Radioprotective Effect of Curcumin on DNA Double Strand Breaks in Human Blood Lymphocytes after *in vitro* γ-Irradiation

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Abstract: *Curcumin is a component of natural spice Curcuma longa. It is known that this polyphenol has been shown to exhibit anti-inflammatory, anticancer, antiviral, antioxidant and antibacterial activities. The mechanism of curcumin effectiveness on both healthy and cancer tissues is still unclear.*
Aims: *In vitro assessment of curcumin effect on both double-strand breaks and chromosomal translocations frequency, after γ-irradiation.*
Methods: *Human peripheral blood samples were pre-treated with different concentrations of curcumin (0.5 μg/ml; 10 μg/ml; 20 μg/ml and 100 μg/ml). Thereafter they were exposed to ^{60}Co γ-rays using various irradiation doses (0.05 Gy; 0.5 Gy; 1 Gy and 2 Gy). Both γ-H2AX/53BP1 foci assay and FISH analysis were used to evaluate DNA double-strand breaks and translocation frequencies.*
Results: *Curcumin pre-treatment exhibited significant lower γ-H2AX/53BP1 foci appearance and reduced translocations frequency in irradiated compared to untreated lymphocytes. At 1 Gy irradiation and 10 μg/ml curcumin, the reduction of total translocations frequency was 42%. We found that at 2 Gy irradiation, the most protective concentration was 0.5 μg/ml curcumin. In this case, translocations declined almost twofold compared to curcumin non-treated cells.*
Conclusion: *The present in vitro study demonstrates that curcumin reduces both γ-H2AX/53BP1 foci and translocations occurrence in peripheral blood lymphocytes, after γ-irradiation.*

Keywords: *Curcumin, γ-irradiation, γ-H2AX/53BP1 foci, Translocations.*

Introduction

Ionizing radiation (IR) induces a variety of DNA lesions. Most of the DNA single strand breaks and other DNA damages are recovered from the cell reparative system. In contrast, DNA double strand breaks (DSBs) can remain non-repaired or misrepaired, that results in either loss or rearrangement of genetic information, cell death or malignant transformation.

DSBs are the most serious lesions as a consequence of IR. It is estimated that a dose of 1 Gy X- or γ-rays would induce about 20-40 DSBs per cell (for review see [14]). It has been shown that histone protein H2AX becomes phosphorylated in the vicinity of DSBs. The phosphorylated H2AX is termed γ-H2AX and is a key factor in DNA damage response. Histone γ-H2AX binds to other reparative proteins (53BP1, BRCA1, MDC1, etc.) forming γ-H2AX foci at the sites of DSBs. The quantity of γ-H2AX foci increases quickly and reaches

peak nearly 30 minutes after irradiation. γ-H2AX immunolocalization is used for assessment of both DNA damage and DNA repair processes [8].

Over the past decades, the scientific interest is focused on natural agents that can protect cells against IR. Recently, Kuefner et al. [7] investigated the effect of various protective molecules (vitamin E, vitamin C, carotenoids, etc.) on the appearance of γ-H2AX foci in X-ray irradiated human lymphocyte. They have detected a significant reduction in γ-H2AX foci when cells were pre-treated with a proper agent before irradiation. On the other hand, curcumin, a component of turmeric (*Curcuma longa*) has been proposed as a radioprotector due to its antioxidant activity [9]. However, in the literature, there are only a few data concerning curcumin effect on γ-H2AX foci occurrence. According to Seong et al. [16], curcumin reduces DSBs in γ-irradiated *D. melanogaster* larvae. Jafarpour et al. [5] have also found a decreased number of γ-H2AX foci when human blood samples were treated with curcumin followed by ^{131}I irradiation. Contradictory, Ogiwara et al. [12] have reported curcumin to suppress main DNA damage response pathways. This agent has been shown to trigger DSBs accumulation [6]. The investigations concerning the radioprotective effect of curcumin are scarce and contradictory.

Non-repaired or misrepaired DSBs induce structural chromosomal aberrations. Some authors have shown the reduced frequency of micronuclei and dicentrics after curcumin treatment followed by irradiation [15, 18]. Others have not found any radioprotective effect or even more, they observed the clastogenic effect of curcumin [1]. All these studies are related to unstable chromosomal aberrations that are eliminated during the cell division cycle. However, translocations are stable aberrations and they could be found in next-generation cells many years after irradiation [10]. The fragments exchange between non-homologous chromosomes could lead to impaired expression of affected genes and increases the cancer risk. That's why our interest is directed at *in vitro* assessment of curcumin effect on both DSBs and chromosomal translocations frequency using peripheral blood lymphocytes irradiated with γ-rays.

Materials and methods

Blood samples handling and in vitro irradiation

Whole blood samples were taken from healthy thirty-four years old female volunteer. Curcumin (Sigma-Aldrich) was added one hour before irradiation at different concentrations (0.5; 10; 20 and 100 μg/ml). The samples were incubated at room temperature in dark. Thereafter they were exposed to ^{60}Co γ-rays using various irradiation doses (0.05; 0.5; 1 and 2 Gy), at a dose rate of 0.246 Gy/min both curcumin non-treated and non-irradiated samples served as controls.

γ-H2AX and 53BP1 immunofluorescence

Thirty minutes after above-mentioned treatment, lymphocytes were isolated using Histopaque (Sigma-Aldrich) according to the manufacturer's protocol and plated on microscope slides (Isolab). Double immunostaining for γ-H2AX and 53BP1 was performed using a standard protocol. Lymphocytes were incubated in a mixture of mouse monoclonal anti-phospho-H2AX (1:500, Millipore) and rabbit polyclonal to 53BP1 (1:400, Abcam) antibodies. To detect DSBs we used mixed secondary antibodies: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit, (1:400, Invitrogen). At last step, the slides were counterstained with DAPI(Sigma-Aldrich)in anti-fading Vectashield mounting solution (Vector Laboratories) and stored at -20 °C until analysis. The yield of γ-H2AX/53BP1 foci

number was estimated using the formula $\Sigma_{foci}/n_{total\ cells}$, where Σ_{foci} shows the total number of foci in the cells scored at each experimental point and $n_{total\ cells}$ presents the total number of cells analyzed at that point. The background frequency of γ-H2AX/53BP1 foci was obtained from untreated cells.

Fluorescent in situ hybridization (FISH)

After proper treatment(see above), whole blood samples were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Sigma-Aldrich), and stimulated with 2% phytohemagglutinin (Gibco-Liftechnologies). The culturing of the cells was carried out at 37 °C in 5% CO_2 for 50 hours. After 48 h culture time cells were arrested in metaphase by demecolcine (0.1 μg/ml; Invitrogen). They were harvested, treated with a hypotonic solution and fixed routinely as described previously [4]. Three-colour FISH was performed using XCyting DNA Probes (MetaSystems), according to manufacturer's protocol. A cocktail of pre-mixed fluorochrome labelled probes was used: chromosome #1 (FITC), chromosome #4 (Texas red) and chromosome #11 (FITC/Texas red). At the last step, metaphase chromosomes were counterstained and mounted with DAPI (Sigma-Aldrich) in anti-fading solution Vectashield (Vector Laboratories). Hundreds of cells for each dose point and each curcumin concentration were scored. Classification of aberrations was carried out using PAINT nomenclature modification proposed by Tucker et al. [19]. Since the FISH analysis of bicoloured aberrations detects only a portion of total aberrations present in a cell, the exchange frequency was converted to a whole-genome equivalent as described by Gray et al. [2]:

$$F_p = 2.05 f_p (1 - f_p) F_g,$$

where F_p is the frequency of translocations detected by FISH, f_p is the painted fraction of the genome, and F_g is the genomic aberration frequency.

Because of using three-colour painted chromosomes we applied calculated formula:

$$F_p/F_g = 2.05[(f_1(1 - f_1) + f_4(1 - f_4) + f_{11}(1 - f_{11})) - (f_1 f_4 + f_4 f_{11} + f_1 f_{11})].$$

Both γ-H2AX foci and FISH analyses were carried out manually using an Olympus BX41microscope equipped with a DP70 digital camera and computerized image analysis system.

Statistical analysis

Data analysis was performed with programs for statistical analysis IBM SPSS 19 and MS Excel. For γ-H2AX foci analysis, non-parametric Mann-Whitney U test was used to compare curcumin-treated/non-treated samples at various radiation doses. p-values < 0.05 were considered as significant. Genomic frequency of total translocations decreasing was calculated by the following formula:

$$\frac{withcurcu\,min - withoutcurcu\,min}{withoutcurcu\,min} \times 100\,.$$

Results

γ-H2AX/53BP1 assay

A total number of 4 500 cells were analyzed for γ-H2AX/53BP1 foci.

Non-irradiated cells

At both concentrations, 0.5 μg/ml and 10 μg/ml, curcumin by itself induced a statistically significant reduction of foci yield, compared to untreated lymphocytes ($p \leq 0.001$). Foci frequency slightly increased to control level at 20 μg/ml and decreased again at 100 μg/ml concentration of curcumin ($p \leq 0.001$) (Table 1, Fig. 1).

Table 1. Numbers of γ-H2AX/53BP1 foci per cell and genomic frequency of translocations ± SE in curcumin-treated/non-treated peripheral blood lymphocytes, after γ-irradiation

Dose, [Gy]	Curcumin, [μg/ml]	Cells analysed	Aberrant cells	Total T Genomic Frequency ± SE	Foci/cell ± SE
0	0	1100	1	0.003 ± 0.002	0.75 ± 0.06
	0.5	1082	0	0	0.45 ± 0.05*
	10	1006	2	0.006 ± 0.002	0.47 ± 0.05*
	20	1026	2	0.006 ± 0.002	0.71 ± 0.06
	100	794	0	0	0.21 ± 0.03*
0.05	0	1022	0	0	1.32 ± 0.11
	0.5	1017	0	0	1.25 ± 0.08
	10	1032	1	0.003 ± 0.002	0.69 ± 0.06*
	20	1014	4	0.018 ± 0.004	0.91 ± 0.07*
	100	1036	2	0.006 ± 0.002	0.46 ± 0.05*
0.5	0	1004	9	0.027 ± 0.005	3.76 ± 0.14
	0.5	1004	8	0.027 ± 0.005	2.28 ± 0.11*
	10	1063	7	0.019 ± 0.004	3.42 ± 0.13*
	20	1023	9	0.026 ± 0.005	2.78 ± 0.12*
	100	695	8	0.034 ± 0.007	2.22 ± 0.11*
1	0	1017	45	0.137 ± 0.012	6.31 ± 0.18
	0.5	863	33	0.113 ± 0.011	6.23 ± 0.18
	10	933	25	0.079 ± 0.009	5.02 ± 0.16*
	20	758	21	0.090 ± 0.011	5.48 ± 0.17*
	100	1022	33	0.101 ± 0.010	5.65 ± 0.24*
2	0	1024	123	0.396 ± 0.020	9.26 ± 0.2
	0.5	1012	59	0.202 ± 0.014	7.47 ± 0.27*
	10	1076	104	0.300 ± 0.017	7.57 ± 0.28*
	20	1087	104	0.297 ± 0.017	6.62 ± 0.18*
	100	541	41	0.230 ± 0.021	

T – translocations; * $p \leq 0.001$; $p < 0.01$ only for concentration 10 μg/ml at 0.5 Gy

Irradiated cells

The yield of foci showed dose depended increasing after in vitro irradiation, ranged from 0.075 ± 0.06 to 9.26 ± 0.03 in curcumin non-treated lymphocytes (Table 1, Fig. 1). At curcumin concentrations of 10 μg/ml, 20 μg/ml and 100 μg/ml a significant lower foci appearance was recorded, compared to untreated cells, at all dose points used ($p < 0.01$).

Our results for 0.5 μg/ml curcumin treatment also showed a decreased number of γ-H2AX/53BP1 foci. In this case we found a statistically significant reduction of foci only at doses of 0.5 Gy and 2 Gy γ-rays ($p \leq 0.001$) (Table 1, Fig. 1).

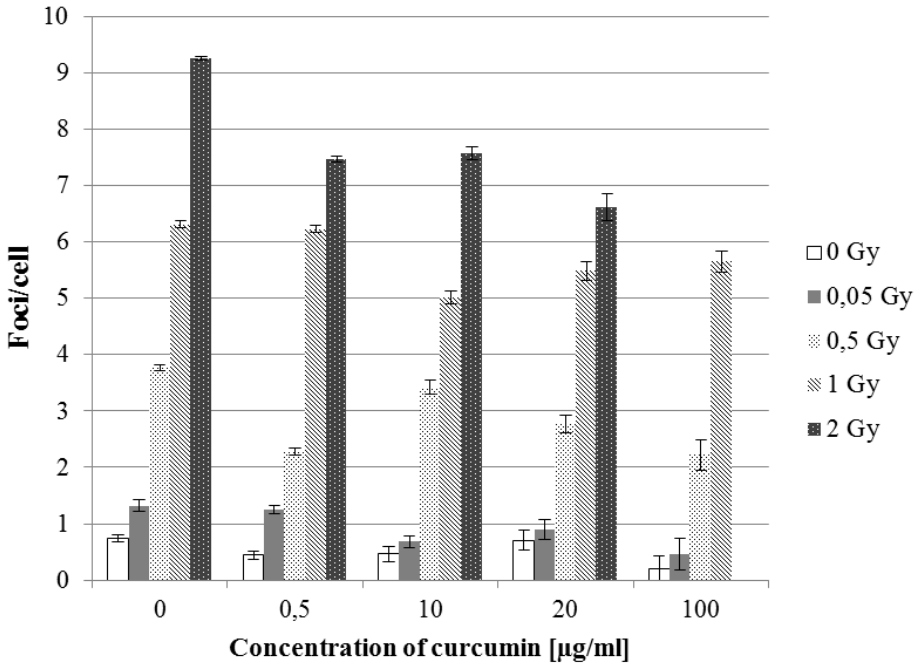

Fig. 1 Reduction of radiation-induced γ-H2AX/53BP1 foci in curcumin-treated peripheral blood lymphocytes. Error bars indicate standard error.

FISH analysis of translocations

A total number of 24 251 metaphases were scored in FISH-stained preparations. Curcumin effect on stable translocations was investigated *in vitro* by three-coloured FISH. The observed genomic frequencies of total translocations (reciprocal + terminal) are represented in Table 1.

Non-irradiated cells

We have not found statistically significant differences in translocation genome frequency between curcumin-treated and non-treated samples. They were all in the spontaneous range of 5-10 translocations per 1000 cells (Table 1, Fig. 2).

Irradiated cells

As expected, we observed dose depended increasing of translocations varying from 3 per 1000 cells in controls to 396 per 1000 cells after *in vitro* exposure to 2 Gy γ-rays (0.003 ± 0.002 to 0.396 ± 0.020). At dose 0.05 Gy γ-irradiation, no curcumin effect was observed for all concentrations used (Table 1, Fig. 2). Our results demonstrated that 0.5 μg/ml curcumin treatment leads to reduced translocation frequencies when lymphocytes were exposed to either 1 Gy or 2 Gy γ-rays. In this case, genomic translocation frequencies decreased with 18% and 49% respectively compared to non-treated cells. The concentration of 10 μg/ml curcumin induced reduction of translocations with increasing the dose. The decrease was 30%, 42% and 24% for doses 0.5 Gy, 1 Gy and 2 Gy respectively. Similar results we found for both 20 μg/ml and 100 μg/ml curcumin at doses of 1 Gy and 2 Gy (Table 1, Fig. 2).

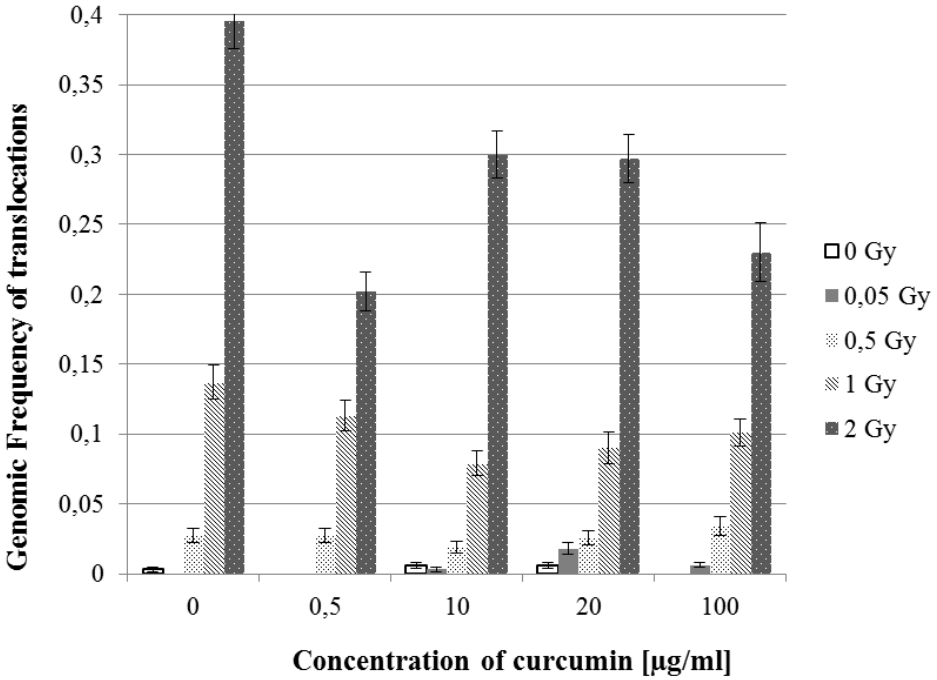

Fig. 2 Curcumin effect on genomic translocation frequency in peripheral blood lymphocytes, after γ-irradiation (ranged 0 Gy - 2 Gy). Error bars indicate standard error.

Discussion

IR is widely used in medical diagnostics, cancer therapy and also has additional industrial applications. That's why scientific interest is directed to find radioprotectors that could limit the deleterious effect of IR. Curcumin could be a potential radioprotective agent because of its high antioxidant activity and ability to neutralize the free active oxygen radicals resulting from various mutagenic factors, particularly IR [9]. The mechanisms underlying the curcumin effect after irradiation are still not clear. Their understanding could have a practical meaning in both radiotherapy and radiation risk assessment. The balance between DSBs induction and repair is considered as fundamental in biological outcome after radiotherapy. Therefore, we investigated the curcumin effect on radiation-induced γ-H2AX/53BP1 foci and translocations after *in vitro* exposure to ^{60}Co source.

Our results in respect to non-irradiated cells showed that all treatment curcumin concentrations decreased spontaneous number of nuclear foci. Jafarpour et al. [5] also observed slightly decreased foci number after treatment with 50 µg/ml curcumin, without irradiation. More detailed studies are necessary to make a general conclusion. On the other hand, the same authors reported reduced numbers of DSBs caused by ^{131}I in curcumin pre-treated human lymphocytes. In this line, we observed a reduction of γ-H2AX foci after curcumin pre-treatment in irradiated cells for all dose points. Our preliminary results are in accordance with Seong et al. [16]. They found that 100 µM curcumin reduces γ-H2AX foci formation. Contradictory, Jiang et al. [6] reported that 30 µM curcumin possesses cytotoxic effect in HCT tumour cell line and induces an increased number of γ-H2AX foci.

Misrepaired DSBs could result in rearrangement of genetic material and chromosomal aberrations appearance. In contrast to non-stable aberrations (dicentrics, rings, micronuclei), translocations are stable and could be found many years after irradiation [11]. On the other hand, translocations are considered to increase cancer risk [3]. That's why our interest is

focused on translocation frequencies in irradiated cells. In this respect FISH analysis is a suitable method for studying curcumin effect after exposure to γ-rays.

The present *in vitro* study shows the protective effect of all curcumin concentrations used in a dose range from 0.5 Gy to 2 Gy. Our findings follow previous reports concerning other types of chromosomal aberrations (micronuclei and dicentrics) [15, 17, 18]. We found that 0.5 μg/ml curcumin causes almost a twofold translocations decrease compared to non-treated cells post-exposure to 2 Gy γ-rays. After 1 Gy irradiation the most protective effect of curcumin we found for concentration 10 μg/ml when translocations frequency declined to 42%. These observations are in contradiction with Srinivasan et al. [18]. The last authors reported that increasing the doses of γ-rays, higher curcumin concentrations are needed to protect lymphocytes against DNA damage. On the other hand, curcumin is known to have a genotoxic effect on tumour cells growth. Thus, Araujo et al. [1] observed an increasing number of chromosomal aberrations in curcumin-treated Chinese hamster CHO cells when exposed to 2.5 Gy of γ-rays. Palanikumar et al. [13] discovered clastogenic activity at very low curcumin concentrations in *Allium cepa* root meristem cells.

Conclusion

Our *in vitro* results showed that curcumin expresses a protective effect on peripheral blood lymphocytes by decreasing both γ-H2AX foci and translocations appearance after exposure to γ-irradiation. More detailed investigations are needed to make a general conclusion.

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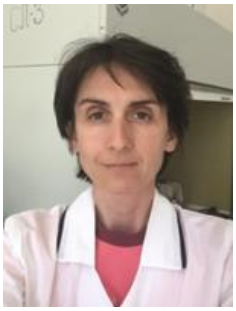

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