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ORIGINAL ARTICLE

Comparison of the in vitro and in vivo effects of retinoids either alone or in combination with cisplatin and 5-fluorouracil on tumor development and metastasis of melanoma

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Abstract
Purpose Retinoids have previously been reported to inhibit proliferation of melanoma cell lines in vitro. However, the relative antimetastatic efficacy of various retinoids on melanoma in vivo is unknown. Therefore, we investigated the effects of different retinoids on the invasion and metastasis of murine melanoma B16-F10 cells in vitro and in vivo. Based on the findings, the antitumor effects of a selected retinoid either alone or in combination with cisplatin were also investigated in a preclinical mouse melanoma model.
Methods Cell proliferation and invasion analyses of murine melanoma B16-F10 cells were assessed in the presence of different retinoids, either alone or in combination with cisplatin (CDDP) or 5-fluorouracil (5-FU). Experimental lung metastasis assay was performed in this study to investigate the antimetastatic efficacy of retinoids. Additionally, a mouse melanoma model was used to assess the antitumor efficacy of a selected retinoid in combination with cisplatin.
Results Retinoids showed significant antiproliferation and anti-invasion effects on murine melanoma B16-F10 cells. Pretreatment with retinoids increased the sensitivity to CDDP but not to 5-FU in in-vitro. Moreover, the number of metastatic colonies formed in the lungs of mice injected intravenously with B16-F10 cells was significantly reduced by injecting the respective retinoid once a day for 10 days. Treatment with a combination of cisplatin and 13-*cis*-retinoic acid resulted in a significant reduction in primary tumor size and the number of lung metastatic nodules in melanoma-bearing mice.
Conclusion These results suggest that retinoids not only exhibit antimetastatic effect, but also enhance the antitumor activity of cisplatin in vivo.

Keywords Retinoid · Cisplatin · Melanoma · Metastasis · Mouse

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Introduction

Metastasis, the major cause of cancer mortality, is a complex series of multi-step processes in which tumor cells invade the basement membrane, penetrate blood vessels and exit vessels at distant sites to form secondary tumors [1]. The degradation enzymes of the basement membrane, matrix metalloproteinases (MMP), play an important role in the invasion and metastasis of human cancer. Although advances in surgery, radiotherapy, and chemotherapy have significantly improved the treatment of primary tumors, the occurrence of metastasis still leads to poor prognosis and death in patients with malignancy. A multi-step process leads to metastasis [2]. The interrelated events of the metastatic cascade include disruption of the basement membrane, cell detachment (separation), cell motility, invasion, penetration of the vascular system, circulating cancer cells, arrest (stasis), and extravasation, and proliferation [3]. Metastatic melanoma involves the escape of melanoma cells from the epidermis, growth, and development of a blood supply, dissemination of melanoma cells to other organs, and their growth at these locations. Excess extracellular matrix (ECM) degradation is an essential step for cell migration [4]. As matrix degradation is so central to the invasive/metastatic behavior of tumors, and this

metastatic ability is life threatening, therapeutic strategies designed to inhibit invasion and keep a tumor localized are attractive. An experimental metastasis mouse model using B16-F10 melanoma cells [5] has been applied to identify possible antimetastatic agents [6–8].

Retinoids as an anticancer agent have been studied in vivo and in vitro using various murine and human cancer cell lines including melanomas for more than 20 years. A number of retinoids have been evaluated as potential therapeutic agents in the prevention and treatment of human cancer [9–11]. It was found that retinoids inhibited growth of many melanoma cell lines of various species [12–14]. Retinoids were also reported to suppress melanoma cells transformed phenotype and modulate their differentiation in vitro and formation of metastases in vivo [15, 16]. The inhibitive effects of retinoids on metastasis may be partly due to retinoid-mediated repression of tumor cell motility [16] and suppression of MMP production [17]. However, not all retinoids inhibit melanoma cells; some have no influence and others even stimulate melanoma cell growth and invasion [18]. Furthermore, some retinoids inhibit various functions in certain melanoma cells, while these functions are not influenced in other melanoma cell lines using the same retinoids. Up-to-date, there is no systematic in vivo study comparing the effects of different retinoids on the metastasis of melanoma. The purpose of this study is to evaluate the potential efficacy of five retinoids, including all-*trans*-retinoic acid (ATRA), 9-*cis*-retinoic acid (9-*cis*-RA), 13-*cis*-retinoic acid (13-*cis*-RA), etretinate, and acitretin, on the growth and invasion inhibition of murine melanoma B16-F10 cell line.

Retinoids have shown a synergistic action in combination with other chemotherapeutic agents in numerous studies in vitro and in animal models [19]. Sacks et al. [20] reported a synergistic interaction between ATRA and CDDP or 5-fluorouracil (5-FU) in a series of squamous cell lines. They inferred that the mechanism of synergism would be based on drug perturbations in DNA synthesis coupled to an increased sensitivity induced by ATRA. Retinoids have also been tested in numerous clinical trials for the prevention of cancer based on their ability to induce cell differentiation and inhibit tumor growth in animal models [19]. Combinations of 13-*cis*-RA and cisplatin or cisplatin and ifosfamide have proven promising in patients with head and neck cancer in phase I/II trials [21]. Objective clinical responses with moderate toxicity have been observed in patients with advanced non-small cell lung cancer treated with 13-*cis*-RA and chemotherapy (carboplatin, vindesine, and 5-FU) [22]. When combined with α-tocopherol or cisplatin, 13-*cis*-RA was effective in two phase II clinical trials in patient with advanced squamous carcinomas of the head and neck or the skin, respectively [23, 24]. Thus, it may be meaningful to evaluate the antitumor and antimetastatic effects of retinoids in combination with CDDP or 5-FU on melanoma in vitro and in vivo.

Materials and methods

Drugs

All-*trans*-retinoic acid (ATRA) was purchased from Tokyo Chemical Industry Company, Ltd. (Tokyo, Japan). 13-*cis*-RA was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). 9-*cis*-RA, acitretin, and etretinate were kindly provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). All retinoids were dissolved in dimethyl sulphoxide (DMSO). They were diluted with culture medium to appropriate concentrations just before use.

Cell culture conditions

The murine melanoma B16-F10 cell line was ordered from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and was tested to be pathogen free by ATCC. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heated-inactive fetal bovine serum and 1% antibiotic solution (penicillin/streptomycin) at 37°C in 5% CO_2 humidified atmosphere.

Cell proliferation study

The inhibitory effect of retinoids on cell proliferation was tested by incubating the B16-F10 cells with different retinoids. The cells were cultured in DMEM and were plated in 6-well plates. After overnight incubation, and thereafter, at every 48 h, the medium was replaced with fresh medium or medium containing retinoids (10^{-6} M), until the total viable cells of the respective wells were counted on days 2, 3, 4, 5, and 6 by trypan-blue dye exclusion method. All experiments were repeated in triplicate.

Combination chemotherapy studies

B16-F10 cells were allowed to grow for 6 days with or without retinoids (10^{-6} M), washed with phosphate-buffered saline (PBS) and then plated onto 96-well microtiter plates (5,000 cells per well). The cytotoxicity of the respective chemotherapeutic agents (cisplatin [CDDP] or 5-FU) was determined using a previously described Fluorometric microculture cycotoxicity assay (FMCA) [25]. Briefly, the plates were incubated at 37°C overnight to allow the cells to adhere to the bottom. The medium of the plates was then changed to 200 µl medium containing

CDDP or 5-FU or control medium. After 3-day incubation at 37°C, the plates were washed with PBS. 200 μl of fluorescein diacetate solution (FDA, 10 mg/mL, Sigma) was then added to each well. The plates were incubated at 37°C for half an hour, and the fluorescence generated from each well was measured by fluorescence spectrometer. In each plate, wells with untreated cells were measured as controls and their fluorescence was defined as 100%. Cell survival was presented as survival index (SI), which was defined as the fluorescence in experimental wells expressed as a percentage of that in the control wells. The 50% inhibitory concentration (IC_{50}) of chemotherapeutic agents was defined as the concentration giving a SI 50%. Experiments were carried out in six replicates and repeated three times. The data were fit into Sigmoid E_{max} model with the software SPSS (version 11.0, SPSS, Chicago, Ill, USA).

In vitro cell invasion assay

B16-F10 cell invasion through reconstituted basement membrane (matrigel) was assayed according to the previously described method [26] with some modifications. Before invasion assay, for each Transwell (pore size, 8 μm; Corning Incorporated, Corning, NY, USA), the upper surface of the microporous polycarbonate membrane that separated the upper and lower chambers was coated with matrigel (10 μg/well). For invasion assay, 100 μl of B16-F10 cells (10^5 cells) was suspended in DMEM supplemented with 0.1% bovine serum albumin (BSA), and seeded in the upper compartments of Transwell Inserts. Conditioned medium (600 μl), obtained by incubating subconfluent human fibroblast cells for 24 h in serum-free medium, was used as a source of chemo-attractants and placed in the lower compartments of the inserts. Cells were allowed to invade through the matrigel for 6 h at 37°C. Cells that remained on the upper surface of the inserts were wiped off by cotton swab. Cells which crossed the matrigel barrier and migrated to the lower side of the inserts were fixed in 70% ethanol, stained by dipping the Transwell into solution of toluidine blue (1% v/v) for 1 min. The Transwell was then blotted dry on a piece of tissue. Ten random unit fields per insert were counted by an inverted microscope (Leica, Germany; 400× magnification). Only invasive cells were detectable on the lower surface of the inserts. Three different experiments in four replicates were performed.

Animals

Experiments were performed on male C57BL/6J black mice, 6–8 weeks old, purchased from Laboratory Animals Center of the National University of Singapore, Singapore. Mice were kept in plastic cages with sawdust bedding and given food and water ad libitum. The room in the Animal Holding Unit, National University of Singapore, Singapore in which mice were kept was environmentally controlled at a temperature of 23 ± 2°C and with a 12:12 light–dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

Experimental lung metastasis assay

To prepare B16-F10 melanoma cells for inoculation, cells in logarithmic growth phase were harvested by exposure to trypsin. Cell viability in single cell suspensions was determined by trypan blue exclusion. A total of 10^5 B16-F10 melanoma cells (>90% viability) in 100 μl of PBS were injected into the lateral tail vein of 6–8 week-old male syngeneic C57BL/6J mice. The mice were divided into four groups. The control group received saline of comparable volume to the drug solution and the treated groups received ATRA, 9-*cis*-RA, and 13-*cis*-RA, respectively, at a dose of 20 mg/kg/day for 10 days. Mice in each group were weighed every other day. The day of inoculation was considered as day zero. After 14 days, mice were killed; their lungs were removed and fixed in 10% formaldehyde. Metastatic foci that appeared as black spots on the lung surfaces were counted under dissection microscope (Leica Co., Inc., Tokyo, Japan).

Effects of retinoids and cisplatin combination on tumor development in melanoma-bearing C57BL/6J mice

On day 0, B16-F10 melanoma cells were harvested from the cultures. 1×10^6 cells in 20 μl PBS were injected subcutaneously (s.c.) into the footpad of the right hind limb of each mouse. The mice were intraperitoneally injected daily saline of comparable volume to the drug solution for 10 days as the control. Other mice received either cisplatin alone (10 mg/kg, i.p., on days 7 and 14), or retinoid alone (13-*cis*-RA or ATRA, 20 mg/kg, i.p. daily for 10 days), or the combination of cisplatin and retinoids. The tumor size was measured on day 21 after the inoculation. Estimated tumor volume was calculated using the formula:

$$\text{Tumor volume } (\text{mm}^3) = (w^2 \times l)/2$$

where w = width and l = length in mm of tumor

After the killing of mice after on day 21, metastatic foci in lungs were examined in the same way as in the experimental lung metastasis assay.

Statistical analysis

The differences between the retinoid-treated groups and the control group or between the respective retinoid-treated

groups for both in vitro and in vivo experiments, were tested by one-way ANOVA with either Dunnett's test or Tukey post-hoc test using the software SPSS (version 11.0, SPSS, Chicago, Ill, USA) A P value of less than 0.05 was considered to be statistically significant. All experiment results were shown as mean ± SD.

Results

Cell proliferation study

To test the inhibitory effect of different retinoids, cells were cultured in medium containing the respective retinoid for 6 days. Cell viability was more than 90% after treatment. The growth curves of cells treated with different retinoids were shown in Fig. 1a. Various inhibitory effects were observed depending on the individual retinoids. When treated at 1 μM, all retinoids significantly suppressed the proliferation of B16-F10 cells after treatment for 6 days ($P < 0.05$). The inhibitory effect of ATRA on the proliferation of cells was not different from that of 9-*cis*-RA and 13-*cis*-RA ($P > 0.05$), while acitretin and etretinate showed less inhibitory effect on proliferation when compared to the first three retinoids ($P < 0.05$). On day 6, growth inhibition in cells treated with retinoids ranged from 24% (acitretin) to 45% (ATRA) as shown in Fig. 1b. The inhibition effects of these drugs could not be due to toxicity, as cell viability was more than 90%. Despite the observed inhibition on proliferation, no significant changes in cell morphology or pigmentation were observed in the cells treated with all retinoids tested in this study (data not shown).

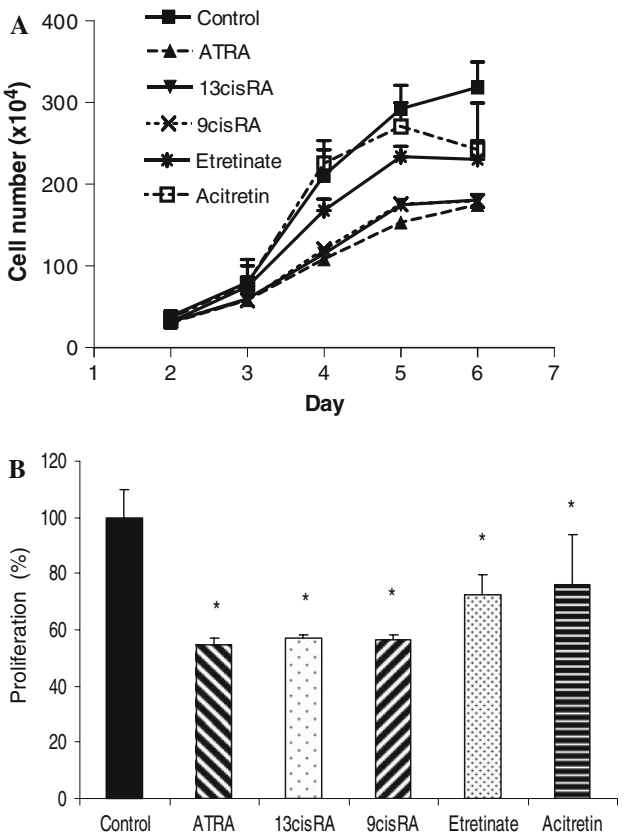

Fig. 1 **a** Growth curves of B16-F10 cells in culture medium and **b** Cell proliferation on day 6 with and without retinoids treatment. Each value was the mean obtained from at least three experiments. Each experiment had three replicates. *Error bars* represent SD ($n = 9$). *$P < 0.001$ significantly different from the value of the control

Combination chemotherapy studies

The melanoma B16-F10 cells were treated with 1 μM of the respective retinoids for 6 days, and then examined for sensitivity to CDDP and 5-FU using FDA assay. Treatment of B16-F10 cells with different concentrations of CDDP (0.5–16 μg/ml) for 72 h led to a marked dose-dependent cytotoxicity. The cells pretreated with retinoids showed different sensitivities to CDDP. The IC_{50} values of CDDP in B16-F10 cells with or without pretreatment of retinoids are shown in Table 1. Comparison of CDDP sensitivities with and without addition of retinoids shows that CDDP was more cytotoxic when combined with ATRA, 9-*cis*-RA or 13-*cis*-RA by at least 1.50-fold, as seen by comparing the IC_{50}s ($P < 0.05$, Table 1). However, combined treatment of CDDP and etretinate or acitretin did not produce a statistically significant reduction in the IC_{50} value of CDDP compared to the treatment of CDDP alone ($P > 0.05$).

Table 1 Comparison of IC_{50} after exposing control or different retinoids pretreated B16-F10 melanoma cells to CDDP and 5-FU

Drug	Pretreatment	IC_{50} value (μg/ml)	Potentiation[a]
CDDP	Control	5.59 ± 1.20	NA[b]
	ATRA	2.52 ± 0.35*	2.33
	9-*cis*-RA	3.07 ± 0.42*	1.91
	13-*cis*-RA	3.93 ± 0.38*	1.50
	Etretinate	3.97 ± 0.53	1.41
	Acitretin	4.02 ± 0.75	1.39
5-FU	Control	0.46 ± 0.14	NA[b]
	ATRA	0.26 ± 0.03*	1.79
	9-*cis*-RA	0.41 ± 0.07	1.12
	13-*cis*-RA	0.40 ± 0.13	1.15
	Etretinate	0.34 ± 0.06	1.35
	Acitretin	0.37 ± 0.18	1.24

[a] Potentiation was expressed as the ratio of the IC_{50} values of the control and pretreated cells. The IC_{50} values given in the table represent the average of at least three independent experiments

[b] Not applicable

* IC_{50} was statistically lower than control ($P < 0.05$)

In contrast to CDDP, the cells pretreated with all retinoids other than ATRA tested in this study did not show significant changes in sensitivity to 5-FU. The IC_{50} values of 5-FU in cells with or without pretreatment of retinoids are listed in Table 1. Since combination of most retinoids with 5-FU did not show much advantage in the in vitro assays, the in vivo efficacy studies were only conducted to examine the combined effects of CDDP and retinoids.

Effects of retinoids on the invasion of B16-F10 cells

The inhibition of retinoids on melanoma invasion was investigated through the Transwell assay. The invasive potential of B16-F10 cells with or without retinoid pretreatment was determined on the basis of the cell's ability to invade a matrigel containing the major components of the basement membrane. Untreated or pretreated cells demonstrated different invasion capacity through matrigel-coated inserts. Data are expressed as cell number/field ± standard deviation. Except acitretin, all other retinoids induced significant decrease in matrigel invasion of B16-F10 cells (Fig. 2). The inhibition of matrigel invasion was 43, 33, 40, and 22% for ATRA, 9-*cis*-RA, 13-*cis*-RA, and etretinate, respectively, in comparison to the control group.

Effects of retinoids on experimental lung metastasis in mice

The retinoids showing in vitro inhibition effect on invasion of B16-F10 cells were tested in vivo using experimental lung metastasis model. All mice received either saline (in control group) or retinoids continuously for 10 days from the time of inoculation of B16-F10 cells. Administration of saline, 13-*cis*-RA, and 9-*cis*-RA did not show any adverse effect on the growth of mice during the study. There were no significant differences in body weight gained among the groups throughout the study. However, administration of

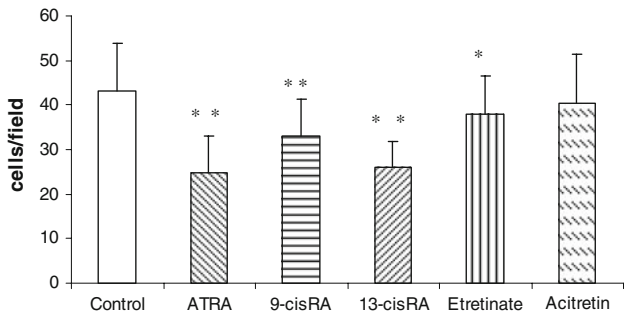

Fig. 2 Effects of retinoids on the invasive ability of B16-F10 cells in the in vitro matrigel assay. Data were expressed as means ± SD of triplicate experiments. $*P < 0.05$. $**P < 0.01$ (compared to the control)

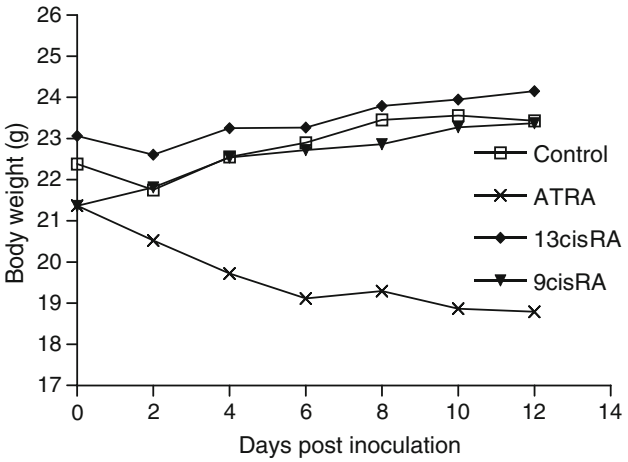

Fig. 3 Body weight changes of mice after inoculation of B16-F10 cells (i.v. injection of 10^5 cells) and treatment with the respective retinoids (20 mg/kg/day for 10 days)

ATRA resulted in a drop of body weight as shown in Fig. 3, indicating the toxic effect of ATRA.

The ability of retinoids to inhibit experimental lung metastasis after intravenous injection of B16-F10 cells in mice was investigated. The incidence and the numbers of metastatic lung tumors are indicated in Table 2. In control mice, 12.1 ± 4.9 colonies of metastasis foci were observed per lung at 14 days after inoculation with B16-F10 cells. Treatment of ATRA and 13-*cis*-RA significantly reduced the overall number of gross lung metastasis and mean number of metastases per lung (Table 2). Despite that 9-*cis*-RA caused lower number of metastatic lung tumors per mouse when compared to the control (8.3 ± 1.9 versus 12.1 ± 4.9), the difference did not reach the level of statistical significance ($P > 0.05$). The relative decrease in the mean number of metastases in the ATRA-treated mice was greater than that in the 13-*cis*-RA or 9-*cis*-RA-treated mice, perhaps reflecting the higher potent inhibition effect of ATRA. The incidence rates of lung metastasis in the control group and the group treated with 13-*cis*-RA or 9-*cis*-RA, were all 100%. However, the frequency of lung metastasis in the group treated with ATRA was 90%.

Effects of retinoids and cisplatin combination on tumor development in mice injected with B16-F10 melanoma cells

ATRA and 13-*cis*-RA showed more potent effects than other retinoids on combination with cisplatin in vitro and on lung metastasis in vivo. These two retinoinds were, therefore, chosen for in vivo test in combination with cisplatin. ATRA (20 mg/kg), 13-*cis*-RA (20 mg/kg), and cisplatin (10 mg/kg), either alone or in combination, were administered to the mice according to the regimens

Table 2 Effects of different retinoids on lung metastasis of tumor bearing mice

Group no.	Treatment	Number of mice examined	Incidence of lung metastasis	Number of metastatic lung tumors/mouse
1	None	10	100% (10/10)	12.1 ± 4.9
2	ATRA, 20 mg/kg	10	90% (9/10)	3.7 ± 1.8[a]
3	13-*cis*-RA, 20 mg/kg	10	100% (10/10)	4.6 ± 2.8[a]
4	9-*cis*-RA, 20 mg/kg	6	100% (6/6)	8.3 ± 1.9

[a] Significantly different from group 1 by one way ANOVA with Dunnett's test ($P < 0.001$)

Table 3 Effects of 13cisRA, cisplatin, either alone or in combination, on melanoma in C57BL/6 J mice

Treatment	No of mice	Tumor size (mm^3)	Metastatic incidence (%)	No. of gross metastasis
Control	6	1,349 ± 712	4/6 (67)	13
13-*cis*-RA	6	1,406 ± 635	1/6 (17)	1
Cisplatin	4	970 ± 431	2/4 (50)	5
Cisplatin + 13-*cis*-RA	5	559 ± 312[a]	1/5 (20)	1

[a] Significantly different from control group by ANOVA with Dunnett's test ($P < 0.05$)

specified in the paragraph under Materials and methods. The combination of cisplatin and ATRA resulted in death of all mice in the group within 14 days of treatment (that group was then excluded from the study), while the combination of cisplatin and 13-*cis*-RA resulted in only one death out of the six mice in that group after 21 days of treatment.

The antitumor effect as judged from the reduction in the tumor size of the combined treatments of 13-*cis*-RA and cisplatin (559 ± 312 mm^3) was higher than that of the control and single-drug treatments (1,349 ± 712, 1,406 ± 635, and 970 ± 431 mm^3, respectively, for the control and 13-cis-RA and cisplatin alone) (Table 3). When 13-*cis*-RA was used alone, it did not show any antitumor effect as compared to the control group. However, the number of pulmonary metastases was significantly decreased by the treatment of 13-*cis*-RA alone, and 13-*cis*-RA combined with cisplatin. Cisplatin alone did not show any inhibitory effect on lung metastasis (Table 3).

Discussion

In this study, we demonstrated that all the tested retinoids (ATRA, 13-*cis*-RA, 9-*cis*-RA, etretinate, acitretin) inhibited B16-F10 cell proliferation in vitro. However, only ATRA and 13-*cis*-RA showed potent inhibition effects on cell invasion in both in vitro matrigel assay and in vivo lung metastasis model. Meanwhile, combination of 13-*cis*-RA and cisplatin treatment showed inhibition effects on tumor growth and metastasis in mouse model with moderate toxicity.

Retinoids exhibit a host of interesting effects on the growth and differentiation of normal, preneoplastic, and neoplastic cells in vitro and in vivo and thus have gained attention in cancer prevention and treatment. Inhibitory effects have been reported in a broad spectrum of different epithelial and mesenchymal tumor cell types and also in mouse and human melanoma cells as reviewed by Lotan [27]. Mechanistically, inhibition has been attributed to cytostasis rather than cytotoxiciy since viability is maintained and growth arrest is reversible [28]. These reports were consistent with our findings in that cell variability could be maintained at more than 90%, despite reduction in proliferation was observed after the 6-day treatment with retinoids.

Several investigators reported that retinoids inhibited the invasion of tumor cells. Wood et al. [15] noted that retinoic acid inhibited the invasiveness of melanoma cells by the down-regulation of gelatinases, tPA, and a motility factor receptor, and up-regulation of laminin receptor. Jacob et al. [29] reported that ATRA inhibited proliferation and chemotaxis in all tested human melanoma cell lines, but invasion was specifically inhibited in the cell lines derived from metastases. Studies of tumor cell invasion into basement membrane have shown that DNA synthesis and cell proliferation are not required, whereas protein synthesis and proteolysis play important roles [30]. These observations suggest that different intrinsic factors in tumor cells mediate the effects of retinoids on proliferation and invasion. It may be the reason why etretinate and acitretin showed significant antiproliferative effects on B16-F10 cells, but showed little anti-invasive effects in this study.

The present study provides the first experimental evidence that both ATRA, and13-*cis*-RA suppressed experimental metastasis of B16-F10 melanoma cells in mice, as revealed by the smaller number of metastatic foci found in treated mice compared with that in untreated mice. The animal model used in this study was developed by direct injection of B16-F10 cells into the bloodstream via the tail vein, which eliminates intravasation but is able

to measure the ability of malignant cells to extravasate into the lungs. The results suggest that retinoids may also inhibit extravasation of malignant cells except the inhibition effect of cell invasion. It should be noted that ATRA, while not 13-*cis*-RA, induced substantial weight loss in tumor-bearing mice (Fig. 3), although ATRA treatment was more effective in inhibiting tumor metastasis (Table 3). This may suggest 13-*cis*-RA, with good inhibition ability and moderate toxicity would be the more likely candidate for use in future clinical trials.

It was reported that retinoid treatment could produce growth inhibition, but allow continuing DNA synthesis [20], which provided a rationale for examining combination therapy with standard cytotoxic agents. Sacks et al. [20] reported the synergistic effect of ATRA and CDDP or 5-FU on cell proliferation of squamous cell carcinoma [20]. However, the combination effects on melanoma cells have not been explored so far. In contrast to the previous report, the results in this study indicated that increased inhibition effects on melanoma cells was only observed for combination of retinoids with CDDP, but not with 5-FU. This suggests that the mechanism for synergism is complex and not just related to the rates or levels of DNA synthesis itself. CDDP and 5-FU are cytotoxic through different mechanisms, but both require DNA synthesis. Further studies on molecular mechanisms by which retinoids enhance effects of cytotoxic agents will allow for selecting more potent and less toxic combination therapies.

Despite the limited efficacy of systemic chemotherapy in the treatment of metastatic melanoma, it remains the gold standard of treatment for patients for whom radical surgery is unsuitable. Unfortunately, the progress in the chemotherapy for metastatic melanoma in the last 20 or 30 years is very limited [31]. Currently, there is no effective therapy to impact overall survival in the management of disseminated melanoma. Findings in our study indicated that combination of 13-*cis*-RA and CDDP could suppress the primary tumor growth and lung metastasis of melanoma cells with moderate toxicity. Thus, this combination could be a potentially effective regimen for the treatment of metastatic malignant melanoma in future clinical trials.

References

1. Fidler IJ (1990) Critical factors in the biology of human cancer metastasis: Twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 50:6130–6138
2. Mareel MM, Bracke ME, Storme GA (1985) Mechanisms of tumor spread: a brief overview. In: Grundmann E (ed) The cancer patient: illness and recovery. Gustav Fischer Verlag, Stuttgart, pp 59–64
3. McKinnell RG (1998) Metastasis. In: McKinnell RG, Parchment RE, Perantoni AO, Pierce GB (eds) The biological basis of cancer. Cambridge University Press, Cambridge, pp 50–78
4. Stetler Stevenson WG, Liotta LA, Kleiner DE (1994) Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J 7:1434–1441
5. Filder IJ (1978) General considerations for studies of experimental cancer metastasis. Cancer Res 15:399–439
6. Takenaga M, Igarashi R, Nakayama T, Mizushima Y (1999) Lecithinized ascorbic acid (PC-AS) effectively inhibits murine pulmonary metastasis. Anticancer Res 19:1085–1091
7. Leyon PV, Lini CC, Kuttan G (2005) Inhibitory effect of Boerhaavia diffusa on experimental metastasis by B16–F10 melanoma in C57BL/6 mice. Life Sci 76:71339–71349
8. Gava B, Zorzet S, Spessotto P, Cocchietto M, Sava G (2006) Inhibition of B16 melanoma metastases with the ruthenium complex imidazolium trans-imidazoledimethylsulfoxide-tetrachlororuthenate and down-regulation of tumor cell invasion. J Pharmacol Exp Ther 317:284–291
9. Niles RM (2000) Recent advances in the use of vitamin A (retinoids) in the prevention and treatment of cancer. Nutrition 16:1084–1089
10. Bertram JS, Vine AL (2005) Cancer prevention by retinoids and carotenoids: independent action on a common target. Biochim Biophys Acta 1740:170–178
11. Pasquali D, Rossi V, Bellastella G, Bellastella A, Sinisi AA (2006) Natural and synthetic retinoids in prostate cancer. Curr Pharm Des 12:1923–1929
12. Siegrist W, Hintermann E, Roggo CN, Apfel CM, Klaus M, Eberle AN (1998) Melanoma cell growth inhibition and melanocortin receptor downregulation induced by selective and non-selective retinoids. Melanoma Res 8:113–122
13. Ohashi E, Hong SH, Takahashi T, Nakagawa T, Mochizuki M, Nishimura R, Sasak N (2001) Effect of retinoids on growth inhibition of two canine malnoma cell lines. J Vet Med Sci 63:83–86
14. Emionite L, Galmozzi F, Raffo P, Vergani L, Toma S (2003) Retinoids and malignant melanoma: a pathway of proliferation inhibition on SK MEL28 cell line. Anticancer Res 23:9–13
15. Wood WR, Seftor EA, Lotan T, Nakajama M, Misiorowski RL, Seftor REB, Lotan R, Hendrix MJC (1990) Retinoic acid inhibits human melanoma tumor cell invasion. Anticancer Res 10:423–432
16. Helige C, Smolle J, Zelinig G, Hartmann E, Fink-Puches R, Kerl H, Tritthart HA (1993) Inhibition of K1735–M2 melanoma cell invasion in vitro by retinoic acid. Clin Exp Metastasis 11:409–418
17. Borden P, Heller RA (1997) Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Expr 7:159–178
18. Edward M, Gold JA, MacKie RM (1989) Modulation of melanoma cell adhesion to basement membrane components by retinoic acid. J Cell Sci 93:155–161
19. Ortiz MA, Bayon Y, Lopez-Hernandez FJ, Piedrafita FJ (2002) Retinoids in combination therapies for the treatment of cancer: mechanisms and perspectives. Drug Resist Update 5:162–175
20. Sacks PG, Harris D, Chou TC (1995) Modulation of growth and proliferation in squamous cell carcinoma by retinoic acid: a rationale for combination therapy with chemotherapeutic agents. Int J Cancer 61:409–415
21. Recchia F, Lalli A, Lombardo M, De Filippis S, Saggio G, Fabbri F, Rosselli M, Capomolla E, Rea S (2001) Ifosfamide, cisplatin, and 13-*cis*-retinoic acid for patients with advanced or recurrent squamous cell carcinoma of the head and neck: a phase I-II study. Cancer 92:814–821
22. Recchia F, De Filippis S, Pompili PL, Rosselli M, Saggio G, Ciorra A, Piccinini M, Rea S (1999) Carboplatin, vindesine, 5-fluorouracil-leucovorin and 13-cis-retinoic acid in the treatment of advanced non-small cell lung cancer. A phase II study. Clin Ther 150:269–274

23. Shin DM, Glisson BS, Khuri FR, Clifford JL, Clayman G, Benner SE, Forastiere AA, Ginsberg L, Liu D, Lee JJ, Myers J, Goepfert H, Lotan R, Hong WK, Lippman SM (2002) Phase II and biologic study of interferon alfa, retinoic acid, and cisplatin advanced squamous skin cancer. J Clin Oncol 20:364–370
24. Shin DM, Khuri FR, Murphy B, Garden AS, Clayman G, Francisco M, Liu D, Glisson BS, Glinsberg L, Papadimitrakopoulou V, Myers J, Morrison W, Gillenwater A, Ang KK, Lippman SM, Goepfert H, Hong WK (2001) Combined interferon-α, 13-cis-retinoic acid, and α-tocopherol in locally advanced head and neck squamous cell carcinoma: novel bioadjuvant phase II trial. J Clin Oncol 19:3010–3017
25. Csoka K, Tholander B, Gerdin E, de la Torre M, Larsson R, Nygren P (1997) In vitro determination of cytotoxic drug response in ovarian carcinoma using the fluorometric microculture cytotoxicity assay (FMCA). Int J Cancer 72:1008–1012
26. Melchiori A, Allavena G, Bohm J, Remy W, Schmidt J et al (1987) Interferons inhibit chemotaxis of transformed cells and their invasion of a reconstituted basement membrane. Anticancer Res 7:475–479
27. Lotan R (1991) Retinoids as modulators of tumor cell invasion and metastasis. Sem Cancer Biol 2:197–208
28. Jeten AM (1984) Modulation of cell growth by retinoids and their potential mechanisms of action. Fed Proc 43:134–139
29. Jacob K, Wach F, Holzapfel U, Hein R, Lengyel E, Buettner R, Bosserhoff AK (1995) In vitro modulation of human melanoma cell invasion and proliferation by all-trans-retinoic acid. Melanoma Res 8:211–219
30. Torgeirsson UP, Lindsay CK, Cottam DW, Gomez DE (1994) Tumor invasion, proteolysis, and angiogenesis. J Neurooncol 18:89–103
31. Mandara M, Nortilli R, Sava T, Cetto GL (2006) Chemotherapy for metastatic melanoma. Expert Rev Anticancer Ther 6:121–130