Inhibition by Retinoic Acid of Type IV Collagenolysis and Invasion through Reconstituted Basement Membrane by Metastatic Rat Mammary Adenocarcinoma Cells

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ABSTRACT

The activity of type IV collagenase, which enables tumor cells to degrade collagen type IV found in the subendothelial basement membrane, has been correlated with the metastatic potential in several tumor types, including the rat 13762NF mammary adenocarcinoma cell line and its clones. In this study, we examined whether all-trans-retinoic acid (all-trans-RA) and other retinoids, which exhibit antitumor activity in vitro and in vivo, affect the collagenolytic activity of metastatic rat 13762NF mammary adenocarcinoma cells. Cells of the highly metastatic lung-colonizing clone MTF7.T35.3, derived from the 13762NF cell line, were treated for 3 days with 0.1, 1, or 10 μM all-trans-RA, harvested, and seeded on [3H]proline-labeled extracellular matrix deposited by cultured rat lung endothelial cells or on a film of purified [3H]proline-labeled type IV collagen. The amount of radioactivity released into the medium during the subsequent 24 to 72 h was measured, and it was found that all-trans-RA treatment inhibited degradation of extracellular matrix and type IV collagen by 50 to 60%. This effect was observed whether the cells had been treated with all-trans-RA in serum-free medium or in medium supplemented with heat-inactivated or acid-treated fetal bovine serum. The growth of the cells was not inhibited under these conditions, except after treatment with 10 μM all-trans-RA in serum-free medium. The reduction in collagenolytic activity was observed in viable cells as well as in conditioned medium. A 24-h exposure of cells to all-trans-RA was sufficient to cause a 30% decrease in the collagenolytic activity, and this inhibitory effect was reversible. The direct addition of all-trans-RA to conditioned medium had no effect on secreted collagenase activity. The apparent molecular weights of the collagenolytic enzymes were determined by electrophoresis of cell extracts and concentrated conditioned medium in type IV collagen-embedded polyacrylamide gels followed by renaturation and activation of the enzymes within the gels. Two major type IV collagenolytic metalloproteinases exhibiting molecular weights of 64,000 and 88,000, respectively, were detected by this method. These two enzymes were also found to have specificity for gelatin. The M, 64,000 enzyme could be extracted from viable cells (presumably from the cell membrane) by 2% 1-butanol. Treatment with all-trans-RA decreased the level of these enzymes in the cellular, cell membrane, and conditioned medium compartments. Other retinoids, including 13-cis-RA, retinol, trimethylmethoxynaphthalene analogue of RA, tetradecaneoxyphenyl naphthalenyl propenylbenzoic acid, and 3,5-di-tert-butyl 4'-chalcone carboxylic acid, also suppressed the level of secreted type IV collagenolytic enzymes. Continuous treatment of the adenocarcinoma cells with 1 or 10 μM all-trans-RA inhibited their ability to invade through a reconstituted basement membrane (Matrigel-coated filters). These results demonstrate the ability of retinoids to suppress the production of type IV collagenolytic metalloproteinases in mammary adenocarcinoma cells and inhibit their invasiveness in vitro. Such an effect in vivo may decrease the invasiveness of malignant tumors.

INTRODUCTION

Metastasis, the process by which cancer cells spread from the primary tumor via the circulation to distant organs, is a complex phenomenon requiring many sequential steps. One of the initial steps is the invasion of primary tumor cells into the surrounding host tissues and blood vessels or lymphatic channels (intravasation). Via the circulation, the tumor cells are disseminated to various organs, where they adhere specifically, or are arrested nonspecifically in capillary beds. Subsequent steps include extravasation by capillary endothelial cells and the subendothelial ECM into the tissues and proliferation of the cells to form secondary tumors. The successful completion of the cascade of events culminating in the formation of metastases depends on the expression of unique properties by progenitor tumor cells, as well as on tumor cell-host interactions (for reviews, see Refs. 1–4).

One tumor cell property that is a prerequisite for metastasis is the ability to degrade connective-tissue ECM and basement membrane components, which constitute barriers for invading tumor cells. Indeed, metastatic tumor cells have been shown to produce enzymes such as proteases and glycosidases that are capable of degrading the various components of the ECM (4–16). One of the proteolytic enzymes, type IV collagenase, which exhibits the ability to degrade type IV collagen, the major structural protein constituent of subendothelial basement membranes, is presumed to play an important role in invasion because its levels correlate with the metastatic potential of various transformed and malignant cells (4, 6, 11–15), including rat mammary adenocarcinoma clones derived from the 13762NF cell line (16).

Retinoids, a group of vitamin A metabolites and synthetic analogues, can suppress the transformed phenotype and enhance the differentiation of many types of tumor cells in culture and in vivo (for reviews, see Refs. 17–19). Some of these compounds are being evaluated for potential use in prevention and treatment of cancer in humans (20). Several retinoids decrease the activity of type I collagenase in various transformed and normal cells, including synovial cells from rheumatoid arthritis patients where the elevated collagenase activity is accompanied by invasion of the nonmalignant synovial cells into articular cartilage (21), cultured rabbit synovial cells treated with 12-O-tetradecanoylphorbol-13-acetate (22, 23), normal...
human skin fibroblasts (24–26), and fibroblasts from keloid tissue (27). Only limited studies have examined the effects of retinoids on collagenases of tumor cells. The induction by RA of differentiation of murine embryonal carcinoma cells into endodermal cells was accompanied by an increased production of differentiation of murine embryonal carcinoma cells into retinoids on collagenases of tumor cells. The induction by RA tissue (27). Only limited studies have examined the effects of human skin fibroblasts (24–26), and fibroblasts from keloid 
retinoids affect the type IV collagenolytic activity and invasive-
ness of metastatic cells. Clone MTF7.T35.3, derived from the 13762NF rat mammary adenocarcinoma cell line, was chosen for this investigation because the 13762NF-derived cells have been shown to produce type IV collagenolytic activity and because an excellent correlation has been established between this activity and the metastatic potential of the cells in vivo (16). Preliminary results of this study were reported previously in abstract form (30).

MATERIALS AND METHODS

Cells. Rat mammary adenocarcinoma cell clone MTF7.T35.3 was selected by in vitro cloning from MTF7 cells derived from a 13762NF mammary adenocarcinoma tumor growing at a local implant site in the mammary fat pad (31). Cells were grown on 100-mm tissue culture plates (Corning Glass Works, Corning, NY) containing α-MEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FBS (Biocell, Carson, CA) and no antibiotics at 37°C in a humidified incubator (5% CO2 and 95% air). In some experiments, MTF7.T35.3 cells were grown in the absence of serum in D-MEM containing 5 Mg/ml of insulin, 5 μg/ml of transferrin, 2 μM ethanolamine, 2 mg/ml glutamine, 25 mM selenious acid, 0.1 mM each of 10 nonessential amino acids, and 10 mM N-2-hydroxyethylpiperazine-N’-propanesulfonic acid buffer, pH 7.5 (serum-free complete medium). RLE were isolated from Fischer 344 rat lungs as previously described (16). RLE cl.8 possessed characteristic endothelial morphology and was found to have a nonthrombogenic cell surface, Ulex europaeus I agglutinin-binding sites, angiotensin-converting enzyme, and underlying ECM that is characteristic of endothelial cells and contains type IV collagen, laminin, and heparan sulfate proteoglycan as its major constituents (16). RLE cl.8 cells were seeded in 1% gelatin-coated plastic tissue culture plates (Corning) containing a 1:1 ratio of D-MEM and Ham’s F-12 nutrient mixture (D-MEM/F-12; GIBCO) supplemented with 10% plasma-derived horse serum (Hyclone Laboratories, Logan, UT) and 100 μg/ml of endothelial mitogen (Biomedical Technologies, Inc., Cambridge, MA). Cells used in this study were at passages 6 to 8.

Retinoids. All-trans-RA, 13-cis-RA, TOTPNA, TNPB, and Ch55 were the gift of BASF Aktiengesellschaft (Federal Republic of Germany); and retinol, retinal, and phenyl analogue of RA, and the TMMP analogue of RA were the gift of Hoffmann-La Roche (Nutley, NJ). The retinoids were dissolved in DMSO and stored as stock solutions of 10 mM under N2 at −70°C. They were added to the growth medium immediately before each experiment.

Subendothelial Matrix Degradation. RLE cells were seeded onto a gelatin-coated well (16-mm diameter; Costar, Cambridge, MA) at 1 x 10^6 cells per well and grown as described above. Confluent cells were incubated for 48 h with 5 μCi/ml of l-[2,3,4,5-3H]proline (100 μCi/ml; ICN Radiochemicals, Irvine, CA) in D-MEM containing 50 μg/ml of ascorbic acid and 10% dialyzed FBS. ECMs were isolated according to the method of Kramer and Vogel (32). The isolated matrices were preincubated in D-MEM containing 10% FBS for 6 h prior to the degradation assay. Tumor cells were harvested from the subconfluent cultures (3 x 10^5 cells per 10-cm plate), and 1 x 10^5 cells suspended in 1 ml of α-MEM plus 10% heat-inactivated FBS were placed onto each well containing radiolabeled matrix and incubated at 37°C in a CO2 incubator. After 24-h incubation, culture supernatants were withdrawn and centrifuged at 30,000 x g for 30 min. The radioactivity (3H) of digested materials in 100 μl of supernatant was measured by liquid scintillation counting using a Beckman Model LS2800 scintillation spectrometer (Irvine, CA) reading with a counting efficiency of 92%.

Type IV Collagenolysis Assay. Type IV collagenolytic activity of mammary adenocarcinoma cells was measured by the methods described previously (16) using [3H]proline-labeled type IV collagen prepared in short-term organ cultures of EHS tumors. Mammary adenocarcinoma cells were pretreated with retinoids for 72 h in the presence or absence of 10% heat-inactivated FBS or 5% acid-treated (α-macroglobulin inactivated) FBS. Cells (1 x 10^5) suspended in 200 μl of medium were placed on a dried [3H]proline-labeled type IV collagen film (20 μg, 8000 cpm), and type IV collagenolysis over a period of 72-h incubation was measured.

In the measurement of secreted enzyme activity, tumor cells were cultured for 3 days in the absence or presence of retinoids, and the conditioned media harvested by centrifugation were assayed for type IV collagenase. A 100-μl aliquot of the conditioned medium was mixed with 5 μg of [3H]proline-labeled type IV collagen suspended in 100 μl of 10 mM CaCl2, 0.1 M NaCl, and 50 mM Tris-HCl buffer, pH 7.5, and incubated at 37°C with gentle mixing. Undigested materials were precipitated by mixing with 50 μl of ice-cold 10% trichloroacetic acid and 0.5% tannic acid and then centrifuged at 18,000 x g, and the enzyme activity was calculated from the radioactivity in the supernatant.

Zymograms. Identification of type IV collagenolytic enzyme secretion by mammary adenocarcinoma cells was performed by electrophoresis of serum-free conditioned media in a type IV collagen-embedded polyacrylamide gel followed by incubation and Coomassie blue staining, based on the methods described by Heussen and Dowdle (33). Type IV procollagen purified from EHS tumors dissolved in 0.5 M acetic acid or bovine lens capsule type IV collagen pepsin fragments (Seikagaku America, St. Petersburg, FL) in HCl at pH 3.0 were neutralized with Tris and immediately dissolved in 2% SDS. After removing undissolved materials by a brief centrifugation at 18,000 x g, SDS-solubilized type IV collagen was copolymerized with 7.5% acrylamide. In some experiments, each of the following proteins was also embedded in SDS-polyacrylamide gels: rat hemoglobin; rat immunoglobulin G; bovine serum albumin; bovine plasma fibronectin; bovine α-casein; and gelatin from swine skin (Sigma Chemical, St. Louis, MO).

Mammary adenocarcinoma cells (1 x 10^6 cells) were seeded in a 10-cm tissue culture plate and cultured for 24 h in serum-free complete medium containing 10% heat-inactivated FBS. The cells were extensively washed with DPBS and then further cultured in 10 ml of serum-free complete medium in the presence or absence of retinoids. In some experiments [35S]methionine (ICN Radiochemicals, Irvine, CA) was added to serum-free complete medium to monitor protein synthesis. The serum-free culture supernatants were withdrawn and sequentially centrifuged at 800 x g and 18,000 x g, and then aliquots of the supernatants were concentrated with Centricon 30 concentrators (Amicon, Danvers, MA) and mixed with SDS sample buffer. Cell butanol extracts and cell extracts in Triton X-100 were also prepared to examine the cell surface-associated activities and total cellular enzyme activities, respectively. Cells (1 x 10^5) were grown as described above, extensively washed with DPBS, and then extracted for 5 min at 22°C with 2% butanol in DPBS (34) or solubilized at 4°C in 0.2% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.5. Cell extracts were centrifuged for 5 min at 18,000 x g, and the supernatant were immediately mixed with SDS sample buffer without β-mercaptoethanol. Electrophoresis was carried out by the method of Laemmli (35) in polyacrylamide gels containing 0.5 mg/ml of type IV collagen. After electrophoresis, gels were rinsed twice with 2.5% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.5, and incubated at 37°C for 16 h in 0.15 M NaCl, 10 mM CaCl2, and 50 mM Tris-HCl buffer, pH 7.5, containing 0.05% NaN3. Gels were stained with 0.05% Coomassie blue, 10% isopropanol, and 10% acetic acid in H2O and then destained with 10% isopropanol and 10% acetic acid in H2O. Type IV collagenolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained slab gels. The stained gels were photographed using Kodak electrophoresis duplicating papers (Kodak, Rochester, NY). Quantitative analysis of the collagenolytic enzyme activity was achieved by photographing
the zymograms using Kodak XAR-5 X-ray films. The active enzyme bands that appeared as positively stained bands on the X-ray films were scanned for absorbance at 560 nm using a Beckman DU-8 spectrophotometer. The area under each peak that represents collagenolytic activity was measured and shown as a relative enzyme activity. In the experiments with [35S]methionine-labeled cells, zymogram gels were dried and subjected to autoradiography using Kodak XAR-5 X-ray films.

**Invasion Assay.** The MICS developed by Hendrix and coworkers (36, 37) was used to evaluate the degree of tumor cell invasion through a reconstituted basement membrane. The assay was performed using polycarbonate filters with 10-μm-diameter pores (Nuclepore, Pleasanton, CA) coated with Matrigel (Collaborative Research, Bedford, MA) (38). Rat mammary adenocarcinoma cells were pretreated for 4 days with 0.1, 1, or 10 μM all-trans-RA in 0.1% DMSO or with 0.1% DMSO alone and harvested by a brief trypsin treatment. Cells suspended in α-MEM containing 10% heat-inactivated FBS and 40 μg/ml of gentamicin (GIBCO, Grand Island, NY) were seeded on reconstituted Matrigel and incubated in the presence or absence of 0.1, 1, or 10 μM all-trans-RA. Cells which passed through the Matrigel-coated polycarbonate membrane were harvested from a bottom chamber and counted as described previously (36, 37). The significance of the difference in data obtained from all-trans-RA-treated cells and controls was determined using an analysis of variance.

**Statistical Analyses.** For the type IV collagenolysis and growth inhibition assays, differences between groups were determined using Student's t test. The least significant differences and Q tests for the significance were also used for the comparison of multiple groups.

**RESULTS**

**Effects of All-trans-RA on the Degradation of RLE-ECM.** We have previously shown that [3H]proline-labeled components of RLE-ECM, composed mostly of type IV collagen, were enzymatically degraded by lung-colonizing metastatic 13762NF cells, and characteristic type IV collagen degradation fragments were solubilized from the matrix (16). The effects of RA on [3H]proline-labeled RLE-ECM degradation by the highly metastatic clone MTF7.T35.3 cells were analyzed in this study. Pretreatment of the cells in the presence of 10% heat-inactivated FBS for 3 days with 0.1, 1, or 10 μM all-trans-RA decreased the release of [3H]proline-labeled components into the medium during a 24-h incubation by 18, 22, and 44%, respectively (Table 1). Similar results were obtained in 3 independent experiments with different batches of tumor cells.

**Effects of All-trans-RA on Type IV Collagenolysis by Rat Mammary Adenocarcinoma Cells.** The results from the RLE-ECM degradation assays suggested that all-trans-RA inhibited mammary adenocarcinoma cell-mediated type IV collagenolysis. Thus, the effects of all-trans-RA on type IV collagenolysis were further investigated directly using purified type IV pro-collagen prepared from the short-term culture of basement membrane-producing EHS tumors that were metabolically labeled with [3H]proline. The specific radioactivity of the [3H]proline-labeled type IV collagen was approximately 400 cpn/μg. Since we found that serum components affect the production, release, and activation of mammary adenocarcinoma type IV collagenolytic enzymes (16), the experiments were performed in the presence or absence of 10% heat-inactivated FBS or 5% acid-treated (α2-macroglobulin-inactivated) FBS. After 1 × 10⁴ MTF7.T35.3 cells were seeded onto a [3H]proline-labeled type IV collagen film (20 μg), type IV collagen was progressively degraded over a 72-h incubation period in the presence of 10% heat-inactivated FBS (Fig. 1). Similar kinetics of collagenolysis was observed in serum-free complete medium alone or medium plus 5% acid-treated FBS (data not shown). The total amounts of type IV collagen degraded during the first 24-h incubation were slightly different: complete medium alone, 1.25 ± 0.12 μg; medium plus 5% acid-treated FBS, 1.45 ± 0.03 μg; and medium plus 10% heat-inactivated FBS, 1.10 ± 0.02 μg. All-trans-RA had almost the same effects on type IV collagenolysis by mammary adenocarcinoma cells in the presence or absence of acid-treated or heat-inactivated FBS (Fig. 1; Table 2). Degradation of type IV collagen was inhibited by more than 50% after treatment of the cells with 10 μM all-trans-RA and by 25 to 30% after treatment with 1.0 μM all-trans-RA (Table 2). A cell growth inhibition was observed only in the serum-free medium containing 10 μM all-trans-RA (P < 0.05) but not in

![Fig. 1](image-url)

**Table 1** Inhibition by all-trans-RA of RLE-ECM degradation by rat mammary adenocarcinoma cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Release of [3H]proline-labeled RLE-ECM components during a 24-h incubation</th>
<th>Mean ± SD</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3699 ± 100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>All-trans-RA, 0.1 μM</td>
<td>3411 ± 89</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>All-trans-RA, 1 μM</td>
<td>2882 ± 99</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>All-trans-RA, 10 μM</td>
<td>2076 ± 155</td>
<td>56</td>
<td>56</td>
</tr>
</tbody>
</table>

* Tumor cells were grown for 3 days in the absence or presence of the indicated concentrations of all-trans-RA, harvested from sub confluent cultures (3 × 10⁴ cells/10-cm plate), and suspended at 1 × 10⁵ cells/ml of α-MEM supplemented with 10% heat-inactivated FBS.

* One-mi portions of the above tumor cell suspension were placed in each well containing [3H]proline-labeled RLE-ECM and incubated at 37°C in a CO₂ incubator. After a 24-h incubation, the radioactivity of the digested materials in 50 μl of the supernatant was measured. Spontaneous release of radioactivity from the matrix in the absence of cells was 1031 ± 48 cpn, and this value was subtracted from the radioactivity released by untreated and treated cells. The values are the mean ± SD of triplicate determinations. Similar results were obtained in 3 independent experiments with different batches of tumor cells.
Collagenolysis and Invasion Inhibition by Retinoic Acid

Table 2: Inhibition by all-trans-RA of type IV collagenolysis by rat mammary adenocarcinoma cells in serum-free and serum-containing media

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum-free medium</th>
<th>Heat-inactivated FBS</th>
<th>Acid-treated FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.0 ± 1.3</td>
<td>90.4 ± 0.8</td>
<td>75.7 ± 0.8</td>
</tr>
<tr>
<td>All-trans-RA, 0.1 μM</td>
<td>11.78 ± 0.44</td>
<td>9.07 ± 0.24</td>
<td>7.60 ± 0.10</td>
</tr>
<tr>
<td>All-trans-RA, 1 μM</td>
<td>17.08 ± 1.56</td>
<td>14.87 ± 0.37</td>
<td>12.30 ± 0.46</td>
</tr>
<tr>
<td>All-trans-RA, 10 μM</td>
<td>42.41 ± 4.3</td>
<td>35.02 ± 3.2</td>
<td>36.5 ± 3.4</td>
</tr>
</tbody>
</table>

* The tumors were cultured in the absence (control) or presence of all-trans-RA in serum-free complete medium or in the same medium supplemented with 10% heat-inactivated FBS or 5% acid-treated (×2-macroglobulin-inactivated) FBS. The cells were harvested after 3 days, when the cultures were subconfluent.

* Cells were seeded at 1 × 10^5 cells/well on a film of [H]proline-labeled type IV collagen (20 μg, 8000 cpm/16-mm-diameter well). After a 24-h incubation, the radioactivity released into the medium was measured as in Table 1. Collagenolysis by the treated cells was expressed as the percentage of the radioactivity released by untreated control cells. The data were corrected for decreases in cell number. Similar results were obtained in 3 independent experiments.

* Mean ± SD.

Table 3: Effects of all-trans-RA on cell growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells (×10^3) after 3 days of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.02 ± 0.33</td>
</tr>
<tr>
<td>All-trans-RA, 0.1 μM</td>
<td>3.98 ± 0.24</td>
</tr>
<tr>
<td>All-trans-RA, 1 μM</td>
<td>3.88 ± 0.16</td>
</tr>
<tr>
<td>All-trans-RA, 10 μM</td>
<td>2.80 ± 0.12</td>
</tr>
</tbody>
</table>

* Cells were suspended at 1 × 10^5 cells/ml in 10 ml of serum-free complete medium without or with the indicated all-trans-RA concentrations and seeded in a series of 10-cm plates. After 3 days, the cells were detached after a brief trypsinization, suspended by repeated pipetting, and counted using an electronic particle counter (Coulter Electronics, Hialeah, FL). Cell viability, expressed as the proportion of cells excluding 0.1% trypan blue, was greater than 90% in all cultures.

* Mean ± SD of triplicate cultures, each counted twice.

* Significantly different from number of cells in control cultures (P < 0.05).

Table 4: Effect on the level of collagenolytic activity of all-trans-RA treatment of rat mammary adenocarcinoma cells before or during the collagen degradation assay

<table>
<thead>
<tr>
<th>Type IV collagen degraded (μg/24 h/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment of cells during growth*</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>10 μM all-trans-RA</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>10 μM all-trans-RA</td>
</tr>
</tbody>
</table>

* The adenocarcinoma cells were cultured for 3 days in α-MEM supplemented with 10% heat-inactivated FBS containing 0.1% DMSO (control) in the presence or absence of all-trans-RA. The cells and the conditioned media were analyzed for collagenolytic activity.

† The adenocarcinoma cell type IV collagenolysis is due to decreasing levels of proteinases or increasing levels of proteinase inhibitors, we examined zymograms of type IV collagen-embedded polycrylamide gels. In this assay, putative proteinase-inhibitor complexes were dissociated by SDS-containing sample buffer and separated during electrophoresis in SDS-polyacrylamide gels. Certain proteinases that are dissolved with SDS can be renatured and activated by removal of SDS in the presence of Triton X-100 (33, 39-41).

When the conditioned media from 48-h cultures were analyzed on zymograms, two major proteinases of apparent molecular weights of 88,000 and 64,000 were detected as clear bands (Fig. 2). These active enzyme bands did not appear when a chelating agent, such as EDTA or 1,10-phenanthroline, was added to the incubation buffer at a concentration of 10 mM, whereas phenylmethylsulfonyl fluoride (2 mM) and N-ethylmaleimide (5 mM) did not inhibit the enzyme activity (data not shown). These results indicate that the M, 88,000 and 64,000 proteinases are both metalloproteinases. These two proteinase bands were also observed in gels embedded with gelatin or with pepsin fragments of type IV collagen instead of intact type IV collagen. However, when polycrylamide gels were embedded with serum albumin, hemoglobin, immunoglobulin G, α-casein, or fibronectin instead of type IV collagen, the M, 88,000 and the M, 64,000 bands did not appear (data not shown). The results indicate that these enzymes are specific for gelatin and might be responsible for the collagenolytic activity.

The activity of M, 88,000 and 64,000 enzymes was reduced by all-trans-RA treatment of cells in a dose-dependent manner (Figs. 2 and 3). Treatment of cells with 10 μM all-trans-RA resulted in the reduction of M, 88,000 and 64,000 enzyme levels by 67 and 86%, respectively (Fig. 3). The M, 88,000 and 64,000 enzymes were also found in the Triton X-100 cell extract, and both activities were dramatically reduced following treatment with 0.1 to 10 μM all-trans-RA (Fig. 4, left). Two minor species with apparent molecular weights of 70,000 and 80,000 were also detected in the Triton X-100 cell extract. To monitor protein synthesis during the incubation with all-trans-RA, 50 μCi/ml of [3S]methionine were added to the cultures, and metabolically [3S]methionine-labeled cell proteins were ana-
COLLAGENOLYSIS AND INVASION INHIBITION BY RETINOIC ACID

Fig. 2. Decreased levels of collagenolytic enzymes in conditioned media of all-trans-RA-treated rat mammary adenocarcinoma cells. MTF7.T35.3 cells (1 × 10⁶) were seeded in each of several 10-cm tissue culture dishes and cultured for 24 h. After extensive washing with DPBS, the cells were further grown for 2 days in serum-free complete medium containing 0.1% DMSO only as control (A); 0.1 μM all-trans-RA (B); 1 μM all-trans-RA (C); 10 μM all-trans-RA (D). A 500-μl aliquot of conditioned medium was removed from each culture, concentrated with a Centricon 30, and then mixed with SDS-sample buffer without β-mercaptoethanol. Samples were immediately subjected to electrophoresis in type IV collagen-embedded SDS-polyacrylamide gels (7.5% acrylamide, 0.5 mg/ml of type IV collagen). After a 24-h incubation, the gels were stained for proteins with Coomassie brilliant blue, and active enzymes were detected as unstained bands. Numbers at left indicate the molecular weight (× 10⁻³) of the proteinase bands estimated in relation to the migrations of molecular weight standards, such as phosphorylase b (Mₚ 94,000), bovine serum albumin (Mₚ 67,000), and ovalbumin (Mₚ 43,000), which are indicated at right.

Fig. 3. Decreased levels of collagenolytic enzymes in conditioned media of all-trans-RA-treated rat mammary adenocarcinoma cells. Zymograms shown in Fig. 2 were negatively photographed using Kodak XAR-5 X-ray film, and the intensities of the Mₚ 88,000 and 64,000 enzyme bands were measured by scanning for absorbance at 560 nm using a Beckman DU-8 spectrophotometer. The integrated intensity averaged from 3 individual experiments was shown as a relative proteinase level.

Fig. 4. Effects of all-trans-RA on cellular type IV collagenolytic enzymes. MTF7.T35.3 cells (1 × 10⁶) were seeded in each of several 10-cm tissue culture dishes and cultured for 24 h. After extensive washing with DPBS, the cells were further grown for 2 days in serum-free complete medium containing 50 μCi/ml of [³⁵S]methionine in the presence of: A and A', 0.1% DMSO only as control; B and B', 0.1 μM all-trans-RA; C and C', 1 μM all-trans-RA; D and D', 10 μM all-trans-RA. Cells were solubilized at 4°C with 0.2% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.5, and cell extracts were mixed with SDS-sample buffer without β-mercaptoethanol and immediately subjected to SDS-polyacrylamide gel electrophoresis with a type IV collagen-embedded polyacrylamide gel (7.5% polyacrylamide, 0.5 mg/ml of type IV collagen). After a 24-h incubation, the gel was stained for protein with Coomassie blue, and active enzymes were detected as unstained bands. Then, the gel was dried and subjected to autoradiography to monitor protein synthesis. A, B, C, and D, zymograms; A', B', C' and D', autoradiograms. Numbers represent molecular weight in thousands.

Fig. 5. Zymogram of cell surface-associated type IV collagenolytic enzymes. MTF7.T35.3 cells were cultured as described in the Fig. 4 legend in serum-free complete medium containing: 0.1% DMSO only as control (A); 10 μM all-trans-RA and 0.1% DMSO (B). Cells were extracted at 22°C for 5 min with 2% 1-butanol in DPBS, and cell butanol extracts were analyzed by zymography using a type IV collagen-embedded polyacrylamide gel. Ordinate, molecular weight in thousands.
tion, cells were able to grow again (cell viability >90%), indicating that the $M$, 64,000 proteinase is localized on the cell membrane outer surface. The activity of cell surface-associated enzyme was diminished when the cells were treated with 10 $\mu M$ all-trans-RA (Fig. 5).

Effects of Various Retinoids on Release of Collagenolytic Enzymes by Rat Mammary Adenocarcinoma Cells. To investigate the relationship between the chemical structures of retinoids and their effects on type IV collagenase production in mammary adenocarcinoma cells, we further tested other retinoids, such as 13-cis-RA, retinal, retinol, phenyl analogue of RA, TTNP, TTNPB, TMMP analogue of RA, and Ch55 (Fig. 6). Since these retinoids were found to have no significant effects on cell growth at 1 $\mu M$, the following experiments were performed with 1 $\mu M$ retinoid. Treatment of MTF7.T35.3 cells for 24 h with any of the listed retinoids except TTNP significantly decreased extracellular levels of proteinases capable of degrading $[^{3}H]$proline-labeled type IV collagen ($P < 0.05$) (Table 5). Similar inhibitory effects on the extracellular levels of two type IV collagenolytic metalloproteinases of molecular weights of 88,000 and 64,000 were also observed on zymograms (Table 5). There was no significant difference in type IV collagenolysis inhibition between all-trans-RA and 13-cis-RA, although they had somewhat different effects on the two metalloproteinases. Ch55 demonstrated the highest inhibitory activity among the 9 retinoids tested, and it reduced the extracellular level of the $M$, 64,000 enzyme by more than 80% (Table 5). Changes in the cell surface-associated enzyme band on the negative photograph of the zymogram and comparison of the areas under the peaks with those of controls.

Effects of Retinoic Acid on Mammary Adenocarcinoma Cell Invasion through Artificial Basement Membrane. Using the MICS assay, we found that a continuous treatment with all-trans-RA at the concentration of 1 $\mu M$ and 0.1% DMSO or in medium containing DMSO only (control) inhibited cell invasion. Since it was difficult to determine the total cell number after the invasion assay, we performed separate experiments to test the effect of all-trans-RA on cell growth under the same conditions. MTF7.T35.3 cells (1 x 10^5) were pretreated for 4 days with 0.1, 1.0, or 10 $\mu M$ all-trans-RA in 0.1% DMSO or 0.1% DMSO alone. Cells were continuously exposed to their respective treatments for an additional 3 days in the MICS assay. Subsequently, the cells that invaded a reconstituted basement membrane were collected and counted. Data were shown as the percentage of invaded cells where the number of cells invaded through reconstituted basement membrane in the presence of DMSO alone is 100%. There were statistically significant differences between the control and the all-trans-RA treatment groups (1 $\mu M$ all-trans-RA, $P < 0.01$; 10 $\mu M$ all-trans-RA, $P < 0.001$). Bars, SD.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of degradation of $[^{3}H]$proline-labeled type IV collagen</th>
<th>Relative proteinase level on zymogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td>76.9 ± 2.4^d</td>
<td>74.7</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>77.5 ± 0.8</td>
<td>64.7</td>
</tr>
<tr>
<td>Retinal</td>
<td>84.7 ± 3.7</td>
<td>88.2</td>
</tr>
<tr>
<td>Retinol</td>
<td>80.0 ± 2.1</td>
<td>58.8</td>
</tr>
<tr>
<td>Phenyl RA</td>
<td>77.3 ± 1.6</td>
<td>58.8</td>
</tr>
<tr>
<td>TTNP</td>
<td>104.8 ± 4.6</td>
<td>100.0</td>
</tr>
<tr>
<td>TTNPB</td>
<td>86.8 ± 4.0</td>
<td>76.5</td>
</tr>
<tr>
<td>TMMP RA</td>
<td>84.8 ± 3.2</td>
<td>64.7</td>
</tr>
<tr>
<td>Ch55</td>
<td>61.2 ± 0.8</td>
<td>64.7</td>
</tr>
</tbody>
</table>

* Cells were cultured for 24 h in serum-free media containing 1 $\mu M$ retinoid and 0.1% DMSO or in medium containing DMSO only (control).

* Conditioned medium (2 ml) was concentrated using a Centricon 30 concentrator, redissolved in 600 $\mu l$ of assay buffer, and then analyzed for type IV collagenolytic activity.

* Relative proteinase levels were determined by densitometric scanning of each enzyme band on the negative photograph of the zymogram and comparison of the areas under the peaks with those of controls.

* Mean ± SD of 3 independent experiments.

Fig. 7. Effects of all-trans-RA on rat mammary adenocarcinoma invasion through artificial basement membrane. Rat mammary adenocarcinoma cells (clone MTF7.T35.3) were pretreated for 4 days with 0.1, 1.0, or 10 $\mu M$ all-trans-RA in 0.1% DMSO or 0.1% DMSO alone. Cells were continuously exposed to their respective treatments for an additional 3 days in the MICS assay. Subsequently, the cells that invaded a reconstituted basement membrane were collected and counted. Data were shown as the percentage of invaded cells where the number of cells invaded through reconstituted basement membrane in the presence of DMSO alone is 100%. There were statistically significant differences between the control and the all-trans-RA treatment groups (1 $\mu M$ all-trans-RA, $P < 0.01$; 10 $\mu M$ all-trans-RA, $P < 0.001$). Bars, SD.

Fig. 6. Chemical structures of retinoids tested for effects on type IV collagenolytic enzyme production by rat mammary adenocarcinoma cells.

Table 5 Differential effects of treatment of rat mammary adenocarcinoma cells with retinoids on secreted collagenolytic activity.
DISCUSSION

This study demonstrated the ability of retinoic acid and certain other retinoids to inhibit the production of type IV collagenolytic activity by metastatic mammary adenocarcinoma cells. Several different, but complementary, approaches have shown that RA modulated type IV collagenolytic metalloproteinases: (a) RA-treated cells exhibited a decreased ability to release [3H]proline from metabolically labeled ECM produced and deposited by RLE; (b) cells treated with RA or with several other retinoids possessed lowered capacity to degrade [3H]proline-labeled type IV collagen purified from EHS tumor tissue, as did the media conditioned by these cells; and (c) the levels of collagenolytic enzymes detected in type IV collagen-containing polyacrylamide gels were lower in retinoid-treated cells and conditioned media than in untreated controls. Several collagenolytic enzymes were detected by substrate gel analyses of the rat mammary carcinoma extracts prepared from untreated cells; the major ones exhibited molecular weights of 64,000 and 88,000. Two minor species with molecular weights of 70,000 and 80,000 were also observed. The M, 64,000 metalloproteinase could be extracted from the cell membrane with 1-butanol, and the conditioned medium contained primarily the M, 64,000 and the M, 88,000 enzymes. Collagenolytic metalloproteinases, such as collagenases, gelatinases, and stromelysin, produced by other normal and tumor cell types, had molecular weights between 25,000 and 95,000 (40–53). Type IV collagenase purified from a metastatic mouse sarcoma has been shown to appear as two bands of molecular weights of 62,000 and 68,000 after trypsin activation (44). Human type IV collagenases with molecular weights of 66,000 to 67,000 were also purified from monocytes/macrophages (47) and H-ras oncogene-transformed bronchial epithelial cells (48). These enzymes are secreted in a latent form, but they may also exist on the cell surface (4, 50).

We previously found that the metalloproteinases secreted from rat mammary adenocarcinoma cells degrade both α-subunits of type IV procollagen and produce characteristic large fragments (16). In this study, type IV procollagen from the EHS tumor, pepsin-treated type IV collagen from the anterior capsule of bovine crystalline lens, gelatin, and other proteins were used as substrates. Only the type IV collagen and gelatin embedded in polyacrylamide gels were susceptible to the two major metalloproteinases (M, 88,000 and 64,000) released from MTF7.T35.3 cells. Thus, these enzymes have a restricted specificity to gelatin and type IV collagen. Recently, Ballin et al. (51) reported that ras oncogene mediated induction of a metalloproteinase with a molecular weight of approximately 92,000 capable of degrading gelatin and type IV collagen, and Yamagata et al. (52) and Morikawa et al. (53) demonstrated the strong association of the M, ~92,000 to 95,000 enzyme expression with metastatic potential of murine and human colon carcinoma cells. These high-molecular-weight enzymes were detected on zymograms without trypsin activation, and their characteristics are very similar to those of the rat mammary adenocarcinoma M, 88,000 type IV collagenase.

RA treatment of the rat MTF7.T35.3 mammary carcinoma cells resulted in a decrease in the amounts of both the M, 64,000 and the M, 88,000 collagenolytic metalloproteinases in the cellular and secreted enzyme pools. Likewise, the amount of the M, 64,000 enzyme extractable from the cells with 1-butanol was diminished in all-trans-RA-treated cells. The decrease in collagenase activity in the mammary adenocarcinoma cells was not a reflection of a general, nonspecific reduction in protein synthesis as shown in Fig. 4.

Most previously reported effects of retinoids on activities of various enzymes in tumor cells, including ornithine decarboxylase, plasminogen activator, transglutaminase, protein kinase (18), and glycosyltransferases (54), were accompanied by modulation of cell proliferation and differentiation. In contrast, the inhibition of collagenase production by the rat mammary adenocarcinoma cells in the present study occurred independently of growth modulation in monolayer culture, because the growth of these cells was not inhibited by all-trans-RA, except when the cells were treated with 10 μM all-trans-RA in serum-free medium. Preliminary results indicate that 1 μM all-trans-RA can suppress by 80 to 90% the ability of the MTF7.T35.3 cells to form colonies in 0.5% agarose without reduction in viability (data not shown). Thus, the transformed phenotype of these cells may be preferentially suppressed by all-trans-RA. Since several studies have demonstrated a correlation between transformation and type IV collagenase production (14, 48), our results suggest that suppression of type IV collagenolytic activity by all-trans-RA may be a consequence of suppression of the transformed phenotype.

The mechanism by which retinoids decrease type IV collagenolytic activity in mammary cancer cells is not known. Retinoids have been shown to modulate the expression of various genes in vivo and in cultured normal and malignant cells (17–19); therefore, we propose that retinoids suppress the expression of the collagenolytic enzyme genes. This contention is supported by indirect evidence based on the lack of inhibitory effect of all-trans-RA on secreted enzymes when added directly into conditioned media from untreated cultures and on the lack of indications for the presence of increased levels of inhibitors in culture media of treated cells when media from untreated and treated cultures were mixed before the degradation assays. More direct evidence is the observation of lower levels of enzymes in substrate-containing gels when cell extracts or media of treated cells were compared to controls. Reduced levels of type IV collagenolytic enzymes may result from either an inhibition of enzyme production, blocking of processing of proenzymes, or both. However, treatment of the samples with metalloproteinase-activating agents, such as 0.1 to 1 mM 4-aminophenylmercuric acetate (48, 55, 56) or mersalyl (57), during the enzyme assay did not significantly affect the zymogram patterns under the conditions used in our experiments (data not shown). Thus, it is likely that the decreased levels of intracellular and extracellular type IV collagenolytic enzyme activity are due to the inhibition of enzyme production.

The secretion of collagenases capable of degrading interstitial collagens (types I to III) has been correlated to the metastatic phenotype of murine mammary tumors (58). Such collagenases have also been found in the plasma membrane of human cancer cells, and it was proposed that the surface enzymes may play an important role in invasion as does secreted collagenase (50). Retinylpalmitate administered in the diet of mice inoculated s.c. with mammary adenocarcinoma cells suppressed tumor growth and metastasis and decreased by 50% the level of tumor-associated type I collagenase (46). It was suggested that vitamin A could be used to inhibit metastasis via suppression of collagenase which results in entrapment of the tumors within a collagen capsule (46). However, this study could not determine whether the collagenase associated with the tumor was derived from the tumor cells themselves or from infiltrating host cells.

Inhibitors of protein synthesis and inhibitors of metallopro-
Carcinoma cells and breast carcinoma lymph node metastases embroyo chorioallantoic membrane by virally transformed rat collagenolysis inhibition, suggesting that the invasion inhibition of native rat lung ECM. However, the levels of invasion inhibition by RA appeared to be higher than those of type IV collagenolysis inhibition, suggesting that the invasion inhibition may also depend on RA effects on various cell activities other than type IV collagenase production (e.g., cell motility).

Previous studies have demonstrated the ability of retinoids to inhibit the growth of murine and human mammary tumor cells in culture (63–66) and in nude mice (66, 67). Retinoids also suppress metastasis of human breast carcinoma transplanted into nude mice (67). Although this study presents results with only one clone of rat mammary adenocarcinoma, our recent studies have demonstrated a similar inhibition of invasion and collagenolysis by RA in several murine and human melanoma cell lines. If the inhibition by retinoids of basement membrane degradation and invasion observed in the present in vitro study can also be achieved in vivo, then retinoids might suppress invasion and metastasis of some tumor cells.

REFERENCES

COLLAGENOLYSIS AND INVASION INHIBITION BY RETINOIC ACID


Inhibition by Retinoic Acid of Type IV Collagenolysis and Invasion through Reconstituted Basement Membrane by Metastatic Rat Mammary Adenocarcinoma Cells

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