Original Article

Effects of Valproic Acid on Radiation-Induced Chromosomal Aberrations in Human Lymphocytes

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ABSTRACT

One of the most widely employed histone deacetylases inhibitors in the clinic is the valproic acid (VA), proving to have a good tolerance and low side effects on human health. VA induces changes in chromatin structure making DNA more susceptible to damage induction and influence DNA repair efficiency. VA is also proposed as a radiosensitizing agent. To know if VA is suitable to sensitize human lymphocytes γ-irradiation *in vitro*, different types of chromosomal aberrations in the lymphocytes, either in the absence or presence of VA, were analyzed. For this purpose, blood samples from four healthy donors were exposed to γ-rays at a dose of 1.5 Gy and then treated with two different doses of VA (0.35 or 0.70 mM). Unstable and stable chromosomal aberrations were analyzed by means of fluorescence *in situ* hybridization. Human lymphocytes treated with VA alone did not show any increase in the frequency of chromosomal aberrations. However, a moderate degree of sensitization was observed, through the increase of chromosomal aberrations, when 0.35 mM VA was employed after γ-irradiation, whereas 0.70 mM VA did not modify chromosomal aberration frequencies. The lower number of chromosomal aberrations obtained when VA was employed at higher dose after γ-irradiation, could be related to the induction of a cell cycle arrest, a fact that should be taken into consideration when VA is employed in combination with physical or chemical agents.

Key words: Biological dosimetry, chromosomal aberrations, histone acetylation, histone deacetylase inhibitors, ionizing radiation, valproic acid

Introduction

Eukaryotic DNA is packaged in a complex and dynamic structure defined as chromatin, where DNA strands are wounded around a core of histone proteins.[1] Changes in chromatin state regulate multiple critical nuclear processes, expanding the repertoire of regulatory factors encoded by the genome such as DNA replication, recombination, transcription, and repair. Moreover, chromatin conformation modulates DNA damage sensitivity a well as DNA damage response, contributing to the genome stability. Besides, it is well known that chromatin state influences genomic imprinting, cell division, and cell death.[2-5] Chromatin remodeling occurs through a cross talk among distinct chromatin remodeling mechanisms including: (i) DNA methylation, a covalent addition of a methyl group to cytosines at CpG dinucleotides, regulating gene expression, (ii) ATP-dependent chromatin remodeling mechanisms (i.e., SWI/SNF, ISWI) that locally dislocate DNA/nucleosome interactions by repositioning nucleosomes, and (iii) a posttranslational histone modifications that act in concert to regulate numerous nuclear processes. The posttranslational histone changes are produced by enzymatically reversible mechanisms, which covalently incorporate and remove different chemical groups to the histone residues, mainly at the amino-terminal histone tails.[5] These epigenetic histone modifications include: (a) Poly-ADP-ribosylation on glutamate and arginine residues of the histone tail;[6] (b) mono- or poly-ubiquitination that covalently attaches one or more ubiquitin moiety to lysine residues and the ubiquitin-like sumoylation adding a small ubiquitin-like modifier group;[7] (c) histone phosphorylation that takes place predominantly on serines, threonines, and tyrosines of the amino-terminal histone tails; (d) histone methylation that mainly

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occurs on the side chains of lysines and arginines;[5] (e) histone biotinylation, by adding the vitamin biotin to the amino group of lysine in histones;[8] and (f) the most common epigenetic histone modification: The histone acetylation produced by the addition of acetyl groups on lysines, mostly on the amino-terminal tails of core histones.[3-5] Acetylation neutralizes lysine's positive charge and decreases histones binding to the negatively charged DNA, resulting in a more relaxed and accessible chromatin conformation. Accordingly, histone acetylation allows gene expression, whereas deacetylation inhibits the expression of genes.[9] A family of nuclear enzymes is responsible for maintaining histone acetylation status, namely histone acetyltransferases (HAT) and histone deacetylases (HDAC). HDAC is better targets than HAT as modulators of protein acetylation (including histones) since the inhibition of HDAC can be used for human cancer and neurological diseases treatments with less effect on normal cells.[10,11] Depending on their structural homologies, enzymatic activities, and subcellular localization, the HDAC can be classified into four classes.[12,13] Class I and Class II HDAC are generally inhibited by most HDAC inhibitors (HDACis).[3] All HDACis differ quite remarkably in their chemical stability or toxicity. In this respect, HDACi can be classified into four different groups, namely, short-chain fatty acids (sodium butyrate and valproic acid [VA]), hydroxamic acids (trichostatin A and suberoylanilide hydroxamic acid [SAHA]), cyclic peptides (trapoxin and depsipeptide), and benzamides (MS-275). Among the most HDACi employed in clinics, the VA inhibits all Class I HDAC and some of Class II HDAC,[14] while HDACi such as SAHA and trapoxin present more global activity, inhibiting all HDAC from Class I and II. Since VA inhibits transamination of the neurotransmitter gamma-aminobutyric acid (GABA), it has been largely employed in the treatment of epilepsy and psychiatric disorders.[15,16] More recently, because of its HDACi property, producing changes in chromatin state and DNA damage response, VA has also been used to sensitize tumor cells to anticancer therapy.[17-20]

Changes in chromatin structure are expected to affect radiosensitivity.[21,22] Transcriptionally active genomic regions, comprising an acetylated and relaxed chromatin structure, have proved to be more sensitive to ionizing radiation.[23-28] On the other hand, cells are also more radiosensitive when the chromatin achieves a high state of compaction during the G2/M phase of the cell cycle although G2/M cells do not get more DNA double-strand breaks (DSBs).[29] Therefore, even though a more relaxed chromatin structure favors the production of radiation-induced DNA strand breaks, DNA repair could be an important component of radiosensitivity as well.[30] Precise dose estimation, by means of a chromosomal aberration scoring, is necessary to determine health risk assessment when people are exposed to ionizing radiation.[31] Dicentric chromosomes as well as translocations are the most widely used biomarkers in biological dosimetry for dose estimation.[32] In addition, radiosensitization induced by HDACi is accompanied by a prolonged nuclear appearance of γH2AX and 53BP1 foci as identified by immunofluorescent staining.[17,33] To know if VA is suitable to sensitize human lymphocytes exposed to γ-radiation *in vitro* toward a possible application in biological dosimetry, two different doses of VA, commonly employed in clinics as reference levels in blood,[11] were added to blood cultures after γ-irradiation, and all types of chromosomal aberrations (stable and unstable) were analyzed on metaphases following of fluorescent *in situ* hybridization (FISH).

Materials and Methods

Blood sampling, lymphocyte cultures, treatments, and slides preparations

Blood samples were obtained from four nonsmoker healthy donors (neither received any pharmacological treatment), between 20 and 50 years old, who gave their informed consent.

Whole blood samples (0.5 ml per culture) were cultured at 37°C during 24 h in 5 ml of RPMI 1640 (supplemented with 2 mM L-glutamine, 1 mM pyruvate, 10 mM HEPES buffer, and 1% penicillin-streptomycin) (Life Technologies, Cergy-Pontoise, France), with the addition of 20% fetal calf serum (FCS; Life Technologies, Cergy-Pontoise, France) and 5% phytohemagglutinin-M (PHA, M form, Life Technologies) to stimulate cells division. To analyze percentages of second metaphases, 1% BrdU was added to the cultures. Whole blood samples were irradiated with a source of γ-rays of Cesium 137 at 37°C, at 1.5 Gy (0.5 Gy/min) in an IBL Irradiator (^{137}Cs, γ-rays; IRSN, Fontenay-Aux-Roses, France), at a distance to the source of 550 mm. After γ-irradiation, cultures were incubated in the presence of VA (0.35 mM or 0.7 mM) at 37°C for the next 48 h until harvesting. Control cultures were processed similarly but without γ-rays and/or VA treatments.

Blood cultures were treated with colcemid (0.1 μg/ml) (Colcemid KaryoMAX; Sigma-Aldrich, France) 2 h before harvesting. After harvesting, they were exposed to a hypotonic solution of KCl (0.075 M) for 10 min at 37°C, prefixed, and then fixed three times with methanol-acetic acid (3:1). For slide preparation, 30 μl of the cell suspensions were dropped onto clean slides in a Thermotron with controlled temperature (20°C) and humidity (43%).

Fluorescence plus Giemsa staining (FPG technique)

Slides were exposed to a Hoechst 33258 (0.05 mg/ml) solution for 10 min. Afterward, they were rinsed in distilled water, covered with 2 × SSC, and exposed to a 20 W ultraviolet lamp (>310 mm) for 60 min. Subsequently, the slides were washed in 2 × SSC, and then with distilled water. Finally, they were stained in a 4% Giemsa solution (in 2 × SSC) for 10 min, rinsed in distilled water, air-dreaded, and mounted.[34] Giemsa staining images were captured with the Metafer4 software (MetaSystems, Germany) on a Zeiss Axioplan microscope, and mitotic index and percentages of second metaphases were estimated for each experimental condition.

Three-color chromosome painting (fluorescent *in situ* hybridization)

FISH technique was performed according to Pinkel *et al*.[35,36] with some modifications as follows. Freshly chromosome spreads were pretreated with pepsin-HCl (0.05%) for

2 min a 37°C and immediately washed in Phosphate Buffer Solution (PBS) (1×) (PBS; Invitrogen, France) for 3 min. Subsequently, they were fixed in formaldehyde (20910.294; VWR, France) and $MgCl_2$ solution (M2670; Sigma-Aldrich, St Quentin Fallavier, France) dissolved in PBS (1×) during 10 min at room temperature and washed in PBS (1×) for 3 min. Subsequently, the slides were dehydrated in 70%, 90%, and 100% ethanol (2 min each) and air-dried. Commercial whole chromosomes probes for chromosomes 2 (Rhodamine), 4 (Fluorescein IsoThioCyanate (FITC), and 12 (both Rhodamine and FITC) (Qbiogen probes, Illkirch, France) were denatured for 5 min at 75°C and then 30 min at 37°C. Slides' preparations were denatured 3 min at 37°C and immediately hybridized with the denatured chromosome probes (overnight, at 37°C). The following day, they were washed with 1 × SSC (SSC; VWR, Fontenay-sous-Bois, France) (2 min, 75°C) and rinsed in 2 × SSC plus 0.01% Tween 20 (28829.183; VWR, Fontenay-sous-Bois, France) (1 min, at room temperature). Finally, they were washed in PBS (1×) for 3 min, counterstained with Vectashield plus DAPI (4, 6-diamidino-2-phenylindole-2-hydrochloride, Qbiogen, Illkirch, France), and mounted with coverslips. Fluorescent images were captured with Metafer system (MetaSystems GmbH, Germany) on a Zeiss Axioplan epifluorescence microscope, and the frequency of translocations was analyzed.

Frequencies of dicentrics and translocations by means of three color painted chromosomes

For both dicentric and translocations scoring, a minimum of 1000 metaphases from the first cell division containing the complete set of chromosome ($2n = 46$) were analyzed. Dicentric and translocations were observed in FISH-three color chromosomes 2, 4, and 12 painting according to Pouzoulet *et al*.[37] and extrapolated to the whole genome following Lucas *et al*.[38] formula. It was considered that chromosomes 2, 4, and 12 represent the 34% of the total genome, covering 19% of the DNA content according to Morton[39] and regarding the exchanges between painted and unpainted chromosomes. The genomic frequencies, FG, were calculated from the respective frequencies (Fp) of painted chromosomes, applying the standard formula: $FG = Fp/2.05\, fp\, (1 - Fp)$, where Fp is the aberration frequency detected by painting and fp is the fraction of the genome painted, i.e., target chromosomes 2, 4, and 12.[38]

Statistical analysis

One-way ANOVA test (with a confidence interval of 95%) was employed to compare the genome equivalent (GE) yields of chromosomal aberrations obtained under different experimental conditions: Control cultures; 1.5 Gy γ-irradiated, VA (0.35 or 0.7 mM) treated blood cultures, and 1.5 Gy γ-irradiated plus VA (0.35 or 0.7 mM) treated cultured human lymphocytes.

Results and Discussion

One of the most widely employed HDACis in clinical is the VA. The capacity of VA to inhibit the transamination of the neurotransmitter GABA, make it one of the most common drugs largely employed in the treatment of epilepsy and psychiatric disorders,[15,16] indicating its good tolerance and low side effects on human health. VA inhibits preferentially all Class I HDACs and only some of Class II HDACs, acting in a more specific way respect to others HDACi,[14,17,18] to induce changes in the chromatin structure, which, in turn, relax DNA conformation and make DNA more susceptible to damage induction.[40,41] In addition, effects of VA on DSB repair could also occur in both normal[17,42] and tumor cells,[43,44] by downregulation the expression of several DNA repair proteins such as Ku70, Ku86, Rad51, and DNA-PK.[45,46] In this respect, VA has been employed to sensitize tumor cells, as a novel strategy for cancer therapy.[19,20]

VA was also proposed as a radiosensitizing agent. Different mechanisms have been associated with radiosensitization following HDAC inhibition, namely: (a) Changes in chromatin structure that increase the accessibility of reactive oxygen species mediating the 60% of radiation-induced DNA damage or can induce transcriptional modifications of genes involved in DNA damage response; (b) changes in chromatin remodeling processes preceding DNA repair that can independently influence repair of radiation-induced DNA injury;[18,23,33] (c) incomplete chromatin restoration at the final step of the DNA repair process; or (d) direct inhibition of radiation-induced DNA damage repair proteins.[42] To know if the addition of VA after the irradiation of blood samples is able to sensitize human lymphocytes for the formation of different types of chromosomal aberrations, blood samples from four healthy donors were exposed to γ-rays, and subsequently subjected to two different doses of VA. These doses of VA (0.35 and 0.70 mM) correspond to the blood reference levels commonly employed in clinics to reach the VA therapeutic efficacy on patients with epilepsy.[11] In this respect, we have demonstrated that treatment of human lymphocytes with VA alone did not produce any increase in the genomic frequency of chromosomal aberrations [Figure 1]. Unstable chromosomal aberrations (UCA: Dicentrics, rings, and fragments) and stable chromosomal aberrations (SCA: Reciprocal and nonreciprocal translocations), analyzed by means of FISH on three painted chromosomes (HSA 2, 4, and 12), were scored on metaphases obtained after irradiation of human lymphocytes with 1.5 Gy of γ-rays in the presence or absence of VA (0.35 or 0.70 mM) added after irradiation and kept for 48 h in blood cultures. Pooled data from donors showed that human lymphocytes exposed to 1.5 Gy of γ-rays plus 0.35 mM VA did not vary significantly from the irradiated cultures alone in terms of the GE frequencies of total chromosomal aberrations [Figure 1a]. However, analyzing separately the frequency of UCA and SCA, there was a significant increase of UCA GE frequency when 0.35 mM VA was present ($P \leq 0.05$). On the other hand, the frequency of all chromosomal aberrations induced by 1.5 Gy of γ-rays decreased significantly ($P \leq 0.05$) when 0.70 mM VA was employed [Figure 1b]. This decrease was due to the significant decrease of both types of chromosomal aberrations, UCA and SCA ($P \leq 0.05$). Contrary to expectations, the higher dose of VA did not sensitize human lymphocytes to γ-irradiation. On the other hand, the lower dose (0.35 mM) of VA only showed an increase of UCA GE frequencies after γ-irradiation, probably related to the effect of VA on the differential efficiency of

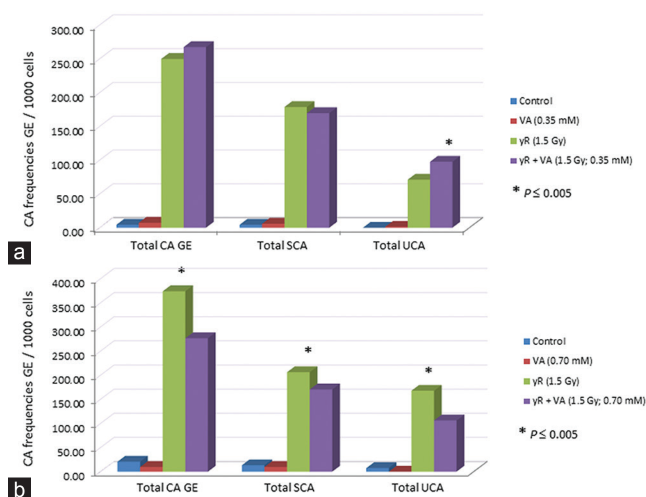

Figure 1: Genome equivalent frequencies of chromosomal aberrations in 1000 cells (CA GE) estimated for the whole genome induced by 1.5 Gy of γR on human peripheral lymphocytes, recovered in the presence or absence of valproic acid during 48 h. SCA: Reciprocal and nonreciprocal translocations and UCA: Dicentrics, rings, and fragments were detected on whole chromosomes probes-fluorescent *in situ* hybridization of HSA 2, 4, and 12. Genome equivalent CA frequencies were estimated applying the Lucas formula from the observation of exchanges between painted chromosomes and unpainted chromosomes. (a) Frequency of total CA, SCA and UCA induced by γR and posttreated with 0.35 mM valproic acid; and (b) frequency of total CA, SCA and UCA induced by γR and posttreated with 0.70 mM of valproic acid. Frequency of CA in unirradiated blood cultures in the presence or absence of different doses of valproic acid is also shown. SCA: Stable chromosomal aberrations, UCA: Unstable chromosomal aberrations, γR: γ-rays

signaling and/or DSB repair proteins involved in processing such chromosomal aberrations.[45] Besides, the analysis of the mitotic index showed that human lymphocytes treated with γ-rays in combination with VA, highly decrease cell division, particularly with the posttreatment of 0.7 mM VA [Table 1]. On the other hand, the analysis of the percentage of second metaphases confirmed the cell cycle arrest produced by the presence of VA after γ-irradiation [Table 1]. It is known that HDACi induces a cell cycle arrest and apoptosis either in tumor cell lines[47,48] or in normal cells such as human lymphocytes,[49,50] which could explain why less damaged cells were observed after the higher VA dose [Figure 1b].

Alternatively, several studies that examined the genotoxicity of VA by analyzing sister chromatid exchanges (SCEs) in human lymphocytes from patients treated with VA have shown opposite results. Hu *et al*.[51] observed a significant increase of SCEs in human lymphocytes from epileptic children treated with VA, whereas Schaumann *et al*.[52] found that VA treatment did not induce SCEs in peripheral lymphocytes of adult male patients. In this respect, results obtained showed a moderate degree of sensitization in terms of chromosomal aberrations when 0.35 mM VA was employed, while a higher dose of VA (0.70 mM) clearly did not increase the frequencies of chromosomal aberrations. VA could produce different effects depending on

Table 1: Mitotic index and percentage of second metaphases of whole blood cultures irradiated with γ-rays (γR: 1.5 Gy) and recovered in the presence or absence of valproic acid (VA: 0.35 or 0.70 mM) for 48 h

	Mitotic index	Second metaphases
Control	7.0	12
VA (mM)		
0.35	4.7	5
0.70	5.0	3
γR (1.5 Gy)	5.5	5
γR (1.5 Gy) + VA (0.35 mM)	3.7	2
γR (1.5 Gy) + VA (0.70 mM)	1.1	0

VA: Valproic acid, γR: γ-rays

the dose, such as the cell cycle block at G1 stage, which could contribute in diminishing the frequencies of chromosomal aberrations.[53] In this respect, it has to be taken into consideration that cell cycle arrest and lower amount of damaged metaphases were when VA is being employed as a sensitizer in combination with physical or chemical agents.

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Conflicts of interest

There are no conflicts of interest.

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