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Curative effects of *all-trans*-retinoic acid on rat sarcomas

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The effects of *all-trans*-retinoic acid (*all-trans*-RA) on benzo(*a*)pyrene [B(*a*)P]-induced malignant sarcomas in Sprague-Dawley female rats were evaluated. Ninety-eight days after B(*a*)P administration, *all-trans*-RA was daily injected to animals with or without clinically palpable tumors. The growth of tumors was slowed down compared with controls. Magnetic resonance imaging analyses showed that *all-trans*-RA-treated rat tumors presented early necrotic areas. Animal survival was slightly increased. Anti-phosphatidylinositol autoantibody levels which were significantly higher in B(*a*)P-treated rat sera were not modified by *all-trans*-RA treatment.

Key words: All-trans-retinoic acid, autoantibodies, magnetic resonance imaging, sarcoma, tumor growth.

Introduction

Retinoic acid (RA), a derivative of vitamin A (retinol), is a key molecule implicated in a wide variety of biological phenomena including development, cell differentiation, and cell proliferation of normal and transformed cells.[1–4] RA has also attracted attention in the clinic because it has effects on proliferative dermatological diseases including skin cancer, leukemia and in the chemoprevention of cancer.[5–8] As a sole agent, it induces *in vivo* remissions in patients with acute promyelocytic leukemia.[9,10]

In vitro, RA has received considerable attention because it induces terminal differentiation of human acute myeloid leukemia cell line HL 60, i.e. a cell line having many of the functional and morphological characteristics of mature granulocytes.[11,12] The differentiation of cultured cells of human neuroblastoma, melanoma, leukemia and teratocarcinoma has also been induced by RA.[13–18]

In vivo, RA has been used in experimental tumor models as a chemopreventive agent for the reduction of cancer incidence and tumor growth.[6,19,20]

Most of the tumor models require multiple treatments with carcinogens.[21] Moreover, except for skin and breast cancers, the cancer incidence cannot be determined prior to sacrifice of the animals.[22] An experimental model of benzo(*a*)pyrene [B(*a*)P]-induced highly malignant sarcomas in Sprague–Dawley (SD) female rats that occurs with 100% efficiency 100–110 days after carcinogen administration has been described previously.[23] This tumor model has the following advantages: (i) a single dose of carcinogen is sufficient to induce a tumor at the injection site and (ii) malignant transformation can be monitored prior to sacrifice of animals by evaluating anti-phosphalidylinositol (PtdIns) autoantibody (autoAb) appearance.[23,24] Consequently, this B(*a*)P-induced sarcoma model seemed appropriate to evaluate potential curative RA effects. In addition, it was possible to observe the evolution of anti-PtdIns autoAb levels in response to *all-trans*-RA treatment. Here we present evidence of increased animal survival and of a time-lag in tumor growth following *all-trans*-RA treatment of our B(*a*)P-induced sarcoma experimental model.

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Materials and methods

Chemicals

B(*a*)P, *all-trans*-RA, PtdIns and thyroglobulin (TH) were purchased from Sigma (St Louis, MO, USA). Dimethylsulfoxide (DMSO) was obtained from Merck-Clevenot Laboratories (Nogent/Marne, France).

B(*a*)P injection and sample collection

Twenty six SD female rats (Janvier, Le Genest-Saint-Isle, France) weighing 180–200 g were housed under controlled conditions (22°C, monitored light–dark cycles with light from 8:00 a.m. to 8:00 p.m.), and were supplied with food (UAR, Versailles, France) and water *ad libitum*. B(*a*)P (2 mg) diluted in 500 μl of sesame oil was s.c. injected under anesthesia in 50–60 day old SD female rats as described by Faiderbe *et al.*[23] Blood from B(*a*)P-treated rats was sampled and assayed at regular intervals.

All-trans-RA treatment

Solutions of *all-trans*-RA were prepared daily as follows: *all-trans*-RA lyophilized powder was dissolved in ethylic alcohol/DMSO (v/v) at room temperature under vigorous stirring. At day 98 after B(*a*)P administration, SD female rats were divided into the following groups:

- SD rats with ($n = 6$) clinically palpable tumors (volume between 0.025 and 0.52 cm^3) or without tumors ($n = 4$) receiving 300 μg *all-trans*-RA.
- SD rats with ($n = 6$) clinically palpable tumors (volume between 0.025 and 0.52 cm^3) or without tumors ($n = 5$) receiving 3 mg *all-trans*-RA.
- SD rats with ($n = 5$) clinically palpable tumors (volume between 0.025 and 0.52 cm^3) or without tumors ($n = 4$) receiving only the carrier solution (ethylic alcohol/DMSO).

Animals were daily injected i.p. with 50 μl of solution containing either 300 μg or 3 mg *all-trans*-RA, or solvent alone. Treatment was continued until the death of each animal.

Immunoenzymatic test for the detection of anti-PtdIns autoAbs in rat sera

Detection of anti-PtdIns autoAbs was performed with an adapted ELISA test as described by Faiderbe *et al.*[23,24] on SD rat sera diluted at 1/1000 in order to follow malignant transformation.

Histological analysis

Some animals were randomly chosen in each group and their tumors were removed, cut in pieces and fixed in Bouin de Hollande.

Tumor growth measurement

B(*a*)P-induced tumors were mechanically measured with a calliper rule as described by Faiderbe *et al.*[23] Their volume was calculated using a standard formula: width^2 × length × 0.52, according to Ingber *et al.*[25], and expressed as cubic centimeters.

Magnetic resonance imaging (MRI) measurements

Some SD female rats randomly chosen in the different groups underwent MRI under anesthesia in the MRI apparatus of the Hôpital Pellegrin of Bordeaux. The imaging device was a Magnetom Siemens with a 1.5 T supraconductive coil. Two kinds of sequence were used, i.e. transverse and sagittal, in two different orientations. The T1-weighted sequence has a 500 ms repetition time and a 15 ms echo time; the T2-weighted sequence has a 2500 ms repetition time, and two echo times of 22 and 90 ms. The two largest dimensions of the tumors were measured.

Results

Effects of *all-trans*-RA on anti-PtdIns autoAb levels

Immunological binding was evaluated on well-plates coated with PtdIns and TH, and expressed as absorbance. B(*a*)P-treated rat anti-PtdIns autoAb levels greatly increased 40 days after B(*a*)P administration. AutoAb levels increased until 60 days and then plateaued.[23] At day 98, B(*a*)P-treated rats were divided into six groups as previously described in Materials and methods. No differences were observed in the profile curve between the different groups. *All-trans*-RA treatment had no effect on anti-PtdIns autoAb levels.

Effects of *all-trans*-RA on tumor growth evolution

From day 98, SD female rats from the three groups with clinically palpable tumors were examined daily. Tumors were mechanically measured. Table 1 shows the increase of tumor growth for the three groups, i.e. 3 mg *all-trans*-RA-treated rats, 300 μg *all-trans*-RA-treated rats and solvent-treated rats, represented as mean tumor volume with their standard error. Five days after detection of clinically palpable tumors, the solvent-treated rat tumor volumes rapidly increased. These animals did not survive until day 20 after the beginning of the experiment. Whatever the *all-trans*-RA dose, the tumor volume increased more slowly than in the control group. However, animals from the 300 μg *all-trans*-RA-treated group endured greater tumors (approximately 470 cm^3) until they died; these were larger than those of the control group (approximately 114 cm^3) and those of the 3 mg *all-trans*-RA-treated group (approximately 215 cm^3). It seemed that a daily administered dose of 3 mg *all-trans*-RA might have a better slowing down effect on tumor volume, i.e. on each day the tumor volume was smaller than that with 300 μg *all-trans*-RA. Survival curves were drawn with the non-parametric Kaplan–Meier test from these data. Figure 1 shows that the mean survival of the 3 mg *all-trans*-RA-treated group and the 300 μg *all-trans*-RA-treated group (curves b and c) was increased by about 25 days relative to the control (only solvent) group (curve a). Moreover, the statistical analysis performed with the log-rank test (Mantel–Cox) revealed that the mean survival of the *all-trans*-RA-treated rats was not significantly different from that of the control group ($p = 0.09$ and $p = 0.24$ for 300 μg and 3 mg *all-trans*-RA-treated rats, respectively).

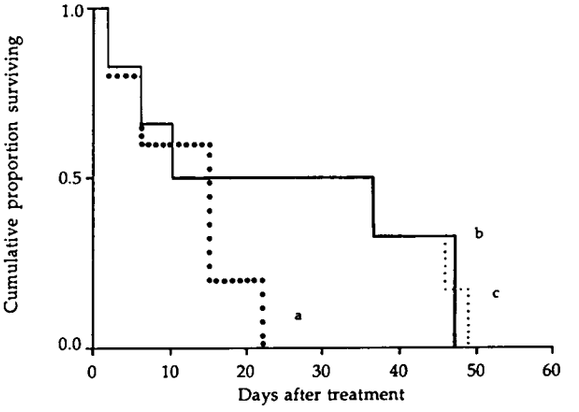

Figure 1. Effects of *all-trans*-RA on animal survival. Cumulative proportion surviving in the RA-treated rat groups and the solvent group with clinically palpable tumors at the beginning of treatment, expressed as percentage of surviving animals during the time course of experimentation. (a) Solvent group; (b) 3 mg *all-trans*-RA-treated rats; (c) 300 μg *all-trans*-RA-treated rats.

Table 1. Mean tumor volumes (±SE) of *all-trans*-RA- or solvent-treated rats with tumors at the beginning of the *all-trans*-RA treatment

Days after RA treatment	Tumor volume[a] of *all-trans*-RA- or solvent-treated rat groups (cm^3)		
	3 mg *all-trans*-RA (*n*)	300 μg *all-trans*-RA (*n*)	solvent (*n*)
1	0.075 ± 0.03 (6)[b]	0.3 ± 0.1 (6)	0.4 ± 0.15 (5)
7	0.2 ± 0.1 (5)	1.5 ± 2 (6)	7 ± 5 (3)
11	0.5 ± 0.15 (4)	4.5 ± 2 (5)	9 ± 7.5 (3)
17	2 ± 1 (3)	24 ± 10 (4)	12.5 ± 7 (3)
22	3 ± 1.5 (3)	58 ± 24 (4)	114 (1)
28	25.5 ± 19.5 (3)	71.5 ± 30 (3)	death
32	47 ± 2.5 (3)	67 ± 30 (3)	
37	52 ± 47 (3)	124 ± 58 (3)	
44	180.5 ± 157	294 ± 173.5 (2)	
47	214 ± 185 (2)	310 ± 157.5 (2)	
50	death	468 (1)	
52		death	

[a] Tumor volume was calculated with the formula: $width^2 \times length \times 0.52$.
[b] No. of animals indicated between parentheses.

Effect of *all-trans*-RA treatment on tumor onset

For SD female rats of groups without clinically palpable tumors at day 98, there was no delay in tumor onset between the *all-trans*-RA-treated groups and the solvent-treated group (Table 2). Survival curves showed that 3 mg and 300 μg *all-trans*-RA-treated rats (Figure 2, curves b and c) died at a date close to that of the solvent-treated group (Figure 2, curve a). Statistical tests showed no significant mean survival differences between the *all-trans*-RA-treated groups and the solvent group, whatever the *all-trans*-RA dose ($p > 0.40$). This could be due to premature death provoked by possible toxicity of *all-trans*-RA, as previously observed by other authors.[22]

Table 2. Mean tumor volumes (±SE) of *all-trans*-RA- or solvent-treated rats without tumors at the beginning of the *all-trans*-RA treatment

Days after beginning of RA treatment	Tumor volume[a] of *all-trans*-RA- or solvent-treated rat groups (cm^3)		
	3 mg *all-trans*-RA (*n*)	300 μg *all-trans*-RA (*n*)	solvent (*n*)
1	0 (5)[b]	0 (4)	0 (4)
7	0.006±0.005 (5)	0.001±0.001 (4)	0.02±0.02 (4)
11	0.0075±0.006 (4)	0.001±0.001 (4)	ND
17	0.0075±0.006 (4)	0.035±0.03 (4)	0.035±0.03 (2)
22	0.01±0.01 (3)	0.6±0.5 (4)	ND
28	0.2±0.2 (2)	1.5±1 (4)	0.5±0 (2)
32	0.2±0.2 (2)	2.7±1.5 (4)	ND
37	ND	6.95±4.5 (4)	1.45±0.2 (2)
44	1.1 (1)	20±11.5 (4)	5±1 (2)
47	4 (1)	27 ± 16 (4)	44 ± 13 (2)
50	6.5 (1)	87 ± 73 (3)	105 (1)
52	9.75 (1)	death	death
54	death		

[a] Tumor volume was calculated with the formula: width^2 × length × 0.52.
[b] No. of animals indicated between parentheses.

Histological analysis

Histological analysis of the tumors showed the presence of (i) highly malignant sarcomas with fusiform cells for solvent-treated rats, as previously described by Faiderbe *et al.*,[23] and (ii) sarcomas with cells presenting no great changes in their morphology, but with wide necrotic and hyperhemorragic areas for *all-trans*-RA-treated rats, whatever the *all-trans*-RA dose used.

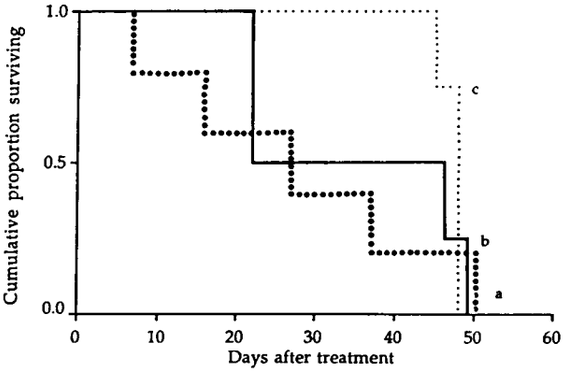

Figure 2. Effects of *all-trans*-RA on animal survival. Cumulative proportion surviving in the *all-trans*-RA-treated rat groups and the solvent group without clinically palpable tumors at the beginning of treatment, expressed as percentage of surviving animals during the time course of experimentation. (a) Solvent group; (b) 3 mg *all-trans*-RA-treated rats; (c) 300 μg *all-trans*-RA-treated rats.

MRI analysis

MRI analysis was performed for two reasons: (i) to evaluate the tumors and to compare them with our mechanical measurements, and (ii) to observe any heterogeneity between *all-trans*-RA-treated rat tumors and solvent-treated rat tumors. Firstly, Table 3 shows that no significant differences were

Table 3. Comparison of MRI and mechanical measurements of tumor volume

Days after RA treatment	MRI measurement (cm^3)	Mechanical measurement (cm^3)
Tumor volume of 3 mg *all-trans*-RA-treated rats		
1	0.2	0.2
7	0.35	0.5
11	0.5	0.6
32	0.6	0.5
Tumor volume of 300 μg *all-trans*-RA-treated rats		
1	0.065	0.065
7	0.065	0.065
17	2.25	3
32	9.5	9.5
Tumor volume of solvent-treated rats		
1	0.045	0.045
7	0.05	0.045
11	0.095	0.1
17	1	1
32	18	18.5

[a] Tumor volume was calculated as described in the text.

Figure 3. T2-weighted image of a 300 μg *all-trans*-RA-treated rat tumor at day 17 after the beginning of RA treatment. A bright area appears indicating a liquid zone in the tumor volume (arrow).

observed between the two kinds of measurements for the *all-trans*-RA- or solvent-treated groups. Our mechanical measurements were thus reliable. Secondly, in the T1-weighted images of *all-trans*-RA-treated animals, dark round images were seen although tumors were small. As tumors grew, the images became more heterogeneous compared with the control group. In the T2-weighted images of *all-trans*-RA-treated animals, bright areas appeared, indicating a liquid zone (Figure 3). Follow-up and histological analyses indicate that these bright signals are due to an increase in necrosis. It seemed that necrotic areas appear earlier in *all-trans*-RA-treated rat tumors.

Discussion

We have used our tumor model induced by a single carcinogen dose for the evaluation of the curative effects of *all-trans*-RA.[23] An increase of anti-PtdIns autoAb titer was seen in all B(*a*)P-treated SD female rats and the animals developed a highly malignant sarcoma at the site of injection of B(*a*)P (top of the right thigh). Thus, tumor incidence and/or growth were easily monitored prior to sacrifice of the animals. This model is thus reliable for the evaluation of *all-trans*-RA effects.

Although PtdIns turnover is implicated in the regulation of cell proliferation, transformation and differentiation,[26] and *all-trans*-RA rapidly decreased PtdIns turnover during neuroblastoma cell differentiation,[27,28] no influence of *all-trans*-RA treatment on anti-PtdIns autoAb levels was observed under our experimental conditions. These levels remained high whichever treatment regime was administered.

Many authors have previously described the chemopreventive efficiency of retinoids against carcinogen-induced skin, urinary bladder, tracheobronchial, pancreas and mammary tumors.[29–32] To study the effects of retinoids on the initiation phase of carcinogenesis, the retinoids were administered before carcinogens until the end of the experiment. In studies on the anti-promotional effects, *all-trans*-RA was injected 1 week post-carcinogen treatment until the end of the experiment. Chemopreventive effectiveness was observed and this was increased by combination with other agents such as hormone antagonists.[33]

Here, we report the evaluation of *all-trans*-RA curative effects before and after the appearance of clinically palpable sarcomas. The onset and growth of the tumors were monitored by palpation and MRI analysis. MRI calculations confirmed the mechanical measurements of tumor volume (Table 3). Morphological changes in tumoral tissues were observed. Early necrotic areas occurred in tumors of *all-trans*-RA-treated rats, but not in solvent-treated rats (control group). Additionally, the necrotic origin of the high-intensity zones seen on the T2-weighted sequence of *all-trans*-RA-treated rats (Figure 3) was confirmed by histological analyses, indicating tissue modifications, particularly vascularization. Curative effects were studied using two experimental *all-trans*-RA treatment conditions. No time-lag was observed in tumor appearance relative to the solvent group at the beginning of *all-trans*-RA treatment (day 98) and no significant survival was observed (Table 2 and Figure 2). Conversely, *all-trans*-RA seemed to be more effective against clinically palpable tumors of small size. We have no clear explanation for this phenomenon. Tumor growth measurements showed a slowing down for *all-trans*-RA treated rats in relation to the control group (Table 1). The

curves in Figure 1 indicate that 300 μg and 3 mg *all-trans*-RA-treated rats survived longer (about 25 days) than solvent-treated rats although this difference is not statistically significant ($p = 0.09$ compared with $p = 0.24$). However, these curves lead us to believe that with larger experimental groups, the results might be more convincing and statistically significant.

RA toxicity might explain small tumor volumes of the 3 mg *all-trans*-RA-treated rats before death, relative to normal evolution of the control group, and their short survival (Table 2 and Figure 2). Some authors have reported lower survival in rats receiving high doses of this compound.[22,34]

Conclusion

All-trans-RA has curative potential (Table 1 and Figure 1); however, our evaluation showed its limits when used alone and at high dose (Table 2 and Figure 2). The curative effects of *all-trans*-RA could be increased if administered together with other agents, such as drugs and antibodies.

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