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Anticlastogenic Effects of 13-*cis*-Retinoic Acid *in vitro*

Zoltán Trizna, T.C. Hsu, Stimson P. Schantz, J. Jack Lee and Waun K. Hong

The anticlastogenic effects of 13-*cis*-retinoic acid were studied in four human lymphoblastoid cell lines and in primary lymphocyte cultures derived from the peripheral blood of 11 study subjects. Cells were pre-incubated with 13-*cis*-retinoic acid in the concentration range of 10^{-8}–10^{-5} mol/l for 24 h and the numbers of chromatid breaks per cell induced by bleomycin were determined. The presence of 13-*cis*-retinoic acid decreased the number of breaks per cell by 13.0 to 59.5% in lymphoblastoid cell lines and by 0 to 57.4% in primary lymphocyte cultures (in the concentration ranges of 10^{-8}–10^{-6} mol/l and of 10^{-8}–10^{-5} mol/l, respectively). Regression analysis showed that there was a statistically significant correlation between the presence of 13-*cis*-retinoic acid and protection against bleomycin-induced clastogenicity. These data give additional information to the knowledge of possible chemopreventive mechanisms of action of 13-*cis*-retinoic acid.
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INTRODUCTION

VITAMIN A IS important for the normal differentiation of epithelial cells; its presence is required for the physiological secretory function of the epithelial lining in the upper aerodigestive tract. There are indications that retinoids have a preventive effect on carcinogenesis in a variety of epithelial tissues, including the oral mucosa and airways in both animals and humans. Recent investigations have suggested that retinoids act primarily by regulating gene expression [1, 2], and through this mechanism retinoids may modulate the growth of premalignant cells or suppress the progression of premalignant cells to neoplastic lesions.

In clinical trials, 13-*cis*-retinoic acid (isotretinoin), a synthetic retinoid, has been shown to effectively suppress premalignant lesions of the oral cavity [3]. Furthermore, high doses were effective in preventing second primary tumours in patients who had been previously treated for an initial squamous cell carcinoma of the upper aerodigestive tract [4].

Chromosomal fragility is an indicator of genetic instability, and is associated with an increased risk of cancer in syndromes such as Fanconi's anemia, xeroderma pigmentosum, ataxia-telangiectasia, and Bloom's syndrome [5]. In the general popu-

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lation, spontaneous chromosome breakage is usually very low. However, chromosome fragility increases following exposures to mutagens. Bleomycin-induced chromatid breakage was used to measure an individual's sensitivity to environmental carcinogens and the greatest sensitivity was found in patients with cancers of tissues directly exposed to the external environment [6].

The purpose of this study was to quantitate the effects of 13-*cis*-retinoic acid on mutagen-induced chromosomal breakage in established human lymphoblastoid cell lines and in lymphocytes of primary cultures derived from peripheral blood samples of head and neck cancer patients.

MATERIALS AND METHODS

Subjects

Cultures of four human lymphoblastoid cell lines, henceforth to be referred to as lymphoid lines [7], and cultures of peripheral blood samples from 11 patients were used in our experiments. Cell line 3498P was derived from a male patient with a previously untreated squamous cell carcinoma of the retromolar trigone. This patient subsequently developed a second primary lung cancer. Cell line 3512P was established from a male patient with squamous cell carcinoma of the pyriform sinus and papillary carcinoma of the thyroid. Cell line 3640P was derived from a female patient with a previous history of multiple primary malignancies, including cancers of the cervix and oral tongue. Cell line 4087P was established from a patient with systemic lupus erythematosus.

Standard primary lymphocyte cultures were derived from peripheral blood samples of 11 study subjects: 9 patients with squamous cell carcinoma of the head and neck (including three oral, three oropharyngeal, one hypopharyngeal and two laryngeal tumours), 1 patient with cervical metastasis of an unknown squamous cell primary, and 1 patient with bronchial metaplasia.

Mutagen-sensitivity assay

The method of inducing chromosomal breakage with bleomycin and the rationale for choosing the doses used have been described in detail [6]. Briefly, all cell cultures were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum (Sigma). Following a 24-h preincubation with 13-*cis*-retinoic acid, bleomycin (Blenoxane, Nippon Kayaku Co.) was introduced to lymphoid line cultures for 2 h to achieve a final concentration of 0.004 units/ml. Bleomycin was added to peripheral blood cultures for 5 h to achieve a final concentration of 0.03 units/ml. 13-*cis*-retinoic acid was obtained through Sigma and was dissolved in ethyl alcohol. Stock solutions were stored at −20°C. Final concentrations of 13-*cis*-retinoic acid of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/l were achieved by adding 0.1 ml from the respective stock solutions to the cultures.

Cell harvest, cytogenetic preparation, and staining followed standard procedures reported by our laboratory. Chromatid breaks were recorded on coded slides by examining 50 metaphases per sample, based on suggested criteria for determining chromosome breakage [6, 7].

The dependency between the 13-*cis*-retinoic acid level and chromatid breakage was studied with regression analysis. The number of breaks per cell was used as the outcome variable. The different concentrations were used as the explanatory variable and were entered on a logarithmic scale where the value for the untreated samples was arbitrarily chosen as 10^{-9}. In cases of the cell lines the average values of duplicate experiments were averaged and used in the regression analysis for each cell line separately. In experiments on peripheral blood lymphocytes, the regression analysis was also done separately for each patient. The model was examined graphically to ensure the adequate fit for the data. The correlation coefficient and slope for each regression analysis were calculated. Both reveal how 13-*cis*-retinoic acid relates to chromatid breakage when the model fits the data. Statistical significance testing was performed on each slope for testing the slope being zero, i.e. there was no dependence between 13-*cis*-retinoic acid and chromatid breakage. One-sample *t*-test was used for testing whether the mean slope in all experiments was zero.

*Table 1. Average number of breaks per cell in four lymphoid cell lines of human origin cotreated with bleomycin (0.004 U/ml) and 13-cis-retinoic acid (13-CRA) in various concentrations**

Concentration of 13-CRA (mol/l)	Cell line 3498P	3512P	3640P	4087P
0.0††	0.60	0.37	0.50	0.46
10^{-8}	0.52	0.28	0.42	0.40
10^{-7}	0.43	0.17	0.33	0.36
10^{-6}	0.32	0.15	0.32	0.22

*Regression analysis showed the slope of all four cell lines being statistically significantly different from zero ($P < 0.05$).

†Baseline value (treatment with bleomycin only).

RESULTS

13-*cis*-Retinoic acid did not affect cellular growth at a concentration of 10^{-6} mol/l and less in the lymphoid lines tested. The highest non-toxic concentration was 10^{-5} mol/l in fresh peripheral blood cultures. These maximal non-toxic concentrations were used in subsequent experiments 24 h prior to bleomycin treatment.

The average number of spontaneous chromatid breaks in control cultures did not exceed 0.02 both in cell lines and in primary lymphocyte cultures in accordance with previous findings [6, 7].

13-*cis*-Retinoic acid was tested in duplicate experiments; concentrations of 10^{-8}, 10^{-7} and 10^{-6} mol/l were used in four lymphoid lines. The averages of the numbers of breaks per cell are shown in Table 1. The highest concentration (10^{-6} mol/l) gave the best protection against bleomycin-induced genotoxicity, and the protective effect was less marked with lower concentrations. The range of inhibition was between 13.0 and 59.5%. Regression analysis showed that protection against mutagen-induced chromosomal damage was statistically significantly different from zero in all four cell lines tested ($P < 0.05$), with the correlation coefficient from −0.961 to −0.997. The mean slope was −0.08 with a standard error of 0.01.

For dose–response experiments on peripheral blood cultures of the study subjects, 13-*cis*-retinoic acid was tested in concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol. For some concentration levels the slides could not be evaluated due to inadequate spread or number of metaphases, but there were at least nine samples for each concentration. Table 2 shows the number of breaks per cell.

13-*cis*-Retinoic acid yielded similar, but somewhat less pronounced effects in primary lymphocyte cultures with regard to

Table 2. Numbers of breaks per cell in lymphocytes from cultures of peripheral blood cotreated with bleomycin (0.03 U/ml) and 13-cis-retinoic acid (13-CRA) in various concentrations in 11 study subjects

Concentration of 13-CRA (mol/l)	Patient number										
	1*	2	3	4	5	6	7	8*	9*	10	11*
0.0^{2}†	0.84	0.90	0.40	0.62	0.34	0.36	0.64	0.22	1.08	0.66	0.38
10^{-8}	0.78	0.88	0.46	n/a	n/a	0.40	0.45	0.22	0.82	0.68	0.36
10^{-7}	0.82	0.92	0.36	0.62	0.30	0.30	0.58	0.16	0.76	0.66	n/a
10^{-6}	0.60	0.64	0.32	0.54	0.36	0.22	n/a	0.12	0.56	0.58	0.26
10^{-5}	0.54	n/a	0.30	0.48	0.26	n/a	0.48	0.14	0.46	0.36	0.22

*$P < 0.05$ for testing to 0 slope.
†Baseline value (treatment with bleomycin only).
n/a = Data not available.

protection from mutagen-induced chromosomal damage, as noted in the cell line experiments (Table 2). The range of inhibition was between 0 and 57.4% and seemed to be dose-related. Regression analysis showed a clear negative correlation in all samples, with the correlation coefficients in the range between −0.52 and −0.99. Even with only five data points for each patient, four of the 11 samples showed that the regression coefficients were statistically significantly different from zero ($P < 0.05$). The mean slope was −0.06, with a standard error of 0.01. The one-sample *t*-test also indicated that the mean slope was statistically significantly different from zero ($P < 0.001$). For both experimental groups presented in Tables 1 and 2, the simple linear regression seemed to fit the data reasonably well from the graphical assessment.

DISCUSSION

Epidemiological data suggest that environmental factors may influence the occurrence of certain types of cancer; for example, alcohol consumption and smoking are two major risk factors for squamous cell carcinoma of the upper aerodigestive tract, but dietary factors can have beneficial effects. In a recent study [8], fruit consumption was thought to provide the strongest and most protective effect against the development of laryngeal cancer. Ascorbic acid and *n*-acetyl-L-cysteine were shown to provide protection against mutagen-induced chromosomal damage *in vitro* [9]. Epidemiological studies have also suggested an association between low dietary intake of vitamin A and the development of lung [10], oropharyngeal and laryngeal [11] and oesophageal [12] cancers. Low vitamin A levels may play a role in the aetiology of second tumours in head and neck cancer patients [13].

Retinoids are known to alter differentiation, immune function, tumour initiation and promotion, and oncogene expression [3, 14–17]. Derivatives of vitamin A, including 13-*cis*-retinoic acid have proved to be effective for chemoprevention in animal models [14]. Topical application of 13-*cis*-retinoic acid inhibited mouse skin tumour promotion, and this inhibition remained constant after cessation of retinoid treatment [15]. In cell lines derived from human head and neck squamous cell carcinomas, retinoic acid inhibited the growth and decreased the level of two differentiation markers *in vitro* [16]. High-dose (2 mg/kg/day) oral isotretinoin (13-*cis*-retinoic acid) was effective in preventing the formation of new skin cancers in patients with xeroderma pigmentosum. This effect was reversed after discontinuation of the drug [17].

Retinoids may modulate the growth of premalignant cells or suppress the progression of premalignant cells to neoplastic lesions. In a randomised, placebo-controlled study of isotretinoin (13-*cis*-retinoic acid), this synthetic retinoid effectively suppressed premalignant lesions of the oral cavity [3]. High doses of 13-*cis*-retinoic acid (50–100 mg/m^2/day) were effective in preventing second primary tumours in patients who had been previously treated for squamous cell carcinoma of the upper aerodigestive tract [4]. Administration of 60 mg/week vitamin A for 6 months to betel nut chewers resulted in complete remission of oral leukoplakias in 57% of this group; formation of new leukoplakias was totally suppressed. This protective effect was maintained for 8 additional months by administration of lower doses [18].

In the present study the effects of 13-*cis*-retinoic acid on mutagen-induced chromosomal breakage were quantitated *in vitro*. Bleomycin was used as it causes chromosomal damage irrespective of the cell cycle [6, 7]. Our results indicate that 13-*cis*-retinoic acid can provide protection against bleomycin-induced clastogenicity in cultures of lymphoblastoid cell lines as well as in peripheral lymphocyte cultures. The mean slope from the regression analysis was −0.08 for the lymphoid lines and −0.06 for the peripheral lymphocyte cultures which means that as the concentration of 13-*cis*-retinoic acid increased by 10-fold in our concentration range, the number of mutagen-induced chromatid breaks per cell could be reduced by 8 and 6%, respectively.

An important consideration in judging our experimental results is how they would relate to the clinical situation, i.e. can the concentrations tested in this study be used *in vivo*? The physiological concentration of vitamin A in human serum is in the range of $1.8–4.6 \times 10^{-6}$ mol/l [13], and this concentration range is similar to the range of the synthetic retinoid (13-*cis*-retinoic acid) used in our experiments.

The mutagen-protective effect of 13-*cis*-retinoic acid, demonstrated *in vitro* in both human lymphoid lines and in lymphocytes from peripheral blood cultures, may play an important role in the chemoprevention of head and neck cancers. The data presented give additional information to the understanding of the chemopreventive effects of 13-*cis*-retinoic acid.

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Serum Osteocalcin in the Management of Myeloma

Adrian T. Williams, Martin J. Shearer, Joyce Oyeyi, Robin G. Aitchison, Adrian C. Newland and Stephen A. Schey

We have measured serum osteocalcin, a vitamin K-dependent glycoprotein synthesised by osteoblasts in 62 patients, 49 with myeloma, 26 at presentation and 23 previously treated, 7 with Waldenstrom's macroglobulinaemia (WM), and 6 with monoclonal gammopathy of uncertain significance (MGUS). Osteocalcin levels were normal in WM and MGUS. High values were found in 5/26 (19%) patients with myeloma at presentation. There was no relationship between serum osteocalcin and stage of disease. Osteocalcin was normal in all patients in plateau phase, falling to low levels in relapsed patients who failed to respond to further treatment. Serum osteocalcin may be a useful indicator of bone metabolism in myeloma.
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INTRODUCTION

LYTIC LESIONS, fractures, osteoporosis and hypercalcaemia are major causes of morbidity in patients with myeloma [1]. Despite increased osteoclast activity in the vicinity of myeloma cells [2], alkaline phosphatase is normal in the absence of fractures due to the lack of osteoblast response. Osteocalcin (bone Gla-protein), is a vitamin K-dependent glycoprotein synthesised by osteoblasts whose function remains unknown; circulating levels, however, are a sensitive indicator of bone turnover in a number of bone disorders [3, 4]. In myeloma, an inverse correlation between serum osteocalcin and stage has been reported [5]. We have measured serum osteocalcin in paraproteinaemias to assess its relationship to disease activity.

PATIENTS AND METHODS

Patients

62 patients with paraproteinaemia were studied, 49 with myeloma (26 at presentation and 23 previously treated), 7 with Waldenstrom's macroglobulinaemia (WM) and 6 with monoclonal gammopathy of uncertain significance (MGUS). 12 patients were stage I, 14 stage II and 23 stage III. The paraprotein was IgG in 40, IgA in 5 and IgD in 1 patient, and there were 3 light chain myeloma cases. Patients were treated with various