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Effects of 1α,25-Dihydroxyvitamin D_3 on Doxorubicin-induced Chromosomal Aberrations in Rat Bone Marrow Cells

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The present study was carried out to evaluate the effects of 1α,25-dihydroxyvitamin D_3 (VD) on chromosomal aberrations induced by doxorubicin (DXR). Wistar rats were divided into eight experimental groups of five animals each. Control group animals were treated with i.p. distilled water. The animals in three VD groups were given only VD for 4, 6 or 8 weeks. In the DXR groups the animals were given only DXR. In the combination groups VD doses were given for 4, 6 or 8 weeks for each group and DXR was injected 24 h before sacrificing the rats. DXR (50 mg/100 g b.w.) was injected intraperitoneally and VD by gavage 3 μg/kg/day twice weekly. Animals treated with both VD and DXR showed a low frequency of chromosomal aberrations and abnormal metaphases when compared with animals treated with DXR alone ($p < 0.0001$). The numbers of both chromosomal aberrations and abnormal metaphases were similar in weeks 6 and 8 ($p > 0.05$) and lower than those in week 4 for the VD groups ($p < 0.0001$). Under the present experimental conditions, the efficiency of VD in protecting cells against DXR-induced chromosome damage was found to be dose dependent. The protective effects of VD on chromosome aberrations induced by DXR are discussed in the light of literature data.

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Doxorubicin (DXR) is one of the most active chemotherapeutic agents for the treatment of solid tumors, such as carcinomas of the breast and lung, and soft tissue sarcomas. The cytotoxic effect of DXR is produced at the cellular level by multiple mechanisms that have not yet been conclusively identified. Key factors are a combination of DXR-induced free radical formation due to metabolic activation, deleterious actions at the level of the membrane, and drug intercalation into DNA (1). DXR induces mutations and chromosome aberrations in normal and tumor cells. In addition, cellular enzymes are capable of converting DXR into free-radical metabolites (2). It is becoming clear that DXR cytotoxicity may be mediated by free radicals derived from this drug. These reactive oxygen species may then oxidize proteins, lipids and nucleic acids and potentially cause DNA strand scission. In this way, DXR could induce mutations and chromosome aberrations in normal and tumor cells (3, 4). Some vitamins may scavange harmful species, free radicals or electrophiles, which damage DNA and other cell targets. Antioxidant defenses against these damages include a few elements and some vitamins such as ascorbate, tocopherol and carotenoids (5, 6). Sardar et al. (7) have documented 1α,25-dihydroxyvitamin D_3 (VD) as an effective antioxidant.

The question is whether administration of VD could modify the DXR-induced clastogenic effect on normal cells. The present study was therefore undertaken to investigate the modulatory effects of VD and of its combination with DXR on the clastogenic action of DXR in Wistar rat bone marrow cells.

MATERIAL AND METHODS

The procedures and animal protocols followed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No: 86-23, revised 1985), and were approved by the Ethics Committee of Kocaeli University, School of Medicine. The animals were supplied by the Animal House of the Faculty of Medicine of Kocaeli.

Doxorubicin (Adriablastina® produced by Carlo Erba) and vitamin D (Rocaltrole® = calcitriol, 1α,25-dihydroxyvitamin D_3, produced by Roche) were purchased on the

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local market. DXR and VD were dissolved in distilled water and in olive oil, respectively, just before the experiments were undertaken.

In this study, 6-week-old albino Wistar rats, weighing approximately 150 g, were used for the experiments. The rats were divided into eight different experimental groups of containing five animals each:

Group 1 (control): Control group, distilled water was given intraperitoneally and olive oil orally (n = 5).
Group 2 (VD_4): Vitamin D was given orally with 3 μg/kg/day twice weekly for 4 weeks (n = 5).
Group 3 (VD_6): Vitamin D was given orally with 3 μg/kg/day twice weekly for 6 weeks (n = 5).
Group 4 (VD_8): Vitamin D was given orally with 3 μg/kg/day twice weekly for 8 weeks (n = 5).
Group 5 (DXR): Doxorubicin was given intraperitoneally with 50 mg/100 g b.w. (n = 5).
Group 6 (VD_4+DXR): Vitamin D was given orally with 3 μg/kg/day twice weekly for 4 weeks and doxorubicin was given intraperitoneally with 50 mg/100 g b.w. on day 27 (n = 5).
Group 7 (VD_6+DXR): Vitamin D was given orally with 3 μg/kg/day twice weekly for 6 weeks and doxorubicin was given intraperitoneally with 50 mg/100 g b.w. on day 41 (n = 5).
Group 8 (VD_8+DXR): Vitamin D was given orally with 3 μg/kg/day twice weekly for 8 weeks and doxorubicin was given intraperitoneally with 50 mg/100 g b.w. on day 55 (n = 5).

The control group was treated with i.p. distilled water. The dose of DXR (50 mg/kg b.w.) was selected on the basis of its effectiveness in inducing chromosomal aberrations. The therapeutic doses of DXR in cancer patients are usually 40–50 mg/m^2 or approximately 1.3 mg/kg. The appropriate DXR amount was adjusted to 0.5 ml/100 g b.w. in distilled water and injected intraperitonally. The dose was chosen because it induced the increase in frequency of chromosomal aberrations necessary for the investigation of the anticlastogenic potential of VD. The full protective efficiency of an agent is elicited when the frequency of aberrations induced is high enough and the concentration of the protector given is sufficient (6). In this study, we decided that the minimum frequency of aberrations induced by DXR should be 30%.

The animals were treated with DXR by the intraperitoneal route as this mode of administration permits a marked exposure of bone marrow cells to the agent tested (8).

The dose of VD was selected on the basis of literature data that presented antimutagenic effects (9, 10). VD was administered orally to the rats at a dose of 3 μg/kg ((7.8 μM/l) dissolved in 100 μl of propylene glycerol) twice a week.

DXR doses were injected into the rats in the DXR, VD_4+DXR, VD_6+DXR and VD_8+DXR groups 24 h before sacrifice.

Preparation of rat bone marrow cell system

Bone marrow cell preparations for the analysis of chromosomal aberrations were carried out by a modification of Ford and Hammerton's 'colchicine–hypotonic citrate technique' (10). In our study, potassium chloride (0.075 M) was used instead of sodium citrate (1%).

All animals were injected intraperitonally with 0.1% (1 ml/100 g b.w.) colchicine 120 min before sacrifice. The rats were sacrificed and then the bone marrow cells flushed from the femora with 0.075 M potassium chloride. The cells were incubated at 36°C for 15 min, and then centrifuged at 1000 rpm for 5 min. The supernatant was discarded carefully; approximately 3 ml fixative (3/1; methanol/acetic acid) was added onto the cell button and fixation was allowed for 15 min at 25°C. The resuspended cells were centrifuged at 1000 rpm for 5 min after fixation. This washing was repeated 3–4 times. The final cell suspension was dropped onto chemically clean slides and stained with 5% Giemsa stain.

The chromosomal abnormalities in the cells were used for analysis on a well-spread metaphase plate with 42 ± 1 chromosomes. One hundred metaphases per animal were analyzed in order to determine the frequencies of chromosomal aberrations in a blind test. The mitotic index was determined by counting the number of mitotic cells in 1000 cells per animal. The chromosomal aberrations were classified according to Savage's classification (11).

Statistical analysis

Statistical analysis for the difference in the number of chromosomal aberrations and mitotic index among groups was performed by a one-way ANOVA test. In cases in which $p < 0.05$, the mean values of each treatment were compared by the Tukey test, in which the calculation of the minimum significant difference for p is 0.05.

Gaps were recorded but not included in the statistical analysis, as their cytogenetic significance is not well established (6).

RESULTS

The results obtained for the different treatments with VD and/or DXR are presented in Table 1. According to Savage's classification (11), seven structural chromosomal aberrations were determined in the control and experimental groups. As expected, animals treated with DXR showed a high frequency of chromosomal aberrations as well as abnormal metaphases when compared with the controls ($p < 0.05$). In treatment with DXR the most frequent chromosomal aberrations observed were chromatid breaks, followed by complex exchanges, gaps and other

Table 1

The mean values of chromosomal structural abnormal metaphases and mitotic index were observed in Wistar rat bone marrow cells after treatment with vitamin D (VD) and/or doxorubicin (DXR)

	MI (%)	Gap	CB	ICB	E	T	AF	TC	Total	AM
Control	3.49±0.23	0.8±0.84	1.4±0.55	0	0	0	0.4±0.55	0	1.8±1.10	1.4±0.55
VD4	2.83±0.40	0.2±0.45	1±0.71	0	0	0	0.4±0.55	0	1.4±1.14	1±0.71
VD6	2.99±0.16	0	1.2±0.84	0	0	0	0.4±0.55	0	1.6±1.14	1.2±0.84
VD8	3.06±0.23	0.2±0.45	0.8±0.84	0	0	0	0.2±0.45	0	1±1.22	0.8±0.84
DXR	1.32±0.21[1]	2±1.22	24.6±4.72	2.4±1.14	5.2±2.28	1±1.22	3.4±1.34	0.4±0.55	37±8.001	28.6±3.581
VD4+DXR	1.59±0.71[1]	1.8±0.84	16.2±3.56	1±1.00	3.4±1.14	0.6±0.89	2.6±1.14	0	23.8±5.932	13.2±1.922
VD6+DXR	1.84±0.26[1]	1.8±0.84	8.2±2.17	0.8±0.84	1±0.71	0	1.4±0.55	0	11.4±3.212	10±1.582
VD8+DXR	2.21±0.38[2]	1.4±0.89	8±1.58	0	2.4±1.14	0	0.6±0.55	0	11±1.872	7.6±0.552

Gap numbers were not included in total chromosomal aberration.
Abbreviations: MI = mitotic index; CB = chromatid break; ICB = isochromatid break; E = complex exchange; T = triradial figure; AF = acentric fragment; TC = translocation chromosome; AM = abnormal metapahase.
[1]Significantly different from the control group ($p < 0.05$).
[2]Significantly different from the DXR group ($p < 0.05$).

rearrangements such as triradial and quadriradial figures. Animals treated with DXR showed a high frequency of gaps, chromatid breaks, isochromatid breaks, exchanges, triradial figures, acentric fragments and translocations of chromosomal aberrations when compared with the control group and the VD groups ($p < 0.05$) (Table 1).

In the combination groups, VD_4, VD_6 and VD_8 doses did not restore the total number of chromosomal aberrations induced by DXR to the control levels.

The rats treated with DXR or with combinations of VD plus DXR (VD_4+DXR, VD_6+DXR, VD_8+DXR) presented a significantly lower mitotic index compared to control and VD groups (VD_4, VD_6, VD_8). The rats treated with VD during 8 weeks and DXR presented a significantly higher mitotic index compared to the DXR group alone ($p < 0.05$) (Table 1).

With respect to chromosomal aberrations and abnormal metaphases, no significant differences were found ($p > 0.05$) when each of the three VD groups was compared with the control group. Total chromosomal aberration counts were significantly higher in the DXR and VD+DXR groups than the VD and control groups ($p < 0.05$).

Animals treated with both VD and DXR showed a low frequency of chromosomal aberrations and abnormal metaphases when compared with animals treated only with DXR ($p < 0.0001$). The numbers of both chromosomal aberrations and abnormal metaphases were similar in weeks 6 and 8 ($p > 0.05$) and lower than those in week 4 for the VD groups ($p < 0.0001$).

DISCUSSION

Like most anticancer drugs, DXR has undesirable side effects. The main genetic effect of DXR and related compounds is binding to DNA. It is known that DXR and other anthracyclines induce peroxide production in various tissues (1). DXR has reactive oxygen species in its cytotoxic mode of action. Cellular enzymes are capable of converting DXR into free radical metabolites. DXR cytotoxicity may be mediated by free radicals derived from this drug (2).

Some vitamins may provide protection from harmful species and free radicals that damage DNA and other cellular targets. Some food-related substances such as vitamins A, C and E are known to cause significant reduction in the incidence of DXR-induced chromosomal changes (3).

In this study, we observed that the clastogenic effect of acute single-dose DXR in bone marrow tissues was maximal 24 h after the last drug injection. These results are in concordance with other studies (6, 12).

In this study, a depressed mitotic index was seen in groups treated with DXR alone or in combination with VD (Table 1). The rats treated with combinations of VD plus DXR (VD_4+DXR, VD_6+DXR, VD_8+DXR) presented a significantly lower mitotic index compared to control and VD groups (VD_4, VD_6, VD_8). The rats treated with DXR and VD_8 presented a significantly higher mitotic index compared to the DXR group ($p < 0.05$) (Table 1). This is related to the cytotoxic effects of DXR on bone marrow cells and similar results have been reported in previous studies (4, 6).

The clastogenic effect of DXR in rodent bone marrow cells was well documented previously by some authors (4, 13). In this study, chromosomal aberrations and abnormal metaphases were similar in each of the three VD groups and in the control group ($p > 0.05$). The total chromosomal aberration counts were found significantly higher in the DXR and VD+DXR groups than in the VD and control groups ($p < 0.05$).

Animals treated with both VD and DXR showed a low frequency of chromosomal aberrations and abnormal metaphases when compared with animals treated only with DXR ($p < 0.0001$). The numbers of both chromosomal aberrations and abnormal metaphases were similar in weeks

6 and 8 ($p > 0.05$) and lower than those in week 4 for the VD groups ($p < 0.0001$). The use of VD for 6 weeks seems to be a sufficiently long period for protective action against DXR toxicity of chromosomes.

Various studies have been carried out to observe the cellular protective effects of VD and different mechanisms have proposed in explanation. The anticlastogenic response of VD was considered to be due to the promotion of excision–repair activity (14). Basak et al. (15) reported that a single dose of diethylnitrosamine (DEN) induced a considerable number ($p < 0.001$) of aberrant metaphase cells in the hepatocytes. DEN-evoked chromosome aberrations consisted mainly of structural aberration, that is gaps, breaks and centric constriction. Supplementation of VD started 4 weeks before the DEN challenge. Synergistic supplementation of both vanadium and VD offered maximum protection against DEN-induced structural aberrations 96 h, 15 and 30 days after DEN injection. Thus, a combination of the effect of vanadium, an essential trace element, and VD, a dietary micronutrient, seems to be beneficial in preventing damage to liver cells upon alkylation induced by DEN.

In a transplantable murine lymphoma model, a steady increase in terms of percentage protection following VD treatment could be seen with respect to time. Two weeks of VD treatment offered the maximum depletion in sister chromatid exchanges (SCE) per cell from Dalton lymphoma mice (16).

Our and other related studies raise more questions about understanding the underlying mechanisms of the chromosome protection effect of VD (9, 10, 15). If the cell is insufficiently protected by enzymatic and non-enzymatic antioxidants, free radicals can react with biomolecules and thus damage cellular structure. Antioxidants may prevent genetic changes by preventing DNA damage directly influenced by free radical attack. An immunoenhancing mechanism of VD could lower immunosuppressive lipid peroxides, stabilize the lysosomal membrane and protect the nuclear structure (9). The ability of VD to inhibit iron-dependent lipid peroxidation has been discussed by Wiseman (17). Wilson (5) has studied the critical role of VD in free radical induced biological damage. Sardar et al. (7) have documented VD as an effective antioxidant. VD was found to be effective in elevating the glutathione (GSH) level to almost 50% in VD-treated rats. As thiol homeostasis is regulated to guarantee basic function and defense mechanisms against xenobiotics, this increase in GSH content indicates a protective role of the vitamin in fighting free radical damage (18). VD lowers glutathione reductase, the enzyme that transfers electrons to oxidized glutathione, thereby maintaining the system in low oxidative stress. Induction of glutathione peroxidase (GPx) activity also strengthens the possibility. GSH can prevent DNA damage caused by certain free radicals. GPx protects cell proteins and membranes against oxidation by inhibiting the initiation of peroxidative attacks on membrane lipids. This has been suggested as the first line of defense against oxidative damage (9).

Antitumor effects of VD analogs have recently become one of the major topics of the vitamin D research field (19). VD was proved by Karmakar et al. (20) to be an effective antitumor drug during the initiation/promotion phases of hepatic carcinogenesis.

Vitamin D upregulated protein 1 (VDUP1) is a VD upregulated protein, and it is induced by various stresses. In human tumor tissues, VDUP1 expression was downregulated. Upon stimulation by VD, its expression was rapidly upregulated as the cell growth was retarded. The transfection of VDUP1 in tumor cells reduced cell growth. The VDUP1 expression was also increased when the cell-cycle progression was arrested. Transfection of VDUP1 induced cell-cycle arrest at the G0/G1 phase, indicating that VDUP1 possesses tumor-suppressive activity. VDUP1 itself suppressed IL-3 receptor and cyclin A2 promoter activity. Taken together, these results suggest that VDUP1 is a novel antitumor gene that forms a transcriptional repressor complex (21).

The exact way in which VD protects the cell from the harmful effects of DXR and other agents is not yet known. New studies on this topic should therefore be designed and performed. In conclusion, VD is an effective protector against DXR-induced chromosomal damages, and further investigations should provide useful data about the treatment dose and schedule.

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