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Retinoids as modulators of metabolism: their inhibitory effect on cyclophosphamide and 7,12-dimethylbenz[*a*]anthracene induced sister chromatid exchanges in a metabolically competent cell line

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Recently, retinoids have been studied for their ability to modify the carcinogenic/mutagenic activity of chemical compounds. Results show that they can inhibit the malignant transformation of cells, the induction of cancer in experimental animals and the mutagenicity of promutagens. Our experiments examine how retinol and retinoic acid can decrease the frequency of sister chromatid exchange (SCE) induced by two indirect mutagens/carcinogens (cyclophosphamide and 7,12-dimethylbenz[*a*]anthracene) in an epithelial liver cell line of male Chinese hamster (CHEL cells). These cells are metabolically competent and activate different classes of promutagens into biologically active metabolites. Our results are consistent with the suggestion that retinoids modulate the genotoxicity of indirect-acting mutagens by altering their metabolic activation or cellular detoxification processes or both.

Introduction

In recent years, particular attention devoted to the study of how chemical carcinogenesis and mutagenesis can be modified has led to the identification of a large number of modifying compounds that are contained in foodstuffs or elsewhere in nature.

Among these naturally occurring inhibitors, several vitamins have been shown to modulate the mutagenic/carcinogenic activity of procarcinogens.

Vitamin A along with its natural and synthetic analogues (referred to as retinoids) are known to prevent or delay the induction of cancer in experimental animals (Moon and Itri, 1984), they inhibit the malignant growth of epithelial human cells (Goodman, 1979; Elias and Williams, 1981), act as anti-promoters (Merriman and Bertram, 1979) and finally, suppress the malignant transformation of radiation-treated cells (Harisiadis *et al.*, 1978).

In vitro studies have shown that retinol inhibits the mutagenicity of compounds requiring metabolic activation in Salmonella tests (Balbinder *et al.*, 1983; Qin and Huang, 1985; Bhattacharya *et al.*, 1987; Victorin *et al.*, 1987), and SCE and chromosomal aberrations induced in V79 cells in the presence of S9 mix (Huang *et al.*, 1982; Qin *et al.*, 1985; Huang, 1987). Both retinol and retinoic acid inhibit 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced UDS in rat hepatocytes (Budroe *et al.*, 1987) and DMBA genotoxicity in CHO cells (Budroe *et al.*, 1988).

In addition, Qin and Huang (1986) have demonstrated that aflatoxin-B_1 (AFB_1) induces more SCE in mice with low liver vitamin A than in those with a high level.

However, retinol does not decrease the mutagenicity of direct acting compounds in Salmonella assays (Baird and Birnbaum, 1979; Busk and Ahlborg, 1980), in V79 (Qin *et al.*, 1985) or in CHO cells (Sirianni *et al.*, 1981), nor does it decrease EMS- and UV-induced UDS (Budroe *et al.*, 1987).

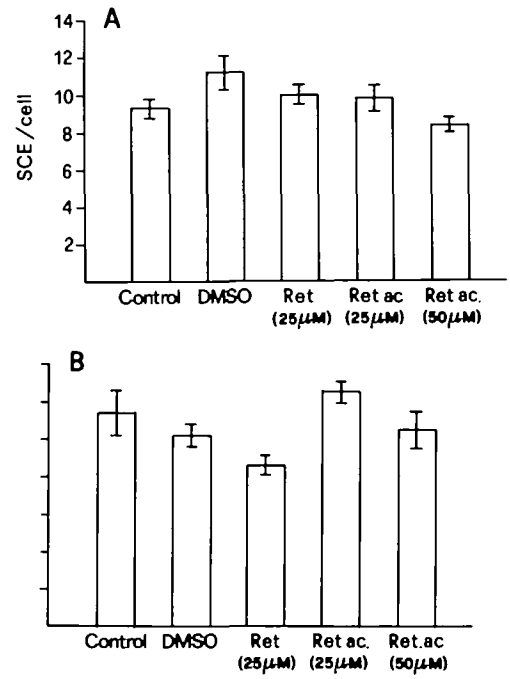

Fig. 1. Effect of retinol and retinoic acid on SCE baseline. (A) Harvesting time: 26 h after chemical treatment. (B) Harvesting time: 32 h after chemical treatment. Bars represent standard error.

Table I. Proliferation and mitotic index of cell cultures treated with retinoids and CP or DMBA

Treatment	26 h[a]				32 h[a]			
	M_1	M_2	M_3	MI (%)	M_1	M_2	M_3	MI (%)
Control	2	62	36	12.7	–	24	76	11.5
DMSO	10	73	17	8.3	3	49	48	8.4
Retinol (25 μM)	16	70	14	6.5	–	23	77	7.5
Retinoic acid (25 μM)	5	77	18	11.3	8	40	52	9.6
Retinoic acid (50 μM)	25	75	–	2.4		ND	ND	4.9
CP + DMSO	5	77	18	10.2	–	37	63	9.7
CP + Retinol	28	52	20	3.0	6	86	8	5.8
CP + Retinoic acid (25 μM)	4	80	16	11.0				
CP + Retinoic acid (50 μM)	11	84	5	8.6	5	65	30	7.5
DMBA + DMSO	10	81	9	9.0		ND		6.8
DMBA + Retinol	26	74	–	5.7		ND		7.5
DMBA + Retinoic acid (25 μM)	2	82	16	10.7				
DMBA + Retinoic acid (37 μM)	3	97	–	5.0				
DMBA + Retinoic acid (50 μM)	19	81	–	3.0		ND		4.0

[a]Harvesting time: 26 and 32 h after chemical treatment.
CP: final dose 10^{-3} M.
DMBA: final dose 0.078 μM.
ND: not detected.
The data are the mean of two to four separate experiments.

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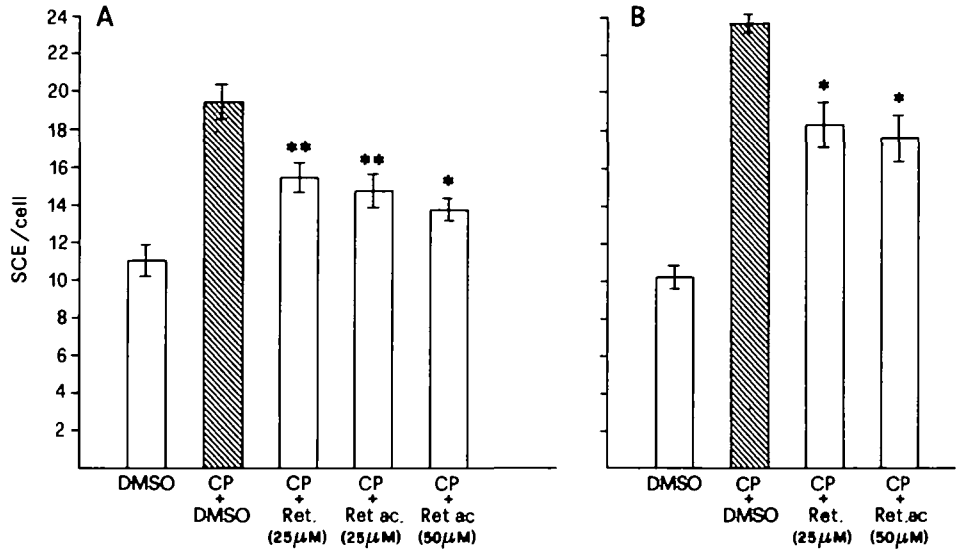

Fig. 2. Effect of retinol and retinoic acid on CP-induced SCE. (A) Harvesting time: 26 h after chemical treatment. (B) Harvesting time: 32 h after chemical treatment. Bars represent standard error. $*P < 0.001$; $**P < 0.01$, when compared to CP+DMSO values.

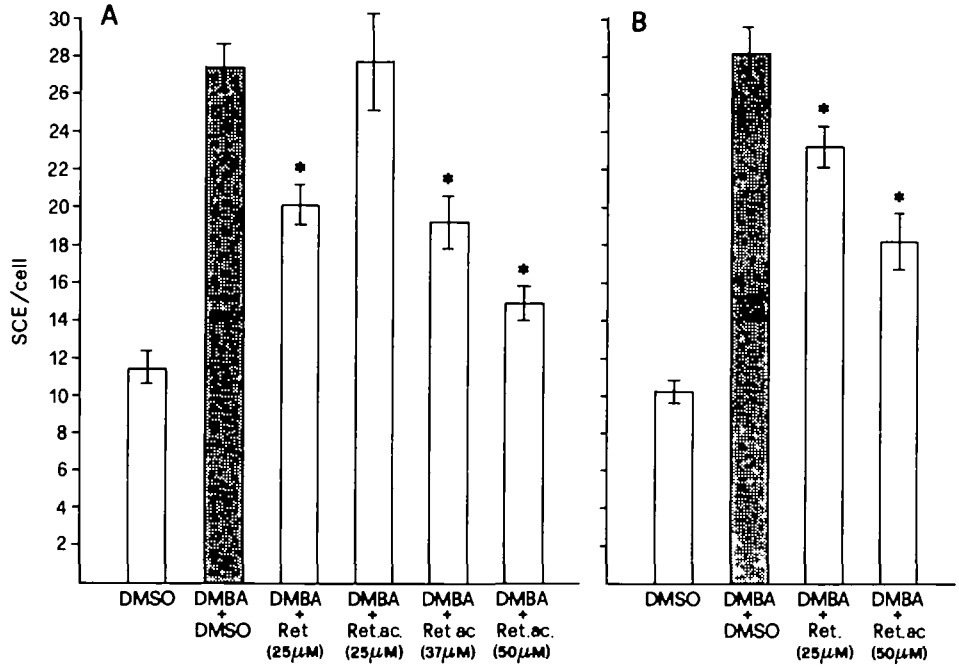

Fig. 3. Effect of retinol and retinoic acid on DMBA induced SCE. (A)Harvesting time: 26 h after chemical treatment. (B) Harvesting time: 32 h after chemical treatment. Bars represent standard error. $*P < 0.001$, when compared to DMBA+DMSO values.

It has recently been shown (Darroudi *et al.*, 1988) that in rats dietary carrot suppresses the mutagenic activity of cyclophosphamide (CP), probably because of the effect of β-carotene on enzymatic activation.

Overall, these results suggest that retinoids may exert their anticarcinogenic effects by inhibiting enzymatic processes required for the activation of certain procarcinogens. Thus, the use of a cell line that accurately mimics these metabolic processes occurring *in vivo* could prove particularly suitable for studying the effect of retinoids on chemically induced DNA damage.

This paper presents data on the ability of retinol and retinoic acid to reduce CP- and DMBA-induced SCE in an epithelial liver cell line of Chinese hamster (CHEL), which was capable of metabolizing some indirect mutagens/carcinogens.

Materials and methods

Chemicals

The chemicals were freshly prepared immediately before use. Retinol and retinoic acid (Sigma) were dissolved in dimethylsulphoxide (DMSO) (concentrated solution: 10 mM). The solutions were dispensed at the final concentrations of 25 and 25–37–50 μM respectively.

CP (Asta Werke) was dissolved in bidistilled sterile water and added to the cultures in complete medium at a final concentration of 10^{-3} M.

DMBA (Sigma) was dissolved in DMSO (concentrated solution: 1 mg/ml) and

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Table II. Effect of retinoids on SCEs induced by CP or DMBA: a 12 h treatment[a]

Treatment	SCE/cell	± SE	Reduction (%)	MI (%)	$M_1-M_2-M_3$
Control	12.7	± 1.1		12.5	4−60−36
DMSO	12.5	± 1.2		11.0	20−62−18
Retinol (25 μM)	15.0	± 1.4		11.4	27−72−1
Retinoic acid (50 μM)	14.4	± 1.1		12.0	18−79−3
CP + DMSO	20.4	± 2.6		13.0	ND
CP + Retinol	16.3	± 1.6	20	11.5	ND
CP + Retinoic acid (37 μM)	17.0	± 1.0	17	10.7	ND
CP + Retinoic acid (50 μM)	16.3	± 1.1	20	10.0	ND
DMBA + DMSO	40.5	± 2.9		11.5	2−90−8
DMBA + Retinol	30.3	± 3.3	25	11.2	9−90−1
DMBA + Retinoic acid (37 μM)	25.6	± 1.7	37	9.8	10−85−5
DMBA + Retinoic acid (50 μM)	25.9	± 2.3	37	10.6	8−91−1

[a]The cell cultures were treated with retinoids plus CP or DMBA for 12 h and then washed and replaced with fresh medium plus BrdUrd.
CP: final dose 10^{-3} M.
DMBA: final dose 0.078 μM.
ND: not detected.
The data are the mean of two to four separate experiments.

added to the cultures at a final concentration of 0.078 μM. The final concentration of DMSO in the culture media never exceeded 1%.

Bromodeoxyuridine (BrdUrd) was added to the cultures from stock solutions stored at −20°C, at the final concentration of 1.5 μg/ml.

All experiments were conducted under dim lighting.

Cell cultures and experiment schedule

CHEL cells were isolated and characterized as previously described (Turchi *et al.*, 1987; De Salvia *et al.*, 1988).

Cell cultures were prepared by seeding 1×10^6 CHEL cells in 5 ml of Williams' E medium supplemented with 10% fetal bovine serum in 25 cm^2 flasks. The flasks were incubated at 37°C in a 5% CO_2 atmosphere to allow cell attachment. After incubation for 4 h, the medium was removed and replaced with fresh medium containing both BrdUrd and the appropriate concentrations of retinoids. CP or DMBA was added to the cultures 1 h after beginning retinoid treatment.

In the first series of experiments (continuous treatment), retinoids and promutagens were present in the medium during the entire period of cell culturing (26 h).

In the second series of experiments, the retinoids and promutagens were removed after 12 h of treatment. The cells were then washed three times with serum-less medium and then incubated with a complete medium supplemented with BrdUrd for a further 14 h.

Colcemid was added to the cultures 3 h before harvesting at a final concentration of 10^{-7} M.

All experiments were carried out in duplicate.

SCE analysis

The cell cultures were washed in Hanks' balanced salt solution, treated with trypsin, subjected to hypotonic treatment for 13 min and then fixed in methanol/acetic acid. The technique of Perry and Wolff (1974), with slight modifications, was used for the differential staining of sister chromatids. Forty metaphases from coded slides were scored for each point and each experiment. Means and standard errors were determined. Statistical analysis (Student's *t*-test) was performed to compare the differences between the cultures treated only with the mutagens and those treated with both mutagens and retinoids.

For each experimental point 1000 cells were scored for mitotic index (MI) and 100 metaphases were analysed for M_1, M_2, M_3 determination.

Results

The data shown in Figure 1 indicate that continuous treatment of CHEL cells with retinol (25 μM) or retinoic acid (25−50 μM) did not increase the SCE rate as compared to the controls or the DMSO-treated cultures. The MI and the frequencies of the first(M_1)−second(M_2)−third(M_3) mitoses were inhibited by retinol and by the highest dose of retinoic acid (Table I). Higher concentrations of retinol were strongly cytotoxic.

Figure 2 shows the effect of retinoids on SCE induced by CP. Treatment with CP at a dose of 10^{-3} M increased the SCE rate 2-fold over the controls. When the cells were exposed simultaneously to CP and retinol or retinoic acid, the decrease in SCE frequencies ranged from 20 to 30%.

Figure 3 shows data on the DMBA treatment. DMBA at a concentration of 0.078 μM induced a substantial increase of SCE. Adding retinol significantly reduced the SCE frequencies to 26%, while the lowest dose of retinoic acid did not affect the SCE rate. Increasing doses of retinoic acid (37−50 μM) reduced SCE frequencies by 30 and 45%.

As the retinoids delayed the cell cycle of CP- and DMBA-treated cells (Table I), we additionally investigated whether highly damaged cells could be detected at a successive sampling time. Cells harvested at 32 h after chemical treatment showed the same SCE induction as in the CP- and DMBA-treated group, plus a comparable pattern of SCE reduction between the mutagen-treated culture and the mutagen with retinoid-treated cells (see Figures 1−3). Furthermore the mitotic and proliferation indices were partially recovered (Table I).

Table II shows the results of the cell cultures that were first treated with mutagens and retinoids for 12 h when, after washing, fresh medium supplemented with BrdUrd was added. The decrease in the SCE rate ranged from 20 to 40%, whereas the MI was restored.

Discussion

Our data show that at the tested doses neither retinol nor retinoic acid caused increases in the SCE rate in CHEL cells. These results agree with those of Huang *et al.* (1982), who reported that retinol is not mutagenic in continuously treated V79 cells in the presence of S9 mix.

In contrast to these findings, other studies (Jhul *et al.*, 1978; Tetzener *et al.*, 1980) have indicated that vitamin A and some related retinoids induce a low level of SCE in human diploid fibroblasts, thereby suggesting that human cells but not S9 mix can activate retinoids to a mutagenic form. Budroe *et al.* (1987) have shown that retinol and retinoic acid are unable to induce UDS in primary rat hepatocytes, which possess metabolic activation enzymes, but they have also noted that the lack of

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UDS induction does not completely preclude genotoxicity. Our cytological assay using a metabolically competent mammalian cell line could provide further support as to the inability of retinoids to affect the genetic material.

The treatment of our cell line with the indirect mutagens CP and DMBA resulted in an increase of SCE frequencies which were more evident in DMBA treatment.

These data confirm previous results (De Salvia *et al.*, 1988) with CHEL cells that showed the ability of this cell line to activate promutagens/procarcinogens measured as the induction of SCE by a series of chemical xenobiotics.

When simultaneously treated with mutagens and retinol, the rate of induced SCE drastically decreased in cell cultures treated with DMBA, whereas the effect was less efficient in those treated with CP. The decrease was found to be all the more evident due to the clear relationship between the doses of retinoic acid used and the percentage of decrease in the SCE rate. It was also demonstrated that cells harvested at a later time interval showed both a similar SCE rate and a corresponding decrease.

In a second series of experiments, cell cultures were treated with mutagens plus retinoids, then washed, and replaced in fresh medium supplemented with BrdUrd. The data show that the decrease in the SCE rate was maintained even when the cells received a shorter treatment. The MI was restored after the retinoids were removed from the culture medium. The delay in the cell cycle caused by the retinoids did not influence the decrease in the SCE rate.

These data agree with Huang *et al.* (1982) who found a similar effect in V79 cells with the metabolic activation of S9, which had been treated with CP and AFB_1.

Conflicting data also exist on the ability of retinoids to reduce DMBA-induced genotoxicity. Qin and Huang (1985) did not detect any inhibition of DMBA-indued mutagenicity by retinol in the Salmonella test with metabolic activation. Furthermore, the same authors (Qin *et al.*, 1985) have reported the absence of an inhibitory effect by retinol on DMBA-induced SCE and chromosome aberrations in V79 cells with S9.

Budroe *et al.* (1987) have found that retinol and retinoic acid inhibit UDS induced by DMBA, but not by EMS or UV in primary rat hepatocytes; they do reduce DMBA-induced genotoxicity in CHO cells with the addition of S9 fraction (Budroe *et al.*, 1988).

In addition, Rocchi *et al.* (1983) have found that retinol palmitate reduces the DMBA-induced mutation frequencies in a metabolically competent epithelial like cell line (EUE). This contrast regarding the inability of retinol to inhibit DMBA mutagenicity might be due to the use of different metabolic activation systems. In fact, all positive results have been obtained with metabolically competent cell lines (like CHEL cells), which can activate procarcinogens without the addition of exogenous metabolic sources (S9).

Our findings are consistent with the suggestion that retinoids modulate the genotoxicity of indirect acting mutagens by decreasing their metabolism to reactive mutagenic species or by increasing the detoxification.

Hill and Shih (1974) have shown that retinol can inhibit polycyclic aromatic hydrocarbon metabolism (like DMBA) by murine liver microsomes. Qin and Huang (1985), however, have found that retinoids may influence the metabolism of some but not all promutagens. Belisario *et al.* (1988) have shown that retinol is more effective than retinoic acid in reducing the enzymatic activity of microsomes from phenobarbital (PB)- or 3-methylcholanthrene (MC)-pretreated animals.

Our data suggest that the enzyme responsible for the metabolism of both CP (BP P450) and DMBA (MC P448) is present in this epithelial liver cell line, which is clearly shown by the significant increase in SCE by these promutagens. Preliminary data in our laboratory indicate that retinol or retinoic acid added to CHEL cells cultivated with DMBA or CP reduces the presence of metabolites in the medium; this is shown by the absence of SCE induction in CHO cells allowed to grow in this medium (unpublished data).

Both retinol and retinoic acid seem equally effective in inhibiting P450 and P448 dependent mixed function oxidase systems.

In conclusion, we feel that it is of overall importance to use both a metabolically competent cell line as well as an S9 mix to assay the antimutagen/anticarcinogen activity of natural compounds that might inhibit the metabolic activation of promutagens.

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