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All-Trans-Retinoic Acid and the Glycolipid α -Galactosylceramide Combined Reduce Breast Tumor Growth and Lung Metastasis in a 4T1 Murine Breast Tumor Model

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Abstract

Vitamin A compounds are promising for cancer prevention and reducing risk of recurrence. Herein we have evaluated the combination of all-trans-retinoic acid (RA), a vitamin A metabolite, and alpha-galactosylceramide (α GalCer), a lipid immune activator, in Balb/C mice inoculated with syngeneic 4T1 breast tumor cells on reduction in breast tumor growth and lung metastasis. In Balb/c inoculated with the syngenic 4T1 primary tumor, and administered dendritic cells treated with RA + α GalCer, the size of the primary tumor and the number of lung metastatic foci were reduced. When 4T1 cells were introduced into the circulation as a model of hematogenous spread of tumor cells and RA and α GalCer were administered directly to mice without dendritic cells, lung metastatic foci were reduced 70% ($P < 0.05$), whereas each agent alone resulted in an intermediate decrease. Concomitantly, the expression of matrix metalloproteinases (MMP), membrane type-1 (MT1)-MMP and MMP3, were reduced by RA + α GalCer in lung. MMP3 protein was also reduced in plasma and culture supernatants from RA + α GalCer-treated 4T1 cells. Together, our results provide new evidence that a nutritional-immunological combination of RA + α GalCer may be promising for preventing or slowing the growth of metastatic foci, and suggest reduced MMP production as a possible mechanism.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer-related deaths in women (1). Many bioactive compounds acquired from the diet or natural sources have received increasing attention for the development of novel agents to prevent the occurrence and progression of cancer. Among such candidates, some agents not only target the cancer cells, but also induce the immune system to greater surveillance and elimination of transformed cells. A combination of treatments may produce additive or synergistic effects, and thus strategies using multiple nutritional or nutrient–drug combinations may prove fruitful for preventing and/or reducing the spread of cancer cells.

All-trans-retinoic acid (RA), an active metabolite of vitamin A, is well-known for its regulatory roles in cell growth and differentiation (2). Numerous animal and human studies

have reported that RA is important in maintaining an efficient immune response (3, 4), and a few clinical studies have also suggested that RA–vitamin A supplementation may be beneficial in immune disorders and cancer (5–8).

α -Galactosylceramide (α GalCer) is a glycolipid that has become the prototype of glycolipid antigens used in experimental studies and clinical trials. α GalCer has entered Phase I/II clinical trials (9, 10). α GalCer is known to stimulate a subset of T cells bearing characteristics of natural killer (NK) cells, referred to as invariant natural killer T (iNKT) cells, through the presentation of α GalCer by a Major Histocompatibility Complex (MHC)-like receptor CD1d (11, 12). The dependence of the antitumor effect of α GalCer on iNKT cells was demonstrated in studies that showed no protective antitumor activity in $Ja18^{-/-}$ mice, which lack iNKT cells (13–16), whereas iNKT mice, which have only iNKT cells and no conventional T cells and NK cells, showed antitumor activity equivalent to wild-type (WT) mice (17). As numerous studies have provided evidence that α GalCer plays a role in the suppression of tumor growth and metastasis, it has become an interesting component of immune therapy (10, 18–22). Furthermore, injection of α GalCer-loaded dendritic cells (DCs) has elicited promising effects in inhibition of tumor growth and metastasis, both experimentally and in clinical trials (23–25), suggesting that the activation of DCs may play an important role the downstream immune responses.

Based on previous observations that RA can markedly increase CD1d gene expression (26, 27), we hypothesized that RA might be effective for enhancing the antitumor activity of the CD1d ligand α GalCer. In the current study, we have used as our model the 4T1 tumor, originally derived from a spontaneously arising Balb/c mammary tumor (28), which has been well characterized and shown to have several advantages, including that the tumor cells are easily transplanted into the mammary gland and multiple distant metastatic foci develop spontaneously from the primary tumor via hematogenous or lymphatic routes, similar to that of human breast cancer. In addition, 4T1 cells are resistant to growth inhibition by 6-thioguanine, which makes it possible to determine the presence of metastatic cells by ex vivo culturing lung homogenates in the presence of this compound, even if the tumor foci are too small to be counted microscopically (28). Using different approaches, we have tested the combination of RA and α GalCer for their ability to reduced 4T1 breast tumor growth and lung metastasis.

MATERIALS AND METHODS

Cell Culture and Reagents

4T1 cells were kindly provided by Dr. Danny Welch (University of Kansas Cancer Center) and maintained in RPMI 1640 supplemented with 10% fetal calf serum and 2×10^{-5} M β -mercaptoethanol to facilitate the cell growth in vitro (Invitrogen, Grand Island, NY).

Splenic DCs were isolated from control Balb/c mice using a dendritic cell isolation kit according to the manufacturer's instruction (Stemcell Technology, Vancouver, Canada); cell purity was up to 87%. DCs were treated ex vivo with RA (20 nM, from Sigma-Aldrich, St. Louis, MO) for 16 h and then treated again with RA, plus TNF α (5 ng/ml, R&D Systems, Minneapolis, MN), an agent used to promote DC maturation (29), and α GalCer (100 nM,

Enzo Life Sciences, Farmingdale, NY) for 2 h, then washed twice with PBS (Invitrogen). We immediately injected 1×10^5 cells per mouse via the retro-orbital sinus.

Animals and Experimental Design

Protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. Eight-wk-old female Balb/c mice (Charles River, Wilmington, MA) were housed in plastic air-filtered cages with free access to water and standard chew diet.

For the orthotopic model, mice were inoculated with 2×10^4 4T1 cells in 100 μ l PBS directly into the inguinal mammary fat pad. RA and α GalCer-pulsed DC were injected retro-orbitally on the same days. Mice were closely monitored and once the tumors reached between 0.5–1.0 cm, about 28 days after 4T1 cell inoculation, all of the mice were sequentially euthanized with carbon dioxide, tumor nodules were dissected and weighed, lungs were subjected to histology and micrometastasis assay, and spleens were collected, weighed, and used for flow cytometry.

In the hematogenous model, 2×10^4 4T1 cells in 100 μ l PBS were injected directly into the circulation to model the spread of tumor cells (30). Twenty-four h later, mice were randomized to receive control (vehicle as Canola oil), RA, α GalCer, or both. All-trans-RA, 37.5 μ g per mouse in Canola oil, was given orally once a day for 10 days. α GalCer, 2 μ g per mouse, was given subcutaneously every other day.

Micrometastasis Assay

As adapted from a previous report (31), lung tissues (25 mg from each individual sample) were minced and digested with 10 mg/ml of collagenase type IV and 10 U/ml of elastase (Worthington Biochemical Corporation, Lakewood, NJ), for 30 min, and a single-cell suspension was prepared by passing the sample through a nylon mesh. Cells were then washed and serially diluted in culture medium containing 60 μ M of 6-thioguanine (Sigma-Aldrich, St. Louis, MO) (28), and cultured for 7 to 10 days in 6-well plates. Colonies were washed with PBS, fixed with methanol, and then stained with 0.1% crystal violet to facilitate the counting of colonies. After counting, the dye was dissolved in 10% acetic acid and the absorbance at 570 nm was measured as an integrated measure of colony size and number.

Flow Cytometric Analysis

Splenocytes were prepared by Histopaque (Sigma-Aldrich, St. Louis, MO) centrifugation and analyzed for cell populations by staining with fluorescently tagged monoclonal antibodies: CD19-PEcy7, CD3-FITC, CD4-PerCP, CD8-APC, NK1.1-PEcy7, CD11c-PEcy7, F4/80-APC, Gr-1-FITC, and CD11b-PE, all from BD Biosciences (San Diego, CA). Five $\times 10^5$ cells were incubated with 0.1 μ g of respective antibody or combination for 1 hour at room temperature (32). The channel selection was optimal for each fluorochrome measured using an Accuri C6 flow cytometer (Accuri, BD Biosciences, San Jose, CA). Appropriate isotype controls were used as negative controls and single-stained samples were used to set the proper compensation.

Cell Proliferation Assay

4T1 cells were plated in 96-well plates, 2×10^4 cells/well/200 μ l, and treated with or without RA (20 nM) or α GalCer (100 ng/ml) for 24 h. [3 H]-Thymidine (PerkinElmer, Inc, San Jose, CA) was added for the last 4 h (0.1 μ Ci/50 μ l/well) for determination of [3 H]-thymidine incorporation as a measure of cell proliferation (32).

Cell Cycle Analysis

4T1 cells were plated in 48-well plates, 1×10^5 cells/well/0.5 ml, and treated as above with or without RA, or α GalCer, for 24 h and then harvested and subjected to propidium iodide staining and cell cycle analysis (32).

Motility Assay

Five $\times 10^4$ 4T1 cells were plated in serum-free RPMI 1640 medium in the top chamber of a transwell plate (Corning, NY), separated from the bottom by a polycarbonate membrane with 8- μ m pores. Medium with 10% FBS containing RA (20 nM), α GalCer (100 ng/ml), or both was placed in the bottom chamber. After a 24-h culture, nonmigrated 4T1 cells were removed with a cotton swab from the upper surface of the membrane. Cells that migrated through and adhered to the lower surface were stained with 0.1% crystal violet and rinsed with deionized water. The dye was eluted with 10% acetic acid and quantified by spectrophotometer as described above.

Quantitative Real-Time PCR (qPCR)

Lung tissues, or 4T1 cells cultured for 24 h, as above, were used to prepare total RNA (Qiagen, Valencia, CA). A SYBR green master mixture (Biorad, Hercules, CA) was used for the quantification of PCR products, and quantified relative to amplification of 18S RNA (27). Primer sequences are listed in Table 1.

ELISA of Matrix Metalloproteinases (MMP) 3

MMP3 protein was measured in mouse plasma samples (1:20 dilution) and 4T1 cell culture medium (without dilution) using an ELISA kit for mouse MMP3 (Abcam, Cambridge, MA) according to manufacturer's instruction.

Statistic Analysis

Results are expressed as the mean $\pm$ SEM. Data was analyzed by *t*-test for 2 groups or analysis of variance followed by Tukey's post-hoc test, using Prism software (Graphpad Software, La Jolla, CA), unless specified in legends. $P < 0.05$ was considered statistically significant.

RESULTS

RA and α GalCer-Treated Syngeneic DC Reduced the 4T1 Cell Growth and Metastasis in a Mouse Orthotopic Breast Tumor Model

It has been reported that α GalCer-loaded DCs can inhibit the growth of several different tumors (23–25, 33). We hypothesized that α GalCer may have a similar effect on inhibiting

the growth of breast tumor cells, and RA may augment the effect of α GalCer-stimulated DCs. In the present study, isolated DC were treated ex vivo with a physiological concentration of RA (20 nM) for 16 h and then loaded with α GalCer (100 ng/ml) for 2 h. The DCs were intravenously injected into Balb/c mice at the same time that 4T1 cells were inoculated into the inguinal mammary gland.

Tumors were palpable at the injection sites 2 wk after injection. By 4 wk, tumors in some mice had grown to ~1 cm and all mice were then euthanized. The 4T1 cells formed a solid tumor mass near the injection site, which often consisted of one or more encapsulated well-defined nodules, without apparent local invasion. Whereas untreated DCs or DCs pretreated with RA or α GalCer alone did not result in a significant change (data not shown), mice treated with DC pretreated with RA + α GalCer had a tendency to bear smaller primary tumor nodules, as shown in Fig. 1A ($P=0.055$).

To test whether injection of DCs also affects the long-distance metastasis of tumors, we processed the lung and performed a colony formation assay. Mice treated with RA + α GalCer-treated DCs tended to have fewer metastatic foci ($P=0.051$, Fig. 1B). Representative histology images (Fig. 1C), showed smaller and fewer metastatic foci, and more normal parenchyma tissue, in the lungs of DC-treated mice than in mice that did not receive DC.

4T1 Cell Inoculation Altered Spleen Size and Cell Population

We also observed that the spleen was markedly enlarged in tumor-bearing mice compared to mice without 4T1 cell injection (Fig. 2A), whereas spleen weight was decreased in mice treated with DCs. The weight of the spleen correlated well with the weight of the primary tumor (Fig. 2B, $P<0.0001$), suggesting a chronic response of the immune system to the growth of the tumor cells (34, 35).

Flow cytometric analysis revealed that the spleen enlargement was due to an alteration of cellular components. CD11b⁺ cells were dramatically increased (Fig. 2C), mainly because of an increase in Gr-1⁺ granulocytes, whereas, conversely, T and B lymphocytes, which normally comprise the major cell populations of the spleen and contribute over 80% of the total cells, were decreased in every subpopulation, especially the B cell subset (Fig. 2D). The percentage of CD11c⁺ dendritic cells was also reduced in 4T1-bearing mice.

RA and α GalCer Decreased 4T1 Cell Metastasis to the Lungs After Intravenous Injection of 4T1 Cells

We next investigated whether RA and α GalCer alter tumor cell metastasis in mice without treatment with DCs. In Experiment 2, mice were inoculated with 4T1 cells directly into the blood stream as a model of the hematogenous spread of tumor cells, and then treated in vivo with multiple doses of RA and/or α GalCer. After 14 days, 4T1 cells in lung tissue were evaluated. With this procedure, the weight of the spleen did not increase nearly as greatly as in the previous experiment (data not shown), which likely reflects the shorter time of this experiment because the number of tumor cell inoculated was the same for both this and our first experiment. However, spleen cell populations were still altered, similar to Experiment 1, with decreased lymphocytes and increased CD11b⁺ cells, most of which were Gr-1⁺ cells

(Fig. 3A and 3B). Treatment with RA and/or α GalCer did not alter the population changes that were caused by the 4T1 tumor cells.

Either RA or α GalCer alone reduced the rate of lung metastasis nonsignificantly, determined both by counting the lung tissue colony number and staining the colonies. However, RA + α GalCer combined decreased lung metastasis significantly ($P < 0.05$, Fig. 3C and 3D).

CD1d Deficiency Attenuated the Effect of RA and α GalCer In Vivo

To further study the mechanisms of RA and α GalCer in the prevention of tumor metastasis, we employed CD1d null mice to determine whether the effect of RA + α GalCer in reducing tumor metastatic growth is CD1d dependent. 4T1 cells were inoculated intravenously in both WT and CD1d-null strains and the lung metastasis rate was determined 14 days later, as in the previous study. After treatment with RA + α GalCer, tumor metastases were lower in WT mice, but not in CD1d-null mice (Fig. 4A).

We then determined if the splenic cellular response observed previously was altered by CD1d deficiency. Both WT and CD1d null mice exhibited a similar marked increase in CD11b⁺ cells and, to a lesser degree, a decrease in lymphocytes, especially B cells. However, those CD1d-null mice treated with RA + α GalCer had higher percentages of CD11b/Gr-1⁺ cells and lower B cells, suggesting that CD1d is also important for limiting the magnitude of CD1b/Gr-1⁺ cell expansion (Fig. 4B and 4C).

RA and α GalCer Decreased the Level of Expression of MMPs in Lung Tissues

It is known that MMPs, a family of zinc-dependent endopeptidases capable of degrading the extracellular matrix, play important roles in tumor invasion and metastasis (36, 37). We first screened the gene expression levels of several MMPs in lung tissue by quantitative real-time RT-PCR (Table 1). Among the several MMPs reported in the literature, the inoculation of mice with 4T1 cells greatly increased the gene expression levels of membrane-type 1 MMP (MT1-MMP) and MMP3 (Fig. 5A). We then assessed MT1-MMP and MMP3 mRNA levels in the lungs of mice with and without treatment with RA and α GalCer. Mice treated with RA and α GalCer showed significantly decreased levels of both MT1-MMP and MMP3 mRNA in lung. To further examine MMP expression, MMP3 protein was measured in plasma. As shown in Fig. 5B, the level of MMP3 in plasma was marginally increased in 4T1 tumor-bearing mice, although it was reduced by treatment with RA + α GalCer. MMP3 protein in 4T1 cell culture supernatant was also lower in mice treated with either RA or α GalCer alone, as well as both in combination (Fig. 5C). These data suggest that inhibition of MMP production by 4T1 tumors may be one of the mechanisms by which RA and α GalCer can reduce the rate of lung tumor metastatic growth.

RA and α GalCer Did Not Alter 4T1 Cell Growth or Migration In Vitro

To determine if RA and α GalCer have a direct effect on 4T1 cells, several properties were tested. In a 24-h assay, cell proliferation did not differ between each treatment and control cells (Fig. 6A). Treatment also did not affect the distribution of cell cycle stages (Fig. 6B). Cell migration in response to RA and α GalCer, tested using a 16-h transwell culture assay, also did not show difference between each treatment and control cells (Fig. 6C).

RT-PCR analysis was used to determine the expression levels of granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF) in 4T1 cells after RA and/or α GalCer treatment in vitro. RA significantly upregulated GM-CSF gene expression of the 4T1 cells (Fig. 6D); however, α GalCer had no effect on the cytokine expression. G-CSF and M-CSF expression were not affected by either RA or α GalCer (data not shown).

We also surveyed the gene expression of several chemokines that have been related to tumor progression, such as C-X-C motif ligand 1 (CXCL1), C-C motif ligand 2 (CCL2), C-C motif ligand 3 (CCL3), C-C motif ligand 4 (CCL4), C-C motif ligand 5 (CCL5), and Kruppel-like factor (KLF). Although each gene was expressed at a different abundance (Table 1), none was affected by the presence of RA and α GalCer, nor were MT1-MMP and MMP3 mRNA (data not shown).

DISCUSSION

In the current study, we used a 4T1 mammary carcinoma model to investigate the potential preventive effects of RA and α GalCer on breast cancer growth and metastasis. In the first model in which 4T1 cells were injected into the mammary fat pads of the mice, the administration of syngeneic DCs pulsed with RA + α GalCer tended to suppress the solid tumor growth, although the reduction was only marginally statistically significant. Because neither RA nor α GalCer had observable effects on 4T1 cell growth in vitro, the reduced tumor growth and lung metastasis is likely to be due to immune stimulation, or other effects mediated by the response of the animals. Because the spread of tumors to the lungs seemed to have been prevented or tumor growth reduced, we then tested the effect of RA and α GalCer when given directly to mice inoculated with 4T1 cells intravenously. In this way, the number of cells introduced could be kept equal among groups, and the effect of RA and α GalCer on lung tumor growth in vivo could be studied directly. This study showed that RA + α GalCer is an effective combination in preventing or reducing the development of micrometastasis in the lungs. CD1d, a receptor for α GalCer (38), was shown to be required, as the effect of RA + α GalCer compared to control treatment that was observed in WT mice was not observed in CD1d-null mice.

It is known that both RA and α GalCer can act on the immune system. RA has a broad range of activities in the regulation of immune competent cells, such as activation of NK cells, inducing differentiation of monocytes, B and T cells. The effects of α GalCer are believed to be mainly due to the activation of NKT cells that can play a crucial role in antitumor responses (12, 33, 39). Moreover, the ability of RA to regulate CD1d gene expression on antigen-presenting cells (26, 27) may be another factor in our results, where the combination of RA + α GalCer was more effective than either agent alone in preventing the growth of lung metastases.

It is reported that the growth of 4T1 tumor can cause a leukemoid reaction, apparent as granulocytosis in which Gr-1⁺ cells are greatly increased in peripheral blood and spleen, accompanied by granulopoiesis in the bone marrow (40, 41). Moreover, the dim staining of the Gr-1 marker suggested most of the increased number of Gr-1⁺ cells were immature

granulocytes (41). We observed a similar reaction in both of the models we tested. In the orthotopic model (experiment 1), which lasted 28 days, the spleen was significantly enlarged, with a significant increase in Gr-1⁺ cells. In the intravenous metastasis model (Experiment 2), which lasted 14 days, the weight of the spleen was not dramatically increased; however, the Gr-1⁺ cells were still increased to a significantly higher level. In both situations, the lymphocyte population was reduced in percentage although the total number of cells was not decreased (data not shown). Because myeloid cells play complex roles in tumor growth and metastasis (42), and the leukemoid reaction with granulocytosis is not a specific phenomenon of the 4T1 tumor, the significance of increased Gr-1⁺ cell is not clear at the present time. It is reported that the CD11b⁺Gr-1^{dim} immature granulocyte population is part of the myeloid suppressor cells that can inhibit T cell-mediated tumor immunity (43, 44). However, the Gr-1⁺ population in our study was mainly CD11b⁺Gr-1^{bright} cells. As RA is known to induce the differentiation of monocytic cells (32, 45), and α GalCer has a mitogenic effect on the activation of immune cells (46), including the myeloid lineage, future studies are needed to characterize the functional activity of the enriched CD11b⁺Gr-1^{bright} granulocytes. Our data showed that treatment of 4T1 cells with RA markedly increases the level of gene expression of GM-CSF. However, whereas GM-CSF can promote granulocytosis, we did not observe that RA, in our animal models, further increased spleen size, or affected the Gr-1⁺ cell population. Thus, the granulocytosis appears to be the consequence of the 4T1 tumor growth in the body. It is reported that the granulocytosis becomes more dramatic with time of tumor residence (41), further suggesting that it is the growth of the tumor cells that causes the change of immune cell populations. Therefore, treatments that reduce tumor growth may be predicted to reduce granulocytosis as well.

Another area that has attracted much attention in cancer prevention concerns MMPs, which, through their ability to degrade extracellular matrix, perform a critical function in processes such as tumor angiogenesis, metastasis, and the release of growth factors and cytokines from the extracellular matrix. The circulating levels of MMPs are currently used as prognostic indicators of metastasis (47, 48). Among several MMPs in the gene family, we found MT1-MMP and MMP3 to be elevated in lung tissue after 4T1 tumor growth. RA reduced the expression of both genes. As determined by ELISA, MMP3 protein in plasma was slightly increased after 4T1 cell inoculation and slightly but significantly reduced after treatment with RA + α GalCer. A direct effect on the tumor cells was evident for MMP3, indicated by reduced MMP3 protein produced and secreted by 4T1 cells treated with RA and/or α GalCer. Although the mechanism of the regulation of MMP expression is not clear at the present time, these data suggest an interesting new potential way through which RA and α GalCer may regulate the tumor invasive characteristics by altering the MMP levels.

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FIG. 1.

Treatment of Balb/c mice with all-trans-retinoic acid (RA) and alpha-galactosylceramide (α GalCer)-treated dendritic cells decreased the primary tumor formation and lung metastasis rate in an orthotopic 4T1 breast tumor model. A: Tumor weight. B: Metastasis colony number in the lung. Data were pooled from 3 independent experiments with $n = 4$ to 6 mice/group in each experiment results of t -test are shown. C: Representative images (hematoxylin and eosin staining) of lung tissue histology.

FIG. 2.

4T1 cell inoculation in Balb/c mice resulted in spleen enlargement and cell population changes. A: Spleen weight. B: Correlation analysis of spleen weight and tumor nodules weight, $P < 0.0001$. C: Staining for CD11b⁺ cell population, most of which were Gr-1⁺ cells in 4T1-inoculated mice. A representative scatter plot showing the population measured is shown in the upper right quadrant of the boxed panel. D: Splenic lymphocytes were determined in Balb/c mice after 4T1 inoculation. Representative scatter plots are inserted to show the CD3, CD4, CD8, and CD19 staining and gating. Data were pooled from 3 independent experiments, as in Fig. 1.

FIG. 3.

All-trans-retinoic acid (RA) and and alpha-galactosylceramide (α GalCer) decreased lung metastasis in a hematogenous model of 4T1 cell inoculation. Spleen myeloid cells (A) and lymphocytes (B) were determined by flow cytometry. C: Micrometastasis was determined by counting the number of colonies on each culture plate. D: Micrometastasis was determined by measuring the absorbance at 570 nm, after crystal violet staining, which represents size and number of colonies. Data were combined from 3 independent experiments with $n = 5$ or 6 mice in each experiment.

FIG. 4.

4T1 tumor metastasis in wild type and CD1d $^{-/-}$ mice. A: Lung metastasis rate represented by the absorbance at 570 nm. B: Splenic myeloid cell population. C: Lymphocyte staining. The data shown were combined from 2 independent experiments with $n = 5$ or 6 mice in each experiment.

FIG. 5.

Matrix metalloproteinase levels in vivo and in cultured 4T1 cells. A: Membrane-type 1 matrix metalloproteinases (MT1-MMP) and MMP3 mRNA levels in lung tissues. B: MMP3 protein in plasma of the animals described in Fig. 3, determined by ELISA. C: MMP3 protein level determined by ELISA in the culture supernatant of 4T1 cells cultured in the presence and absence of all-trans-retinoic acid (RA; 20 nM) and alpha-galactosylceramide (α GalCer; 100 ng/ml) for 24 hr. The data shown represents 2 independent experiments each conducted in triplicate.

FIG. 6.

Effects of all-trans-retinoic acid (RA) and and alpha-galactosylceramide (α GalCer) on 4T1 cell growth, cell motility, and gene expression in vitro. 4T1 cells were cultured as in Fig. 5C. A: Cell proliferation was measured by [3 H]-thymidine incorporation. B: 4T1 cell cycle distribution was determined by flow cytometric analysis. C: 4T1 cell motility was measured in a transwell assay and cells that migrated cells were quantified as the absorbance at 570 nm after staining with crystal violet. D: The level of GM-CSF mRNA in 4T1 cells was measured by q-PCR. The data shown represents 2 independent experiments each conducted in triplicate.

TABLE 1

Primers used in the qPCR analysis, and the gene expression level in 4T1 cells determined as threshold cycle (CT) number

Name	Primer sequence	# of CT
G-CSF (granulocyte colony stimulating factor)	F: 5-ATGGCTCAACTTTCTGCCCA R: 5-ACATCCAGCTGAAGCAAGTCCA	16.70 ± 0.08
M-CSF (macrophage colony stimulating factor)	F: 5-ATAAAGGCACGCAGCCCAAA R: 5-AAACCTGGCTTGCTTGGCTA	21.67 ± 0.13
GM-CSF (granulocyte macrophage colony stimulating factor)	F: 5-TCCGGAACGGAAGTGTGAAACA R: 5-TGCCACATCTCTTGGTCCCTTT	19.88 ± 0.31
CXCL1 (C-X-C motif ligand 1)	F: 5-AACGCTGGCTTCTGACAACA R: 5-AAACACAGCCTCCACACAT	19.20 ± 0.07
CCL2 (C-C motif ligand 2)	F: 5-AGGTGTCCCAAAGAAGCTGT R: 5-TGCTTGAGGTGGTTGTGGAA	20.22 ± 0.17
CCL3 (C-C motif ligand 3)	F: 5-TGAAACCAGCAGCCTTTGCT R: 5-ATGCAGGTGGCAGGAATGTT	25.43 ± 0.16
CCL4 (C-C motif ligand 4)	F: 5-AACACCATGAAGCTCTGCGT R: 5-AGCTGCTCAGTTCAACTCCA	26.70 ± 0.11
CCL5 (C-C motif ligand 5)	F: 5-TGCCCACGTCAAGGAGTATT R: 5-CAGGACCGAGTGGGAGT	18.93 ± 0.05
KLF13 (Kruppel-like factor 13)	F: 5-TGGCAGGAGTGCAACAAGAA R: 5-CTCAAGTTCGCTCAGCTTTCCT	19.20 ± 0.12
MMP2 (matrix metalloproteinase 2)	F: 5-GACATCAAGGGATCCAGGAGCTC R: 5-TCAGCAGCCAGCCAGTCTGATTTG	UD
MMP3 (matrix metalloproteinase 3)	F: 5-GGAACCTGAGACATCACCAATGTGC R: 5-AGCTATTGCTCTTCAATATGTGGGT	20.22 ± 0.08
MMP9 (matrix metalloproteinase 9)	F: 5-CAGCCCCTGCTCCTGGCTCTCCTG R: 5-ACTCGTCGTCGTCGAAATGGGCAT	UD
MT1-MMP (membrane-type 1 matrix metalloproteinase)	F: 5-GGAACCCGGGTACGCCAACACATTA R: 5-TCAGACCTTGTCAGCAGCGAACGC	19.32 ± 0.12
HPRT (hypoxanthine-guanine phosphoribosyltransferase)	F: 5-AAGGACCTCTCGAAGTGTGGATA R: 5-CATTTAAAGGAAGTGTGACAACG	25.27 ± 0.21
18S (18S ribosomal RNA)	F: 5-AATGGTGCTACCGGTCATTC R: 5-ACCTCTCTTACCCGCTCTCC	15.31 ± 0.37

CT was set at the same level for every gene. HPRT and 18S served as reference genes. UD: Undefined. UD in 4T1 cells, whereas the mRNA level in lung was not changed after treatment.