Retinoic acid inhibits the fixation of initial transformational damage in X-irradiated Balb/3T3 mouse fibroblasts *in vitro*

Hans Peter Rutz[1] and John B.Little[2]

Harvard School of Public Health, Department of Cancer Biology, 665 Huntington Avenue, Boston, MA 02115, USA

[1]Present address: Laboratoire de Radiobiologie, Service de Radiothérapie, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland
[2]To whom correspondence should be addressed

We have examined the effects of all-*trans* retinoic acid (RA) on confluent holding recovery (cell survival) and on the fixation of initial transformational damage expressed as the ultimate yield of transformed foci following X-irradiation of density-inhibited cultures of Balb/3T3 cells. Non-cytotoxic concentrations of RA suppressed both recovery of potentially lethal damage and neoplastic transformation in a dose-dependent manner when added for 24 h during post-irradiation confluent holding after a dose of 5 Gy. At 100 μM, RA inhibited the fixation of initial transformational damage by 80%. These findings are discussed in terms of the hypothesis that retinoids may allow a selective enhancement of the inactivation of certain irradiated tumor cells *in vivo* while reducing the risk of secondary malignancies in successfully treated patients.

Introduction

Vitamin A and certain of its natural and synthetic analogs, collectively called retinoids, are potent inhibitors of carcinogenesis at many tissue and organ sites in both rodents (1—3) and humans (4—6). They also inhibit chemically (7—9) and radiation-induced (10,11) transformation *in vitro*, as well as transformation by transfection with human oncogenes (12). The use of retinoids in cancer prevention has therefore become a promising field of investigation (4—6). The mechanisms of this inhibition, however, are not yet fully understood.

Malignant transformation develops in two distinct phases: the first is the production and fixation of initial transformational damage as a heritable cellular property, and the second is the phenotypic expression of this damage as a morphologically altered cell (13). Several studies have reported effects of retinoids on cells in culture which result in a stabilization of the non-transformed phenotype; these include effects on cell growth, adhesion to the culture substrate, cell morphology, cytoskeleton, protein-phosphorylation, expression of cellular proto-oncogenes, expression of receptors, differentiation, membrane function, inhibition of the protein kinase C cascade system, and blocking of the G_0 to G_1 transition in the mitotic response of initiated cells to growth factors which act as endogenous promoters of transformation (14—23). However, little is known about effects of retinoids on cellular recovery mechanisms underlying the fixation of initial transformational damage.

Cellular recovery processes mitigate the cytotoxic (repair of potentially lethal damage or PLD* repair) and clastogenic (repair of chromosomal aberrations) effects of carcinogen exposure; they

*Abbreviations: PLD, potentially lethal damage; RA, all-*trans* retinoic acid

are involved in the fixation of DNA sequence alterations resulting in mutants as well as in neoplastic transformation through processes leading to the fixation of initial transformational damage (24). Cellular repair can be studied in confluent holding recovery experiments with density-inhibited, confluent cultures of mammalian cells (25,26).

In such experiments, DNA repair processes can act to remove damage in the absence of ongoing DNA replication. Subculture to low density at various times after exposure stimulates the initiation of DNA synthesis, allowing resumption of active traversal of the cell cycle. When confluent cultures are immediately subcultured to low density after exposure to radiation, a dose-dependent induction of effects such as cell killing, mutagenesis, transformation and chromosomal rearrangement occurs. These toxic effects are reduced when post-irradiation recovery periods of 24 h or longer are allowed prior to subculture (24—29). Such recovery has not been observed in certain repair-deficient cell strains (30—32), suggesting the involvement of DNA repair in the confluent holding recovery phenomenon.

In the present investigation, we have examined effects of a 24 h post-irradiation exposure to all-*trans* retinoic acid (RA) on confluent holding recovery (PLD repair) and on the fixation of initial transformational damage expressed as the ultimate yield of transformed foci in Balb/3T3 mouse fibroblasts.

Materials and methods

Cells and culture conditions, irradiation, confluent holding

The Balb/3T3 cell system and the procedures for the maintenance of these cells, as well as the radiation source, have been described in detail elsewhere (33). The cells were grown in Eagle's minimum essential medium supplemented with 10% serum. This serum was Biocell VSP neonate calf serum lot no. 211200 for experiments I—III, Biocell VSP neonate bovine serum lot no. 36211A182 for experiment IV, and Gibco heat-inactivated calf serum, cat. no. 230-6170AJ for experiments V—VII.

All experiments were carried out with density-inhibited, confluent cultures. Three daily medium changes after reaching confluence allowed the cells to approach a steady state. They were irradiated in conditioned medium 24 h after the last medium change with a dose of 5 Gy. Survival was determined by a routine colony-formation assay (33). The cloning efficiency in these experiments ranged from 83.5 to 97.5%. Survival and the transformation frequency were determined immediately following irradiation. An additional set of similarly treated cultures was used to examine the effects of post-irradiation incubation with RA on confluent holding recovery and on the fixation of initial transformational damage.

RA, received from Sigma (cat. no. R-2625), was dissolved in ethanol (10 mM stock solution) and stored at 4°C in the dark. From this solution, RA was diluted into complete medium. The conditioned medium was removed immediately after irradiation and replaced with fresh medium containing various concentrations of RA up to 100 μM. After 24 h, the cells were subcultured into medium without RA at low density (200 viable cells/dish) to determine survival and at a higher density (10 000 viable cells/dish) for the transformation assay. The effect of a 24 h treatment with RA on the cloning efficiency of non-irradiated cultures was determined in a parallel set of dishes. The number of colonies with >50 viable appearing cells was scored after 8—10 days.

Transformation assay

Cell numbers were adjusted in each treatment group such that ~10 000 viable (colony forming) cells from the same confluent cultures as those used to measure survival were seeded in each of 20—60 100-mm Lux Petri dishes. The nutrient medium was renewed on the third or fourth day after irradiation. In experiments
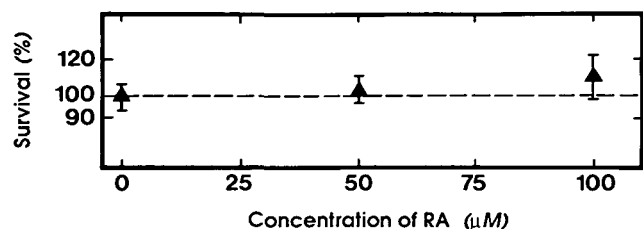
Fig. 1. Survival of density-inhibited, confluent cultures of Balb/3T3 mouse fibroblasts following a 24 h incubation with RA. Data points are the mean of three independent experiments. Error bars indicate one SD.
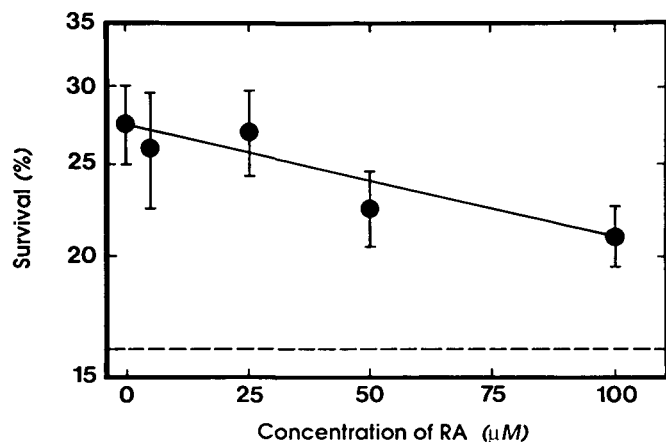

Fig. 2. Inhibitory effect of RA on confluent holding recovery in Balb/3T3 cells irradiated with a dose of 5 Gy. RA was present only during the 24 h post-irradiation recovery period. Dashed line: survival of cells subcultured to low density immediately following irradiation. The data points were calculated from six independent experiments. Error bars indicate one SD.
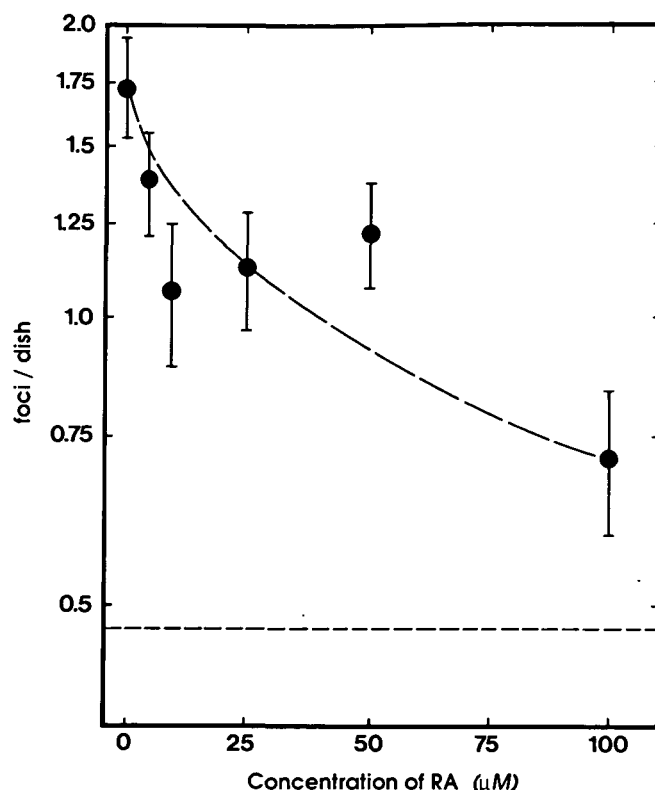

Fig. 3. Effect of RA on the fixation of initial transformational damage expressed in terms of the ultimate yield of transformed foci/dish in Balb/3T3 mouse fibroblasts irradiated with a dose of 5 Gy and held under confluent holding conditions during a 24 h post-irradiation recovery period. Dashed line: background transformation frequency of untreated controls. Data points were calculated from the pooled data of experiments II−VII as described elsewhere (36,37). Error bars indicate one SD.

I−III, the medium was subsequently changed every 10 days; in experiments IV−VII, twice each week. After 4 weeks, the cultures were fixed and stained. Transformed foci were scored as described by Kakunaga (34). Since previous transformation studies have shown that the number of foci appearing per dish is independent of the number of cells initially seeded (33,35), the results are expressed in terms of the number of foci per dish (35).

Statistical analysis

For the estimation of the effects of RA on PLD repair and on survival of unirradiated Balb/3T3 cells, we calculated the mean ± one SD from six (PLD repair) or three (RA toxicity) independent experiments. The effects of RA on the fixation of initial transformational damage were calculated from the fraction of dishes without foci of transformed cells arising from irradiated cells which were exposed to various concentrations of RA during 24 h of post-irradiation confluent holding. Statistical analysis was calculated as described by Han and Elkind (36) and Balcer-Kubiczek *et al.* (37). The data from experiments II−VII were pooled to calculate the transformation frequencies. The results of experiment I were excluded from this study because of an unusually high background frequency of transformation; foci of transformed cells appeared in 18 of 19 non-irradiated control dishes. These results (in terms of the actual number of foci per dish), however, were qualitatively similar to those of the other six experiments.

Results

Effects of RA on confluent holding recovery

Treatment of non-irradiated confluent cultures with RA alone for 24 h had no significant effect on survival for all concentrations studied (Figure 1). Figure 2 shows the inhibitory effect of RA on confluent holding recovery. Survival of cells subcultured immediately after irradiation (initial survival) was 16 ± 3% (dotted line in Figure 2). During the 24 h confluent holding period, survival increased to 27.5 ± 2.5% in the absence of RA, reflecting the repair of PLD. RA suppressed the recovery in a

Table I. Number of dishes, transformation frequencies, and the percentage inhibition of the fixation of initial transformational damage by retinoic acid in X-irradiated Balb/3T3 mouse fibroblasts[a]

Treatment	Concentration of retinoic acid (μM)	Dishes without foci/ total no. of dishes	Foci per dish[b] ($\pm$ 1 SD)	Inhibition (%)
control	−	74/118	0.47 ± 0.07	−
5 Gy, i.s.	−	31/129	1.42 ± 0.15	−
5 Gy, d.s.	0	20/113	1.73 ± 0.20	0
5 Gy, d.s.	5	27/108	1.39 ± 0.17	27.7
5 Gy, d.s.	10	20/58	1.06 ± 0.18	52.2
5 Gy, d.s.	25	25/77	1.12 ± 0.16	48.4
5 Gy, d.s.	50	31/105	1.22 ± 0.15	45.2
5 Gy, d.s.	100	35/71	0.71 ± 0.12	80.9

[a]Cells in density-inhibited, confluent cultures were X-irradiated in the absence of RA and subcultured immediately after irradiation (i.s.) or after a post-irradiation confluent holding period of 24 h (d.s.) in the presence of various concentrations of RA. The fixation of initial transformational damage was expressed as the ultimate yield of foci per dish. The inhibition is calculated in terms of percentage inhibition of the transformation frequency observed in cells reincubated without RA, after subtracting the background transformation frequency observed in non-irradiated controls.
[b]Calculated from the number of dishes without foci as described elsewhere (36,37).

dose-dependent fashion. This trend was evident in all six experiments, and is consistent with previous observations in human cells (38).

Effects of RA on the fixation of initial transformational damage

Figure 3 shows the inhibitory effect of RA on the fixation of initial transformational damage when it is present during the 24 h period of post-irradiation confluent holding. Table I shows this suppression in terms of percentage inhibition of the transformation frequency observed in cells reincubated without RA, after subtracting the background transformation frequency observed in non-irradiated controls. At 100 μM, RA suppressed the ultimate yield of transformed foci by 80%. Table I also shows the total number of dishes from which these data were calculated.

A 24 h exposure of non-irradiated cells to RA did not affect the spontaneous formation of foci of transformed cells (data not shown). Hence, the suppression by RA of PLD repair leading to enhanced killing of non-cycling cells (Figure 2) is accompanied by a suppression of the fixation of initial transformational damage leading to a reduction in the induced frequency of transformation (Figure 3, Table I).

Discussion

Non-cytotoxic concentrations of RA (Figure 1) suppressed both recovery (Figure 2) and neoplastic transformation (Figure 3) when added to the medium during a 24 h post-irradiation confluent holding period. These observations suggest that RA is not only suppressing later events in neoplastic transformation involved with the expression of a transformed phenotype as reported by other investigators (7–11, 14–23), but also inhibits an early event. This may be caused by a relatively simple interaction such as a change in chromatin structure (39) or may involve more complex events, as for example an inhibition of sister chromatid exchanges which has been observed after exposure to cytotoxic drugs (40), or the inhibition of molecular error-prone mechanisms for the repair of X-ray-induced DNA damage (38).

The activity of such a mechanism in the fixation of initial transformational damage has been postulated based on the kinetics of transformation during confluent holding recovery in X-irradiated C3H 10T1/2 mouse cells (27). An inhibition of error-prone repair may be a causal molecular link between the inhibition of PLD repair by RA and the suppression of transformation. However, an alternative explanation for these results can be derived from the hypothesis that ionizing radiation causes two types of damage, namely potentially lethal and potentially transforming damage. RA could be causing decreased transformation, not by suppressing an error-prone repair mechanism but by decreasing the number of cells with transforming damage that escape the cytotoxic effects of potentially lethal damage.

The findings of this study are of particular interest as regards a possible application of retinoids in radiation therapy: they may not only allow a selective enhancement in the inactivation of certain irradiated cancer cells as compared to normal cells (38), but also reduce the risk of secondary tumors (41) in successfully treated patients. On the other hand, the toxic side-effects of RA (4–6) may limit the use of this drug as modifier for radiotherapy, since effective concentrations in the present study were well above physiological levels. Hypervitaminosis A also prohibited the use of clinically effective doses of RA for the systemic treatment of dermatological disorders (42–45). However, it may be possible to identify retinoids with a better therapeutic index for clinical use as a biological response modifier for radiation therapy, as

has been successfully done in the search for effective retinoids in dermatology (46).

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