SHORT REPORTS

Effect of Retinoic Acid on the Infiltration of Murine Melanoma Cells into the Type I Collagen Gel

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Two lines of murine melanoma cells (B16 and Cloudman S91) were cultured on type I collagen gel and the effects of all-trans-retinoic acid on the growth and infiltration into the gel were assayed. In both lines, proliferation and the degree of infiltration were suppressed by the addition of all-trans-retinoic acid. The infiltration-inhibiting effect was expressed very rapidly and was dose-dependent at concentrations ranging from $10^{-7}$ to $10^{-4}$ M of all-trans-retinoic acid. These results suggest the anti-invasive effects of all-trans-retinoic acid on melanoma cells.

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Tumour cells tend to invade surrounding normal tissue by penetration or infiltration with active movement. However, the precise mechanism is not well elucidated because of the absence of simple in vitro experimental systems (1, 2).

We and other authors have reported that cells cultured in or on type I collagen gel resemble, as closely as possible, those in vivo (3-7). Recently, Schor et al. (8, 9) and our group (10-13) have cultured melanoma cells on and in type I collagen gel and have suggested melanoma cell-to-collagen interactions. Schor et al. first reported the penetration or infiltration phenomenon of tumour cells into collagen gel (8). On the other hand, all-trans-retinoic acid (RA) has been known to be an anti-proliferative agent against melanoma cells (14, 15).

In the present study, we cultured two lines of murine melanoma cells (B16 and Cloudman S91) on type I collagen gel and investigated the effects of RA on growth and infiltration into the gel.

MATERIALS AND METHODS

Cell culture

A B16 BBC7 clone was derived from B16 C3M cells (16) (kindly donated by Dr A. Okada, Tohoku University School of Medicine, Sendai, Japan) and maintained in our laboratory. Cloudman S91 melanoma cells were obtained from the Dainippon Pharmaceutical Co. (Osaka, Japan). These lines were grown in Eagle's medium supplemented with 10% fetal calf serum (FCS).

Preparation of the type I collagen gel

Hydrated type I collagen gel was prepared using a modified method of Eshleman & Bard (3) as previously described (5-7, 10-13). Briefly, appropriate volumes of 0.3% papainized type I collagen solution (Nitta Gelatin Co., Osaka, Japan), concentrated Eagle's medium, 200 mM HEPES/0.08 N NaOH solution and FCS were mixed rapidly at a temperature below 4°C to a final collagen concentration of 2.0 mg/ml. One milliliter of the mixture was poured into a 35-mm bacteriological dish (Falcon) and incubated at 37°C. The collagen gel formed within 10 min. Before use, cells were trypsinized, washed and then inoculated onto the gel at a cell density of 10^5 cells/dish.

Assay of total cell growth and infiltration into the collagen gel

The total number of cells both on and in the gel and that on the surface of the gel were determined by the modified method of Schor (8). The collagen gel with cells on its surface was washed three times with Ca2+-, Mg2+-free phosphate-buffered saline (PBS2) and incubated in 2 ml of 0.05% trypsin/2 mM EGTA in PBS2 for 30 min. The trypsin solution was vigorously stirred by pipetting to release the cells. The cells released were recovered as completely as possible and stored in a tube. The gel was incubated in 2 ml of 0.4 mg/ml collagenase (Sigma) in serum-free medium for 20 min at 37°C. The collagenase solution was stirred vigorously by pipetting. The detached cells were recovered and added to the tube containing cells previously released by trypsinization and the cell numbers were counted using a hemocytometer (the number of cells on the surface of the gel; A). By the treatment with trypsin and collagenase, the cells on the surface were almost completely detached and the intact gel was left.

The gel was minced with scissors and incubated again in 2 ml of 0.4 mg/ml collagenase for 5 h to dissolve the gel. Cells which infiltrated and grew in the gel were released,

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cell infiltration into the gel was remarkably suppressed as early as day 1 of culturing in both lines ($p < 0.01$).

**Dose-dependency of the suppression of cell infiltration by RA**

The effects of various concentrations of RA on the infiltration is shown in Table I. The effects were found to be dose-dependent in both lines. At concentrations of $10^{-7}$ and $10^{-8}$ M, the suppression rate of infiltration of S91 cells was lower than that of B16 cells ($p < 0.01$).

**DISCUSSION**

In the present study, rapid infiltration into the gel was observed in both melanoma cell lines (Fig. 1C, D). Because the growth rates of melanoma cells in the collagen gel were the same as those on the gel (11, 13), the increase in the number of cells in the gel was thought to be due mainly to cell infiltration. RA inhibited both cell growth and infiltration into the gel in both lines (Fig. 1), and the anti-infiltrating effect of RA was dose-dependent (Table I). The effects of RA were expressed from day 1 or 2 of culture.

The anti-proliferative effect of RA against melanoma cells has been reported by Lotan (14) and Lauharanta et al. (15). They have reported that the most sensitive cell line is Cloudman S91 and that the cell-specific differentiating function, melanogenicity, of this cell line is enhanced by the addition of RA. As shown in Fig. 1A, the growth of B16 cells was also inhibited by RA. However, the degree was lower than that of S91 cells (Fig. 1B). In contrast to S91 cells, the melanogenetic activity of B16 cells was suppressed by RA (data not shown) as previously reported by Hosoi et al. (17). On the other hand, the inhibitory effects on infiltration were observed in

**Table I. Effects of various concentrations of RA on melanoma cell infiltration into the collagen gel, expressed as % of cells in gel (vs. control)**

<table>
<thead>
<tr>
<th>Line</th>
<th>Concentration of retinoic acid</th>
<th>Cone large of cells (%)</th>
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<tbody>
<tr>
<td>B16 CBC7</td>
<td>$10^{-9}$ M</td>
<td>42.5±0.8</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$ M</td>
<td>31.4±0.3</td>
</tr>
<tr>
<td></td>
<td>$10^{-7}$ M</td>
<td>21.2±0.5</td>
</tr>
<tr>
<td>S91</td>
<td>$10^{-9}$ M</td>
<td>43.2±0.1</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$ M</td>
<td>38.1±0.7</td>
</tr>
<tr>
<td></td>
<td>$10^{-7}$ M</td>
<td>30.5±0.2</td>
</tr>
</tbody>
</table>

recovered and the cell numbers were counted (B). The total number of cells was the sum of A and B.

RA (Sigma) was added at the indicated concentration from day 0 of culturing. In the control, the same volume of solvent (ethanol) as that of RA was added.

Statistical analysis was performed using Student's t-test.

**RESULTS**

**Growth-inhibitory effect of RA**

Fig. 1 shows the time course of the growth (A, B) and the infiltration into the gel (C, D) of B16 (A, C) and S91 (B, D) melanoma cells. With the addition of RA ($10^{-8}$ M), growth was suppressed in both lines. The suppression was shown to be stronger in S91 than in B16 cells (% of growth inhibition at day 5 was about 58% in B16 and 81% in S91 cells). The growth inhibition was reversible, since the growth rate recovered when the medium was changed to an RA-free one (data not shown).

**Inhibition of cell infiltration into the gel by RA**

As shown in Fig. 1C and D, melanoma cells rapidly infiltrated into the collagen gel and this process progressed in a time-dependent manner. The degree of

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both lines, and the suppression rate of S91 cells was lower than that of B16 cells (Fig. 1 C, D and Table I).

The mechanism of infiltration into the gel remains to be clarified. Some authors have pointed out the important role of proteolytic enzymes in metastasis (18, 19). However, Schor et al. (9) and Bogenmann et al. (20) found no correlation between the collagenolytic activity of cells and the invasion or infiltration process. RA has been reported to be involved in the synthesis and secretion of fibronectin (FN) (21), which is known to have stimulatory (22) and inhibitory (23) effects on cell migration in plastic dishes. Schor et al. have also reported that FN is involved in cell migration in collagen gel (24). FN or another extracellular matrix, such as glycosaminoglycan, may be related to the process (25).

Recently, the anti-invasive effect of RA in mammary tumour cells has been reported (26, 27). The present study also shows that RA not only has anti-proliferative potential but also has an anti-infiltrating effect into collagen gel against melanoma cells.

As previously reported (3–8), a culture system of type I collagen gel has been considered to be a reconstituted dermis model. However, other components of the dermis including proteoglycans or fibroblasts, which will influence the behaviour of the melanoma cells, must be introduced to this system. Such an attempt has been in progress in our laboratory. The culture system using type I collagen gel as a substrate may be a useful and simple model for the in vitro study of tumour metastasis and invasion.

REFERENCES


Dose and Timing Studies for the Optimization of Contact Sensitivity in the Mouse

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We investigated the effectiveness of very low doses of the contact sensitizer dinitrofluorobenzene in sensitizing BALB/cJ mice. Surprisingly, the ear swelling reactions were greater with lower dinitrofluorobenzene doses, down to one-twentieth of doses commonly used. Although it is common practice to use much lower doses at challenge than at sensitization, we found greater reactions with lower doses at sensitization than at challenge. We also studied the timing of the development and waning of reactivity to dinitrofluorobenzene, dinitrochlorobenzene and oxazolone. Reactivity peaked at day 5 for dinitrofluorobenzene and dinitrochlorobenzene, and at day 3 for oxazolone. Reactivity waned by 3 weeks with dinitrofluorobenzene and oxazolone, and by day 7 with dinitrochlorobenzene. Pretreatment with cyclophosphamide caused a delay in the development and waning of reactivity. Key words: Dinitrofluorobenzene: Dinitrochlorobenzene: Oxisolone: Ear swelling.

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Contact sensitivity reactions in mice are often used in dermatological research, for example to study the effects of treatments such as UVB irradiation on the immune system. Since contact sensitivity reactions are used as a tool in such analyses, it is important to know the optimum doses and timing of the reactions. It is common practice to use sensitizing doses of contact sensitizers that are greater than the doses used at challenge. However, in our studies on the contact photosensitizer tetrachlorosalicylanilide (TCSA), we found that optimum reactions were achieved with larger doses of TCSA and UVA at challenge than at sensitization (1). Because of this surprising finding with a photosensitizer, we decided to study low doses of the contact sensitizer dinitrofluorobenzene for this effect as well. We also investigated the optimum timing between sensitization and challenge, the time taken for sensitivity to wane, and the effect of cyclophosphamide on the development and waning of reactivity.

MATERIALS AND METHODS

Female BALB/cJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A. We obtained 2, 4 dinitro-1-fluorobenzene (DNFB), 1-chloro-2, 4-dinitrobenzene (DNCB) and 4-ethoxy-methyl-2 phenyloxazol-5-one (OX) from Sigma Chemical Company, St. Louis, MO, U.S.A. Cyclophosphamide (Cy) was purchased from Procytox, Montreal, Canada.

Mice were anesthetized with ethyl ether and a patch of fur 2x2 cm was removed from the dorsal skin with electric clippers. For dose-response studies, we sensitized groups of mice with various quantities (1 μl to 150 μl) of 0.5% DNFB in acetone applied to the shaved back skin on days 0 and 1. The total dose was therefore 10 μg to 1500 μg. For studies