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Journal of Cell and Molecular Research (2019) 10 (2), 52-58 DOI: 10.22067/jcmr.v10i2.75890

Research Article

Investigating the Genotoxic Effect of Gamma Irradiation on L929 Cells after Vinblastine Treatment Using Micronucleus Assay on Cytokinesis-blocked Binucleated Cells

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Received 10 October 2018 Accepted 20 January 2019

Abstract

There are several studies suggesting the role of aneuploidy in tumor formation. Aneuploid cells are different from normal ones in term of gene expression and proteome. Cells with different amount and kind of proteins might act differently to external stimuli, including ionizing irradiation. Currently, radiotherapy is one of the main methods in fight against cancer, therefore, it is important to understand the response of the aneuploidy-tumor cells to irradiation. To investigate the chromosomal effect of gamma irradiation on aneuploid cells, L929 cells were treated with 1.5 $ng.ml^{-1}$ of vinblastine to induce aneuploidy. Vinblastine-treated cells were left to recover for 72 h and irradiated with 1 Gy of gamma radiation. Induced chromosomal damages were investigated using micronucleus (Mn) assay. Data showed that vinblastine and gamma irradiation both were able to significantly increase micronucleated-binucleated cells (MnBi) frequency. However, 1 Gy gamma irradiation of the cells after 72 h of vinblastine treatment led to the lower frequency of MnBi compared to irradiated cells. Results of this study suggest that vinblastine treatment of cells before irradiation not only did not sensitize the cells to radiation-induced chromosomal abnormalities, but also had radio-protective effect for these cells. This result could be useful in planning cancer therapy regimes.

***Keywords*:** Gamma, Vinblastine, L929 cells, Micronucleus assay, Binucleated cells

Introduction

Aneuploidy is an important incidence in cancer formation. Even low rates of chromosome mis-segregation lead to tumor formation (Silk et al., 2013). It has been reported in all solid tumors (Tanaka K, 2016). Aneuploidy by disturbing the genetic balance of the cell, affects several fetal cellular pathways involved in maintaining the integrity of genetic materials such as response of the cell to DNA damage and monitoring mechanisms of chromosome segregation during cell divisions (Nicholson and Duesberg, 2009). Carcinogens cause progressive anomalies in chromosomal integrity of the cells (Bloomfield et al., 2014). Therefore, aneuploid cells are more prone to genetic damages which may lead to transformation and cancer.

Agents capable of inducing chromosomal loss or non-disjunction do so by inducing anomalies in chromosome segregation during cell division. One of the strong aneugens is vinblastine, a Vinca alkaloid with strong aneugenic capability which has been suggested in several in vivo and in vitro studies (Cammerer et al., 2010; Leopardi et al., 2002; Zijno et al., 1996). It confers its effect by blocking tubulin dimers and preventing spindle fiber formation. It is able to induce aneuploidy even at very low doses (Cammerer et al., 2010).

Radiotherapy and chemotherapy are currently frequent approaches to cancer treatment (Blank et al., 2017; Franklin et al., 2017). Radiotherapy of cancer cells leads to harmful damages to the cells. Ionizing irradiation used in radiotherapy does so by inducing profound genetic damages to the cells. The harmful effect of ionizing radiation has been known for several years. It is able to induce damages and cuts to DNA which could end up as chromosomal breaks. It imposes damages to chromosomes by its direct effect on DNA or producing free radicals with high tendency to react with it (Ward, 1988).

Investigating the combined effects of radiation with other agents is advised by UNSCEAR (UNSCEAR, 2000). Chromosomal unbalanced cells may have different responses to other harmful agents compared to the normal ones. Because of the

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important role of aneuploidy in cancer induction, it is important to know more about the reaction of aneuploid cells to other stimuli. A previous study showed that radiation-induced aneuploid cells showed higher sensitivity to radiation (Bakhoum et al., 2015). In this study we investigated the response of the vinblastine-induced aneuploid cells to ionizing radiation, which is a most common way of cancer treatment. It is for the first time that the co-treatment of two aneugen and clastogen agents is investigated on L929 cells.

Materials and Methods

Vinblastine treatment

L929 cell line was used in this experiment. Cells were cultured in DMEM LG (Gibco) supplemented with 10% FBS (Gibco) in 5% CO2 and 37ºC. Sub-culturing was taken place every 72 h in 1:5 ratios. Vinblastine sulfate (GedeonIchter Ltd.) was dissolved in distilled water to the stock concentration of 1µg.ml^{-1}. L929 cells have been treated with three doses of 0.5 and 1.5 and 2 ng.ml-1 of vinblastine 24 h post culture initiation for next 24 h. For the second part of the experiment, cells have been treated with 1.5ng.ml^{-1} of vinblastine for 24 h. Culture medium was replaced with fresh vinblastine-free medium at the end of treatment. Cell harvest was performed at different time points of 24, 48 and 72 h post vinblastine removal. Cytochalasin-b (Cyto-b) was added to the cells at final concentration of 4ng.ml^{-1} 20 h before harvest for each time point.

Gamma irradiation

Gamma irradiation of the cells was performed with the rate of 0.99Gy/min at the final dose of 1Gy in T25 flask (60CO radiation therapy, Therateron, Canada). Cells which have been recovered at 72 h post vinblastine removal as well as non-treated cells were irradiated. Cyto-b was added to the cells at final concentration of 4ng.ml^{-1} 2 h after irradiation for 20 h.

Cell harvest and giemsa staining

Cell harvest was performed according to Fenech(Fenech, 2000) with some modifications. Briefly, 20 h post Cyto-b treatment, cells were detached from culture flasks by trypsin and centrifuged at 100g for 10 min. Cells were washed twice with 9:1 methanol: acetic acid fixator. Cell suspension was dropped on clean slides from height of 15-20 cm and left to air-dry.
Dried slides were placed in 10% giemsa solution for 20 min. They were washed with buffer phosphate and left to dry before scoring.

Scoring

Cell scoring took place at a 1000x magnification. In this study, cells with two detached nuclei with visible shared cytoplasm were scored as binucleated cell. From each culture flask at least three slides were coded, and on each slide at least 500 Bi as well as all mononucleated cells which were encountered were scored. In all Bi scored, cells harboring one or more small unattached nuclei considered as micronucleated-binucleated cell (MnBi). The frequency of MnBi was calculated as:

% MnBi= NO of MnBi/ All Bi scored x100

The Binucleated index (Bi index) was calculated as:

Bi index= NO of all Bi/ All Mono and Bi-nucleated cells scored x100

MTT test

Treated as well as untreated control cells were cultured in 96 wells culture plates. Cell viability test was performed for control and vinblastine treated cells harvested 24, 48 and 72 h post vinblastine removal. 100µg of tetrazolium salt (Sigma), dissolved in PBS, was added to 200 µl of cell culture medium and left for 6 h in 37◦C. Medium of each well was replaced with 150 µl of DMSO. Light absorbance of each well at 545 nm wavelength was recorded using ELISA reader (AWASENESS). Light absorbance value of each well was compared to control and its graph was prepared.

Statistical analysis

Statistical analysis was performed using MINITAB software version 14. The differences between control and treated cells as well as between treated groups were analyzed by one-way analysis of variance (ANOVA). For MTT analysis, the SD for all treatments was calculated using MINITAB software and the graphs were plotted using EXCEL software version 2013.

Results

Cyto-b blocked the cytokinesis of the cell division. Treatment of the cells with Cyto-b resulted in cells with two detached nuclei in one shared cytoplasm (Figure 1). Any break to chromosome structure which led to chromosome fragment or any chromosome loss resulted in binucleated cell harboring micronuclei (Mn) (Figure 2).

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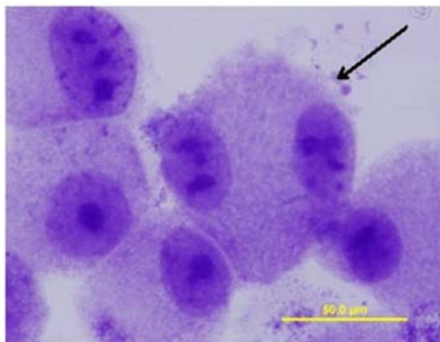

Figure 1. Binucleated cell

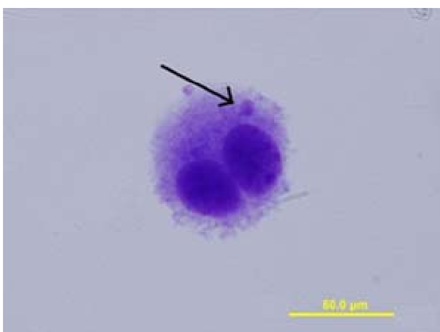

Figure 2. Binucleated cell harboring one micronucleus (MnBi)

Vinblastine treatment

Vinblastine treatment of the cells led to statistically significant increase in the frequency of MnBi (Table 1) compared to control at two highest doses used in this experiment (P<0.05). The highest increase was for 1.5 ng.ml^{-1} of vinblastine. The Bi index showed that vinblastine treatment significantly reduced the cell division activity at all doses used in this experiment (P<0.05) (Table 1).

Table 1. Vinblastine treatment of the L929 cells with different doses of 0.5, 1.5 and 2 ng.ml^{-1}

Doses of Vinblastine	Bi index ±SD	Frequency of MnBi±SD
0.0 ng.ml^{-1}	53.14 ± 3.62	1.89 ±1.64
0.5 ng.ml^{-1}	29.11 ± 2.46^{a}	5.48 ±4.52
1.5 ng.ml^{-1}	27.90 ± 4.91^{a}	15.26 ±7.58^{a}
2 ng.ml^{-1}	26.82 ± 6.94^{a}	10.63 ± 0.85^{a}

^{a}: Statistical difference with control (P<0.05)

The lowest dose that was able to induce the significant increase in MnBi frequency in this experiment was 1.5 ng.ml^{-1}. To minimize the probability of cell damage, we used this dose throughout the rest of the experiment.

Cell recovery after 1.5 ng.ml^{-1} vinblastine treatment

L929 cells were treated with 1.5 ng.ml^{-1} vinblastine for 24 h and cell harvest performed at different time intervals after cell culture replacement. The results of MnBi analysis are presented in Table 2. Vinblastine treatment of the cells led to statistically significant increase in the frequency of MnBi right after vinblastine removal compared to control (P<0.05). However, the frequency decreased in time dependent manner till reached to the control level at 72 h post culture replacement. Bi index although showed significant decrease compared to control right after vinblastine removal, but in all time points after vinblastine removal did not show any significant differences with control (Table 2).

Table 2. Effect of 24 h of vinblastine treatment at different time points after cell wash

		Binucleated index (Bi I)±SD	Frequency of MnBi±SD
control		44.89 ± 9.60	2.22 ± 1.06
Time points after vinblastine removal	0 h	27.90 ± 4.91^{a}	15.26 ±7.58^{a}
	24 h	39.56 ± 2.11	6.85 ± 0.39^{a}
	48 h	44.04 ± 5.21	5.96 ± 2.92^{a}
	72 h	35.78 ± 3.27	2.14 ± 0.51

^{a}: Statistical difference with control (P<0.05)

Figure 3 represents the result of MTT assay after vinblastine treatment for 24 h at different time points post culture replacement. The results revealed that cells were able to gain their survival ability from harmful effect of vinblastine treatment 72 h post vinblastine removal. The cell viability reached to normal after 48 h.

Gamma irradiation of untreated and vinblastine treated cells

The results of gamma irradiation of L929 cells are represented in Table 3. Gamma irradiation of L929 cells caused significant increase in the frequency of MnBi compared to control (P<0.05). Recovered cells from 1.5 ng.ml^{-1} vinblastine treatment after 72 h of vinblastine removal did not show any significant difference in the frequency of MnBi compared to control. Irradiation of the

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recovered cells 72 h after vinblastine treatment led to the significant increase in the frequency of MnBi in comparison to control ($P<0.05$), however, they also showed a significant decrease of MnBi frequency compared to non-vinblastine treated irradiated cells ($P<0.05$).

In all treatment regimes, Bi index did not show any significant difference in comparison to control (Table 3).

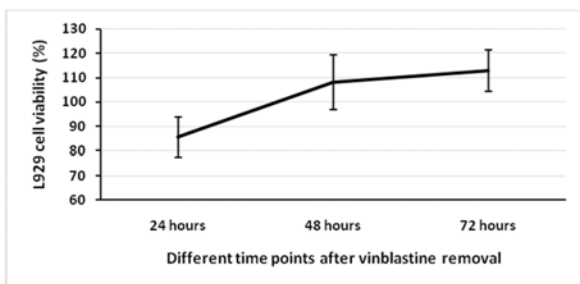

Figure 3. Cell viability after 24 h of 1.5ng.ml^{-1} vinblastine treatment at different time points post vinblastine removal

Table 3. Gamma irradiation of untreated and recovered cells 72 h after treatment with 1.5 ng.ml^{-1} vinblastine

	Binucleated index (Bi I)±SD	Frequency of MnBi±SD
Control	42.19 ± 12.06	2.45 ± 1.23
Cells recovered 72 h post vinblastine treatment	40.33 ± 6.30	2.63 ± 0.98
1 Gy gammairradiation of non-vinblastine treated cells	45.07 ± 5.96	10.40 ± 1.82[a,b]
1 Gy gamma irradiation of cells recovered 72 h post vinblastine treatment	48.83 ± 2.65	6.13 ± 1.01[a]

[a]: Statistically significant difference with control ($P<0.05$)

[b]: Statistically significant difference with vinblastine recovered cells after Irradiation ($P<0.05$)

Discussion

There are different conclusions about the role of aneuploidy and structural chromosomal aberrations in inducing cell transformation. Various kinds of numerical and structural chromosomal abnormalities have been reported in tumor cells. Although there is no doubt about the role of these two mechanisms in tumor formation, but the way these two react at the same time and how tumor cells which are involved in genetic instability react to therapy needs to be explained. Changes in the genetic composition of the cells leads to modification in time and quantity of gene expression and synthesis of proteins involve in cell division control and DNA repair, which would have profound consequences on the cell life(Nicholson and Duesberg, 2009). Ionizing irradiation of cancer cells is widely used in cancer therapy. It is suspected that cells with abnormal genetic equilibrium might act differently to the irradiation in comparison to normal cells. Aneuploid cells exhibit profound modifications in their gene expression profiles (Roschke et al., 2008) which may lead to different responses to other stimuli. In this study, we tried to investigate the effect of gamma irradiation in vinblastine-induced aneuploid cells.

Strong aneugenic capability of vinblastine has been investigated in several studies. It can induce chromosome loss or non-disjunction even at very low doses (Cammerer et al., 2010). Vinblastine is able to bind to DNA and in much higher affinity to tubulin (Pandya et al., 2014) and direct its effect through preventing tubulin polymerization during mitosis. Vinblastine treatment of the cells at the two highest doses used in this study were able to induce micronucleus formation. Judged by the nature of vinblastine action, it is possible to say that the increase in the frequency of micronucleus formation was because of high incidence of chromosome loss in treated cells. In other studies, also vinblastine treatment increased kinetochore positive micronuclei, which supports this argument (Marshall et al., 1996).

L929 cells are aneuploidy in nature. Chromosome analysis of this cell line has been revealed the chromosome number of 62-64 chromosomes (Sorokina et al., 1988). In current experiment, vinblastine treatment of these cells was performed to produced extra chromosomal instability in these cells.

Results of this experiment showed that treatment with vinblastine led to increase in the micronuclei frequency, although, it reached the base line frequency 48 h post treatment. The reduction in the frequency of induced micronuclei was started 24 h post vinblastine removal and reduced in a time dependent manner till reached the control level 72 h post vinblastine removal. Cell viability test also revealed that damaged cells were able to recover from vinblastine effect 48 h post vinblastine removal.

Reduction of the frequency of micronuclei after several cell divisions might be the result of

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reintegration of micronuclei into main nuclei or simply by losing them during cytokinesis (Leach and Jackson-Cook, 2004). In both suggested reasons it is possible to expect that the result would be creation of aneuploid cells either by losing or gaining one or more chromosomes. Therefore, most of the recovered cells in this experiment could be considered as aneuploid cells.

Ionizing radiations are able to induce structural chromosomal damages in exposed cells (Shi et al., 2012). Cells which have been treated with doses of gamma irradiation showed a significant increase in the frequency of chromosomal damages which in micronucleus assay were represented as micronuclei (Hosseinimehr et al., 2009; Rao et al., 2011). Ionizing radiation imposes its harmful effects on chromosomes of the cells either through direct cuts of double or single strands of DNA or its indirect effect by increasing the free radicals capable of inducing breaks in DNA. Ionizing radiation by passing through water, produces highly active free radicals capable of attacking macromolecules of the cells including DNA. Induced one or double strand cuts in DNA molecule eventually lead to chromosomal structural aberrations including chromosome or chromatid breaks (Hall and Giaccia, 2012).

In several studies increase in the frequency of micronucleus in binucleated cells was proceeded the gamma irradiation of human peripheral lymphocytes (Hosseinimehr et al., 2009; Rao et al., 2011). In this study gamma irradiation of L929 cells also led to significant increase in the MnBi. The induced frequency of MnBi in our study was lower than the frequency of MnBi in human peripheral lymphocytes irradiated in vitro in both studies mentioned earlier (Hosseinimehr et al., 2009; Rao et al., 2011). This difference might be related to the higher dose of gamma-ray used in those studies.

On basis of the nature of damages imposed by ionizing radiation it is possible to expect that high frequency of micronuclei in exposed cells in the present study was mainly the result of chromosome or chromatid breaks. Those chromosomal parts were left behind in the cytoplasm during cell division and form micronuclei.

Cells already treated with vinblastine when exposed to 1 Gy gamma irradiation showed a lower frequency of MnBi compared to untreated cells. The vinblastine treated cells are expected to have unbalance composition of chromosomes and genetic materials. It is possible to consider the protective effect of aneuploidy against ionizing radiation through alteration in transcription and changing the amount of proteins and gene transcripts involved in radiation response of the cells. The gene expression modification in aneuploid cells could affect different aspects of cell response to external stimuli. The role of chromosomal instability in response to cancer therapy has been reviewed extensively (Rangel et al., 2017). Changes in the cell transcriptome by aneuploidy leads to increase in expression of a large group of genes appropriate for cancer formation (Gao et al., 2007). From these large group of genes some might be responsible for resistance to irradiation. These cells produce the higher amount of proteins with capability of radioprotection. Change in gene expression profile of the vinblastine-induced aneuploid cells results in producing higher amount of proteins involved in free radical scavenging and/or DNA repair mechanisms. The reduced frequency of MnBi in those aneuploid cells could be a result of alteration in quantity of gene products of those cells. The result of this study is in contrary with another study that suggests the higher sensitivity of aneuploid cells to ionizing radiation compared to normal cells (Bakhoum et al., 2015). The difference could be related to the way of inducing aneuploidy in those cells compared to ours. In that study aneuploidy was induced by irradiation whereas in ours it was induced by vinblastine treatment. Aneuploidy induction by irradiation would not be the only consequence of irradiation. Irradiation most definitely would be able to induce some forms of gene mutation which might reduce the resistance of the cells to subsequent irradiation. In our study aneuploidy was induced by vinblastine, which no evidences show its mutagenic capability. Therefore, it is possible to imagine that vinblastine induced aneuploid cells have no gene mutation which reduce their resistance to irradiation.

This lower frequency of chromosomal damages also could be the result of ability of vinblastine to reduce the harmful effect of ionizing radiation by its free radical scavenging capability. The radio-protective ability of vinblastine has been already suggested (Rajagopalan et al., 2003). Hence, despite vinblastine removal, the amount of vinblastine inside the cells could act as an antioxidant and protect the cells from clastogenic activity of gamma irradiation. To be able to understand this, the time interval between vinblastine removal and irradiation must be extended to several days.

Data of this study suggest that vinblastine treatment is able to protect the cells from ionizing radiation induced damages. The lower frequency of damages

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induced by gamma irradiation in aneuploid cells reveals the need for more studies to create more effective cancer treatment protocols. The authors suggest longer interval time between vinblastine treatment and ionizing irradiation to reduce the probability of direct protection of vinblastine.

Acknowledgements

This work was supported financially by the Ferdowsi University of Mashhad under the grant No. 3/31476.

Conflict of Interest

The authors report no conflicts of interest.

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