankinen *et al.*, 1996; Visvardis *et al.*, 1997). The studies on repair kinetics indicate that only minutes after the end of *in vitro* radioactive treatment there was a significant reduction in radiation-induced DNA migration. Until now there have been only a small number of investigations in which the Comet

Table II. Distribution of the grades of DNA damage in the hyperthyroidism patients

Patient code	Grade of damage[a]																	
	Pre-treatment						Post-treatment (1 week after)						Post-treatment (1 month after)					
	A	B	C	D	E	g.d.	A	B	C	D	E	g.d.	A	B	C	D	E	g.d.
302	74	22	3	1	0	31	52	41	7	0	0	55	35	57	7	0	1	75
319	67	33	0	0	0	33	36	46	12	4	2	90	59	22	16	3	0	63
333	68	27	4	1	0	38	59	23	14	2	2	65	11	42	38	9	0	145
290	61	34	5	0	0	44	30	60	7	0	3	86	32	62	6	0	0	74
261	79	19	0	1	1	26	79	17	3	1	0	26	63	32	4	0	1	44
284	54	35	7	4	0	61	75	19	5	0	1	33	7	81	11	0	1	107
262	45	32	19	3	1	83	65	28	7	0	0	42	27	47	23	3	0	102
282	45	52	3	0	0	58	41	44	13	2	0	76	45	41	11	0	3	75
348	82	12	5	0	1	26	24	42	29	4	1	116	35	52	10	2	1	82
314	9	72	17	2	0	112	64	19	13	1	3	60	71	17	9	2	1	45
283	58	38	3	1	0	47	90	5	4	1	0	16	39	50	10	0	1	74
289	68	25	4	1	2	44	50	39	9	1	1	64	73	24	3	0	0	30
291	74	17	5	1	3	42	46	29	13	5	7	98	57	42	1	0	0	44
254	14	39	41	4	2	141	73	23	4	0	0	31	45	41	14	0	0	69
359	68	20	12	0	0	44	3	50	39	7	1	153	37	48	14	1	0	79
265	73	18	9	0	0	36	64	27	8	0	1	47	55	23	10	8	4	83
Total	939	495	137	19	10		851	512	187	28	22[b]		691	681	187	28	13[c]	
Mean ± SE						54.13 ± 8.02						66.13 ± 9.06						74.44 ± 7.01

[a]Data based on 100 cells/sample.
[b]$P < 0.01$; [c]$P < 0.001$ (χ^2 test); comparison with the pre-treatment distribution.
g.d., mean of DNA grade of damage.

assay was applied for biomonitoring of human populations exposed to ionizing radiation; in particular, Plappert et al. (1995) investigated patients subjected to radioiodine therapy and a Chernobyl worker. They observed that while all the patients showed a significant increase in DNA damage that is repaired in 7–10 days, the DNA damage induced in the Chernobyl worker persisted over time.

From the results of our study it appears that although there is an increase in the proportion of damaged cells in the sample obtained 1 week after ^{131}I treatment, no significant augment DNA migration was detected. If we assume that ionizing radiation induces genetic damage measurable in the Comet assay, this finding would indicate that most of the DNA damage had been repaired at this sampling time, which is in good agreement with the results reported by Plappert et al. (1995).

On the other hand, the results obtained in the sample taken 1 month after ^{131}I exposure clearly indicate a significant increase in both the extent of DNA migration and the ratio of damaged to non-damaged cells. This increase in the response over time is in accordance with our findings previously obtained in the same patients using the micronucleus assay (Gutiérrez et al., 1997), where induced cytogenetic damage persisted in the last sampling period, 3 months after treatment. The kinetics of the response found in the present study with the Comet assay, showing a significant increase in genetic damage 1 month after exposure to a relatively low dose of ^{131}I, can be explained by taking into account that ^{131}I is a radionuclide with a half-life and an effective half-life of ~8 and 3 days respectively (Metller and Guiberteau, 1991) and that the thyroid gland might act as an internal source of irradiation. Therefore, when blood cells of hyperthyroid patients subjected to ^{131}I therapy pass through the irradiated gland they could be damaged by β-rays. In this context it can be considered that these patients are chronically exposed to decreasing doses of ^{131}I and that the increase in the average comet tail length

observed 1 month after ^{131}I therapy reflects an accumulation of non-repaired DNA damage.

Thus we can assume that after ^{131}I administration highly damaged cells are induced that are subject to repair. After ~1 week such repair mechanisms have been efficient enough and have almost eliminated the damage induced by the first 'acute' exposure; nevertheless, due to the later 'chronic' nature of the exposure and perhaps to the relative saturation of the repair mechanisms, non-repaired DNA damage can be accumulated over time, as observed in the sample obtained 1 month after treatment. Although this hypothesis could be reinforced by analysing some samples in the first week after treatment, the radioactivity of plasma and cells during this first week makes manipulation of such blood samples dangerous.

As previously reported by different authors, we also observed great variability in the extent of DNA migration at both the individual and cellular levels. This variability could be explained by differences in susceptibility between cell types (Holz et al., 1995; Tice and Strauss, 1995), inter-individual differences in DNA repair (Oesch et al., 1987) and possible sensitivity of blood cells to physical, physiological and nutritional status (McKelvey-Martin et al., 1993; Ross et al., 1995).

The poor induction of DNA damage measured by the Comet assay after ^{131}I administration does not correspond with our previous results (Gutiérrez et al., 1997) using the micronucleus assay, where a clear effect and a positive relationship between ^{131}I activity and frequency of binucleated lymphocytes with micronuclei was observed. This apparent discrepancy could be explained by taking into account that the Comet data reported in the present work are based on the response of leukocytes, while the micronucleus results were based on lymphocytes only, as indicated by other authors (Van Goethem et al., 1997).

On the other hand, it must be pointed out that we did not find a relationship between DNA migration and age, neither before ^{131}I therapy nor after it. This lack of effect of age in

modulating DNA migration confirms previous observations (Betti *et al.*, 1995; Frenzilli *et al.*, 1997). In contrast, Singh *et al.* (1991) reported higher levels of DNA damage in subjects older than 60 years, but in this case the electrophoresis time was increased to 40 min to achieve higher resolution in DNA migration.

Longitudinal analysis in biomonitoring studies, as conducted in this work, is a very good approach, since exposure effects are compared in the same individual with values observed before exposure. Nevertheless, given the fact that seasonal variation can apparently affect comet formation (Betti *et al.*, 1995), it cannot be excluded that the observed effects in post-treatment samples could be modified by this seasonal effect. Although this effect could be evaluated using measurements from control individuals over time, it would perhaps be difficult to discriminate between inter-individual differences and real seasonal effects.

In conclusion, our data indicate that the Comet assay is sensitive enough to detect the genotoxic effects induced *in vivo* by ^{131}I treatment. Nevertheless, taking into account the high variability found between and within individuals and that the response is less clear than that obtained with the well-established micronucleus test, further knowledge of the fundamental aspects of the Comet assay and on the kinetics of formation and disappearance of comets after *in vivo* exposure are needed for correct application of this technique in human biomonitoring.

Acknowledgements

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