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Inhibition of Epithelial Cell Adhesion by Retinoic Acid

Relationship to Reduced Extracellular Matrix Production and Alterations in Ca^{2+} Levels

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Human squamous epithelial cells maintained in growth factor-deficient medium were examined for sensitivity to all-trans retinoic acid (retinoic acid). Under conditions of low external Ca^{2+} (0.15 mmol/l [millimolar]), or high external Ca^{2+} (1.4 mmol/l), retinoic acid stimulated proliferation. Concomitantly, cell–substrate adhesion was decreased. Enzyme-linked immunosorbent assays were used to assess production of two extracellular matrix components, ie, fibronectin and thrombospondin. In the presence of retinoic acid, production of both was decreased. Because both fibronectin and thrombospondin serve as epithelial cell adhesion factors, the decreased production of these moieties could contribute to reduced adhesion. Using $^{45}Ca^{2+}$ to measure total cell-associated Ca^{2+} and the Ca^{2+}-sensitive dye Indo-1 to measure intracellular free Ca^{2+}, it was found that concentrations of retinoic acid that altered cell–substrate adhesion in the squamous epithelial cells had no effect on total, cell-associated Ca^{2+}, but reduced intracellular free Ca^{2+} by 50% to 60%. Because Ca^{2+} is a regulator of adhesion, the ability of retinoic acid to modulate Ca^{2+} levels in the squamous epithelial cells may explain, in part, how retinoic acid influences their adhesiveness. (Am J Pathol 1991, 138:887–895)

Topical treatment with all-trans retinoic acid (retinoic acid) reverses several features of sun-damaged skin.[1–7] This is associated with histologic changes in both the epidermis and the dermis. Epidermal changes include thickening and compaction of the stratum corneum, due in part to increased keratinocyte proliferation, and increased deposition of alcian blue-staining material within the stratum corneum. In contrast, systemic retinoid therapy of psoriasis ameliorates the lesions,[8,9] and this is associated with decreased keratinocyte proliferation. Retinoic acid also may stimulate or inhibit epithelial cell proliferation in culture, depending on the condition of the cells at the time of treatment.[10] Interestingly retinoic acid treatment is associated with a reduction in epithelial adhesiveness. *In vivo*, this is expressed as sensitivity to intraepidermal blister formation and reduction in the water vapor barrier. Both of these effects have been observed after systemic treatment for psoriasis.[8,9,11,12] *In vitro*, one sees increased sensitivity to trypsin/ethylene diamine tetraacetic acid (EDTA)-mediated release from the substratum after retinoic acid treatment.[10] The cellular and molecular basis for these effects on epithelial cell adhesion is not completely understood. The present study was conducted as part of our effort to understand how retinoic acid influences epithelial cell behavior. We show here that retinoic acid inhibition of epithelial cell adhesion is associated with a reduction in the synthesis of two extracellular matrix components, ie, fibronectin and thrombospondin, that serve as epithelial cell adhesion factors.[13–15] Concomitantly retinoic acid reduces the level of intracellular Ca^{2+} in the same cells. Both of these effects could have a profound influence on epithelial cell adhesion.

Materials and Methods

Cells

An established human squamous epithelial cell line derived originally from a tumor of the floor of the mouth was

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used in this study. These cells, designated UM-SCC-1, were provided by Dr. Thomas E. Carey (Department of Otorhinolaryngology, University of Michigan) and have been described in a past report.[16] The cells were maintained in monolayer culture using minimal essential medium (MEM) of Eagle supplemented with Earle's salts, nonessential amino acids, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of fungizone (MEM), and 10% fetal bovine serum (FBS) as culture medium. The cells were grown at 37°C and 5% CO_2 and subcultured by trypsinization as required. Based on reported values for retinoic acid in FBS, cells grown in medium containing 10% FBS are exposed chronically to approximately 5×10^{-9} mol/l (molar) retinoic acid.

For use in experiments, the cells were plated in MEM supplemented with 10% FBS and allowed to attach and spread. They were then washed two times in keratinocyte basal medium (KBM, modified MCDB-153, Clonetics, Inc., San Diego, CA) and incubated in this medium. Keratinocyte basal medium normally contains 0.15 mmol/l (millimolar) Ca^{2+}, and we routinely compared KBM containing 0.15 mol/l Ca^{2+} (low-CA^{2+}) with KBM containing 1.4 mmol/l Ca^{2+} (high-Ca^{2+}). We used MEM supplemented with 10% FBS for initial plating, as we obtained a higher and more uniform plating efficiency in this culture medium.

Retinoic Acid

Retinoic acid was obtained from Ortho Pharmaceutical Co. (Raritan, NJ). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (20 mg/ml) and stored frozen, protected from light. Working solutions were prepared in the appropriate culture medium at the time of use. When cells were treated with retinoic acid, they were protected from light during the incubation period. The final concentration of DMSO in the cultures treated with 1.0 μg/ml of retinoic acid (highest concentration used) was 0.005%. This concentration of DMSO had no detectable effect by itself.

Proliferation Assay

Epithelial cell proliferation was measured by seeding 35-mm (diameter) culture dishes with 1.5×10^5 cells per dish in MEM supplemented with 10% FBS. The cells were allowed to attach and spread in this medium. The dishes were then washed and incubated in either KBM containing 0.15 mmol/l Ca^{2+} (the normal amount present in KBM) or in KBM supplemented with 1.4 mmol/l Ca^{2+}. Cells in each of the groups were left without further treatment or treated with 0.01, 0.1, 0.25, 0.5, or 1.0 μg/ml retinoic acid and incubated at 37°C and 5% CO_2 for an appropriate period. After incubation, the cells were harvested with 0.25% trypsin and counted using an electronic particle counter. Before counting, the trypsinized cells were examined under phase-contrast microscopy to confirm that they were in a single-cell suspension. In addition, samples from each group were stained with trypan blue and counted with a hemocytometer to determine cell viability.

Measurement of Cell–Substrate Adhesion

Sensitivity of the cells to trypsin/EDTA-mediated release from the substratum was used as a measure of adhesion. Cells grown in 35-mm (diameter) dishes were washed two times and exposed to a solution of 0.05% trypsin/0.01% EDTA at 37°C. At various times later, the detached cells were gently harvested and counted. A cell count made when all of the cells had been released was used to determine the total cell count, and the percentage of cells released at each time point was determined from this. In addition to measuring the rate of release from the substratum, samples of the released cells were examined on a hemocytometer slide. This was done to determine the number of single cells and the number of aggregates. We were able to accurately assess single cells, cells in small clumps (2 to 10 cells), and cells in large clumps (greater than 10 cells).

Extracellular Matrix Molecules and Antibodies to Extracellular Matrix Molecules

Extracellular matrix molecules used in these experiments included fibronectin and thrombospondin. Human plasma fibronectin was obtained from GIBCO (Grand Island, NY). When examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, a single band at approximately 200 kd was seen. Thrombospondin was purified in our laboratory from outdated human platelets using heparin–Sepharose affinity chromatography.[15] The purified thrombospondin migrated in SDS-PAGE as a single band with a molecular weight of 170 kd under reducing conditions. Rabbit polyclonal antibodies (gamma-globulin fractions of whole sera) to fibronectin and thrombospondin also were used in these studies. These were obtained and characterized as described.[10,15] Each antibody reacted with its appropriate antigen in enzyme-linked immunosorbent assay (ELISA), but showed no cross-reactivity with the other extracellular matrix component. Normal rabbit globulin was used as a control.

ELISA

Enzyme-linked immunosorbent assays were used to quantify amounts of immunoreactive fibronectin and thrombospondin produced by control and retinoic acid-treated cells. Briefly, cells grown in 35-mm (diameter) culture dishes were washed two times and then incubated for a period in the appropriate culture medium. At the end of the incubation period, the cells were again washed and incubated for an additional 2 hours in the same culture medium. The 2-hour culture fluids were harvested, clarified by low-speed centrifugation, and added to wells of a 96-well plate (Falcon Plastics, Oxnard, CA) from lots that had been prescreened for acceptability in ELISAs. Normally 0.1 ml of culture medium was used per well and the wells were incubated for 4 hours at 37°C. Culture medium alone served as control. Purified fibronectin or thrombospondin (0.5–0.0005 μg per well) in the same medium were also added to each assay plate to serve as standards. After the 4-hour incubation, the culture medium from the cells, the control culture medium, and the standards were removed from the wells and the ELISAs performed as described previously.[17]

Total Cell-associated Ca^{2+}

Total cell-associated Ca^{2+} was determined using $^{45}Ca^{2+}$. $^{45}CaCl_2$ was obtained from ICN (Irvine, CA). Cells were plated at 2.5×10^5 cells/35-mm (diameter) dish in MEM supplemented with 10% FBS. After the cells had a chance to attach and spread, they were washed three times in KBM and incubated in KBM or KBM supplemented with 1.4 mmol/l Ca^{2+}. $^{45}Ca^{2+}$ (0.1 μCi/0.15 μmol) was added to each dish and the cells were then incubated at 37°C and 5% CO_2. At various times later, the culture fluid was removed and the cells rapidly washed three times with cold KBM. The final wash contained 5 mmol/l EDTA. The cells were lysed in phosphate-buffered saline containing three detergents (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) (PBS-TDS), and the total amount of $^{45}Ca^{2+}$ taken up by the cells determined by β-scintillation counting. In some experiments, retinoic acid was added directly to the wells without first removing nonincorporated $^{45}Ca^{2+}$. At various times later, the cells were washed three times with cold low-Ca^{2+} or high-Ca^{2+} KBM (with or without retinoic acid) and lysed in PBS-TDS. The total amount of $^{45}Ca^{2+}$ associated with these cells was determined by β-scintillation counting.

Intracellular Free Ca^{2+}

The Ca^{2+} indicator Indo-1/acetoxymethyl ester was purchased in 50-μg aliquots (special packaging, Molecular Probes, Junction City, OR) and stored desiccated at −20°C. At use, an aliquot was brought up to 1 mmol/l concentration with DMSO, and mixed periodically for 10 minutes to allow complete dissolution. The cells were then loaded with dye; ie, healthy cultures of UM-SCC-1 cells were trypsinized and resuspended in either KBM with 0.15 mmol/l Ca^{2+} or KBM with 1.4 mmol/l Ca^{2+} to a concentration of 2×10^7 cells/ml (note: samples of 10^6 cells were removed and suspended in 2 ml of KBM with 0.15 mmol/l Ca^{2+} or KBM with 1.4 mmol/l Ca^{2+} at this point to use for measurement of autofluorescence). To the remaining cell suspension was added Indo-1 solution to a final concentration of 5 μmol/l (micromolar). The suspensions were mixed and incubated in a 37°C waterbath for 7 minutes, immediately after which the cells were washed twice by dilution 10-fold in KBM with 0.15 mmol/l Ca^{2+} or KBM with 1.4 mmol/l Ca^{2+} and centrifugation at 150*g* to remove unincorporated dye. The labeled cells were resuspended in KBM with 0.15 mmol/l Ca^{2+} or KBM with 1.4 mmol/l Ca^{2+} to a concentration of 1×10^7 cells/ml. These cells were added to polypropylene tubes containing KBM with 0.15 mmol/l Ca^{2+} or KBM with 1.4 mmol/l Ca^{2+} to a final concentration of 10^6 cells in 2 ml, and subjected to various treatments (see Results) before measurement of intracellular Ca^{2+}. No significant leakage of dye was detectable from cells stored up to 1.5 hours at 37°C. Intracellular Indo-1 fluorescence was monitored by placing the cell suspensions in a Farrand System 2 fluorometer (Optical Technology Devices, Valhalla, NY) fitted with monochromators in a T-configuration and a thermostated 1-cm cuvette with stirring at 37°C. The dye was excited at 355 nm and exhibited emission peaks at 405 nm and 485 nm. Because the chelation of Ca^{2+} by the dye shifts the relative intensities of the emissions (405 increases and 485 decreases on binding), the ratio of the two intensities may be used to quantitate cytosolic (Ca^{2+}) independent of the actual dye concentration by the method of Grynkiewicz, Poenie, and Tsien[18]; ie, the basic relationship: $Ca^{2+} = K_dS([R - R_{min}]/[R_{max} - R])$ was used, where R is the ratio of emission (405/485) for the Indo-1–loaded cells after the individual emissions of autofluorescence have been subtracted from the values. In other words,

$$R = \frac{(\text{experimental } 405) - (\text{autofluorescent } 405)}{(\text{experimental } 485) - (\text{autofluorescent } 485)}$$

In like manner R_{min} and R_{max} are calculated using the same cells after lysis with digitonin (final concentration: 40 μmol/l). R_{max} was measured in the presence of 1.5 mmol/l Ca^{2+}, whereas R_{min} was measured with 12.5 mmol/l ethylene glycol tetraacetic acid (EGTA). Of special note: the autofluorescence of cells varies slightly in the presence of digitonin or EGTA; therefore these autofluores-

cence values had to be determined for each experiment in addition to the autofluorescence of cells alone. S is the ratio of $485_{min}/485_{max}$ after subtraction of the appropriate autofluorescence. The published value for the effective dissociation constant of the dye (K_d) of 250 nmol/l was used in calculation.

Results

Suitability of UM-SCC-1 Cells as a Model for Studies on Retinoic Acid Effect

In the first series of experiments, we sought to determine if the UM-SCC-1 cells were similar to normal epithelial cells in their response to retinoic acid. Previously it had been shown that epidermal keratinocytes proliferate when cultured in the presence of a serum-free, low-Ca^{2+} (0.15 mmol/l) growth medium. Addition of excess Ca^{2+} (1.4 mmol/l, final concentration) to the culture medium induced morphologic features of differentiation but did not significantly affect proliferation. In contrast, removal of the growth factors from the culture medium led to a complete cessation of growth coupled with induction of differentiated features.[10] Retinoic acid stimulated proliferation of keratinocytes maintained under growth factor-deficient conditions but did not overcome the effects of elevated Ca^{2+} on differentiation.[10]

Figures 1 and 2 summarize data obtained with the UM-SCC-1 cells. Like their normal counterpart cells, features of differentiation were induced in the UM-SCC-1 cells by elevation of the extracellular Ca^{2+} concentration to 1.4 mmol/l (Figure 1), whereas proliferation was not inhibited (Figure 2). In addition (and most important), retinoic acid stimulated proliferation of UM-SCC-1 cells cultured in KBM in the presence of either low-Ca^{2+} (0.15 mmol/l) or high-Ca^{2+} (1.4 mmol/l) (Figure 2). It thus appears that the UM-SCC-1 cells are similar to normal epidermal keratinocytes in their ability to respond to alterations in the extracellular Ca^{2+} concentration as well as to treatment with retinoic acid.

Effects of Retinoic Acid on Cell–Substrate Adhesion in UM-SCC-1 Cells

Retinoic acid was examined next for effects on UM-SCC-1 adhesion to the substratum. Using sensitivity to trypsin/EDTA-mediated release from the substratum as a measure of adhesiveness, treatment with retinoic acid

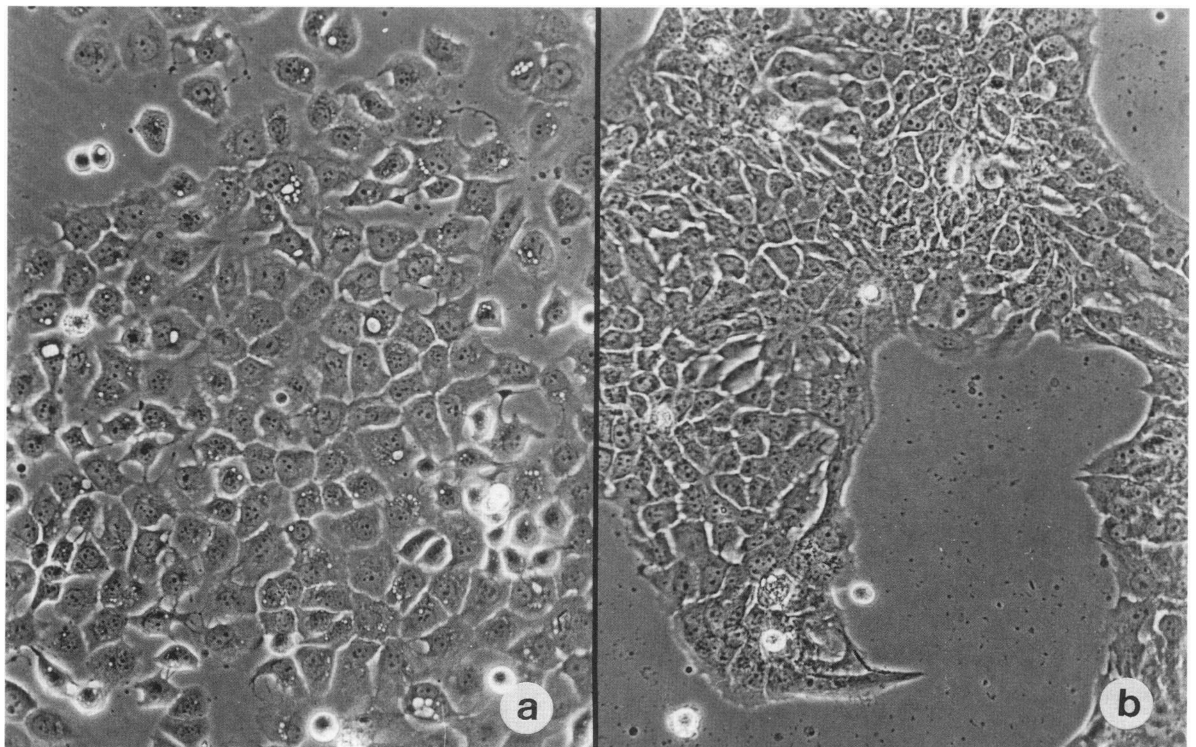

Figure 1. *Morphologic appearance of UM-SCC-1 cells grown in KBM* (**A**) *and KBM supplemented with 1.4 mmol/l Ca^{2+}* (**B**). *Phase-contrast photomicrographs were obtained 1 day after the beginning of the incubation period (magnification × 230).*

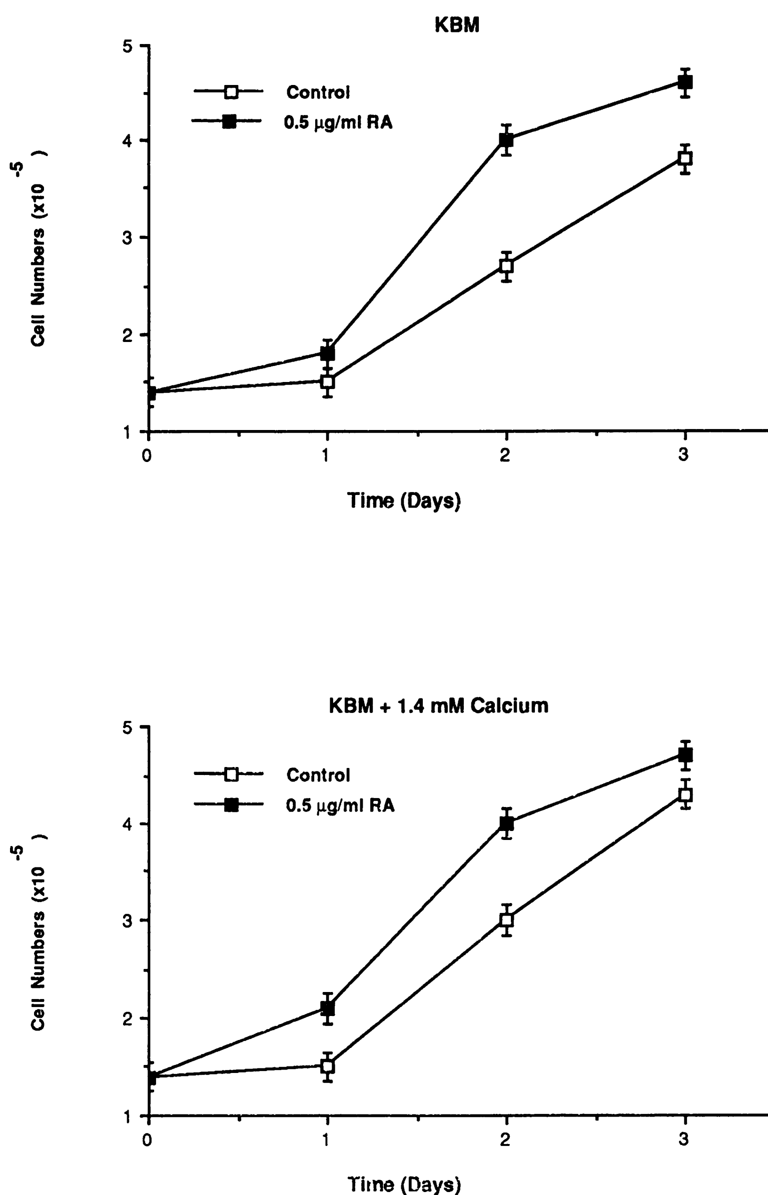

Figure 2. *Effect of retinoic acid on UM-SCC-1 cell proliferation in KBM and KBM containing 1.4 mmol/l Ca^{2+}. Proliferation was measured as described in Materials and Methods. Values shown represent averages ± standard deviations based on triplicate samples in a single experiment. The experiment was repeated four times with similar results.*

(0.5 or 1 μg/ml) was found to decrease adhesion significantly (Figure 3). Increased sensitivity to trypsin/EDTA was evident as early as 4 hours after treatment. Interestingly it can be seen in Figure 3 that the cells not treated with retinoic acid demonstrated increasing resistance to trypsin/EDTA-mediated release from the substratum as a function of time in culture. It was actually the increase in resistance that was blocked by retinoic acid.

In additional studies, cells were incubated in KBM containing 1.4 mmol/l Ca^{2+} for the same periods and then examined for sensitivity to trypsin/EDTA. In the presence of the elevated Ca^{2+} concentration, responsiveness to trypsin/EDTA was retarded slightly. Even in the presence of the higher Ca^{2+} concentration, however, retinoic acid was still able to increase the sensitivity of the cells to trypsin/EDTA-mediated release from the substratum (not shown). Although increasing the Ca^{2+} concentration had little effect on sensitivity of the cells to trypsin/EDTA-mediated release from the substratum, the concentration of Ca^{2+} in the external environment had a dramatic effect on cell–cell interactions. In the presence of 0.15 mmol/l Ca^{2+}, virtually all of the cells detached as single cells (greater than 90%). The remainder were released in clumps of 2 to 10 cells. In contrast, when the Ca^{2+} concentration was increased to 1.4 mmol/l, most of the cells (approximately 70%) were released in clumps. The presence or absence of retinoic acid had no effect on this. Thus, it appears that retinoic acid and Ca^{2+} both influence UM-SCC-1 cell adhesion, but primarily affect different aspects of the adhesion process.

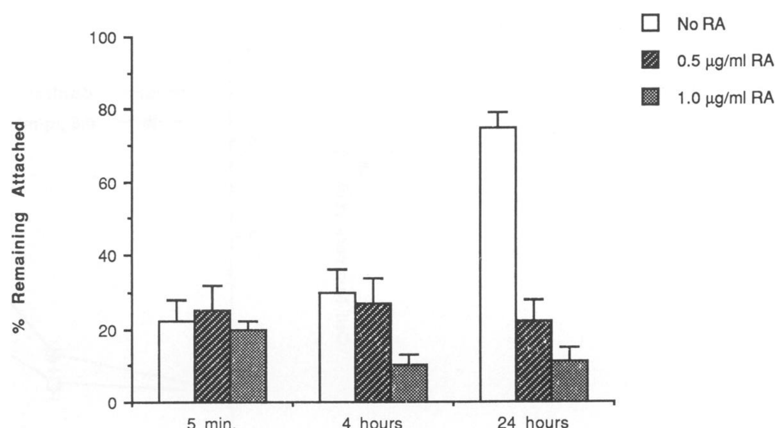

Figure 3. *Effects of retinoic acid on sensitivity of UM-SCC-1 cells to trypsin/EDTA-mediated release from the substratum. Trypsin/EDTA-mediated release from the substratum was assessed at various times after treatment with retinoic acid as described in Materials and Methods. Values represent the average percentage of cells remaining attached ± the differences between individual values and averages 8 minutes after exposure to trypsin/EDTA. The values are based on duplicate samples in a single experiment. The experiment was repeated six times with similar results.*

Effects of Retinoic Acid on Extracellular Matrix Production by UM-SCC-1 Cells

Next we examined UM-SCC-1 cells for production of two extracellular matrix components (fibronectin and thrombospondin) under control conditions and after treatment with retinoic acid. As shown in Table 1, fibronectin and thrombospondin production was reduced in the presence of retinoic acid. Time-course studies showed that reduction in fibronectin and thrombospondin synthesis could be seen as early as 4 hours after retinoic acid treatment (not shown). Thus the decrease in extracellular matrix production after retinoic acid treatment parallels the loss of adhesiveness after the same treatment.

Modulation of Total Cell-associated and Intracellular Ca^{2+}

Because both retinoic acid and extracellular Ca^{2+} influenced UM-SCC-1 adhesion, studies were carried out to determine if these treatments altered cell-associated or intracellular Ca^{2+} levels. Cell-associated Ca^{2+} was assessed as the amount of $^{45}Ca^{2+}$ bound to monolayers of UM-SCC-1 cells when incubated with the radioactive Ca^{2+} for 1 day in KBM containing either 0.15 mmol/l or 1.4 mmol/l Ca^{2+}. As indicated in Table 2, when the extracellular Ca^{2+} concentration was elevated from 0.15 mmol/l to 1.4 mmol/l (a ninefold increase), cell-associated Ca^{2+} increased from 0.51 nmoles/10^6 cells to 3.73 nmoles/10^6 cells (a sevenfold increase). In contrast, incubating the cells with 0.5 μg/ml of retinoic acid for 1 day had virtually no effect on total cell-associated Ca^{2+} (Table 2). In additional studies, it was shown that retinoic acid had no effect on cell-associated Ca^{2+} when examined over a range of concentrations from 0.1 to 1.0 μg/ml and also had no effect when examined in the presence of KBM containing 1.4 mmol/l Ca^{2+} (not shown).

In addition to measuring cell-associated Ca^{2+}, we also examined the effects of altering the extracellular Ca^{2+} concentration and adding retinoic acid to the culture medium on intracellular free Ca^{2+} levels. Intracellular Ca^{2+} levels were measured using the Ca^{2+} indicator Indo-1 as described in Materials and Methods. When the extracellular Ca^{2+} was increased from 0.15 to 1.4 mmol/l, the intracellular free Ca^{2+} level increased from 77 nmol/l, to 220 nmol/l, a 2.9-fold increase (Table 2). The data for

Table 1. *Effects of Retinoic Acid on Fibronectin and Thrombospondin Production by UM-SCC-1 Cells*

	ng/ml†	
Treatment group*	Fibronectin	Thrombospondin
KBM	1900 ± 150	500 ± 70
+0.01 μg/ml RA	Not Done	510 ± 50
+0.1 μg/ml RA	800 ± 45	320 ± 20
+0.25 μg/ml RA	750 ± 30	100 ± 10
+0.5 μg/ml RA	700 ± 10	<10
+1.0 μg/ml RA	750 ± 25	<10

* Cells were plated at 1 × 10^5 cells/well in MEM containing 10% fetal bovine serum and allowed to attach and spread. They were then washed two times, incubated for 1 day in KBM, and treated with retinoic acid. The cells were then washed two times and incubated for an additional 2-hour period. The 2-hour culture supernatant fluids were harvested and assayed for fibronectin or thrombospondin by ELISA.

† Values represent ng of fibronectin or thrombospondin produced ± differences between individual values and averages based on duplicate samples in a single experiment. The experiment was repeated three times with similar results.

Table 2. *Effects of Retinoic Acid and Extracellular Ca^{2+} Concentration on Cell-associated Ca^{2+} and Intracellular Free Ca^{2+} in UM-SCC-1 Cells*

Treatment	Cell-associated Ca^{2+} (nmol/10^6 cells)*	Intracellular free Ca^{2+} (nmol/l)†
KBM	0.51 ± 0.01	77 ± 28
KBM + 0.5 μg/ml RA	0.53 ± 0.05	36 ± 17
KBM + 1.0 μg/ml RA	0.63 ± 0.06	40 ± 20
KBM + 1.4 mmol/l Ca^{2+}	3.73 ± 0.34	220 ± 80

* Total cell-associated Ca^{2+} was measured as described in Materials and Methods. The values shown represent averages ± standard deviations based on three samples in a single experiment. The experiment was repeated three times with similar results.

† Intracellular free Ca^{2+} was measured as described in Materials and Methods. The values shown represent averages ± standard deviations based on three separate experiments.

this table is based on three independent experiments in which cells were incubated for 1 day in the desired amount of extracellular Ca^{2+} and then assayed. To confirm this difference, cells that had been incubated for 1 day in KBM (0.15 mmol/l) were harvested with trypsin and examined for intracellular free Ca^{2+} in the normal manner. When stable emission intensities were attained at 405 and 485 nm, Ca^{2+} was added directly to the cells in the cuvette to bring the extracellular Ca^{2+} concentration to 1.4 mmol/l. Emission intensities at 405 and 485 nm were measured until they were again stable. The experiment was concluded at that point and intracellular free Ca^{2+} levels estimated for both external Ca^{2+} concentrations from the cells in the single cuvette. The data from this experiment showed that the level of intracellular free Ca^{2+} increased by approximately fourfold when the external Ca^{2+} concentration was increased from 0.15 to 1.4 mmol/l (42 nmol/l to 170 nmol/l). Furthermore this experiment showed that the re-equilibration occurred very rapidly (within 7 to 10 minutes) after addition of Ca^{2+} to the cuvette (Figure 4).

The effect of retinoic acid on intracellular free Ca^{2+} in the UM-SCC-1 cells was examined next. Cells were maintained for 1 day in KBM as described above and then harvested with trypsin. The harvested cells were aliquoted into separate tubes and treated for 1 hour with KBM alone or with KBM containing 0.5 μg/ml retinoic acid. Intracellular free Ca^{2+} levels were then determined in the normal manner. As seen in Table 2, treatment of cells for 1 hour with 0.5 μg/ml of retinoic acid resulted in a decrease in the intracellular free Ca^{2+} concentration. In additional experiments, cells maintained for 1 day in KBM alone were harvested with trypsin and assayed for intracellular free Ca^{2+} in the normal manner. When emission intensities at 405 and 485 nm had stabilized, retinoic acid (0.5 μg/ml, final concentration) was added directly to the cuvettes containing the Indo-1–loaded cells. When the emission intensities at 405 and 485 nm had again stabilized, the experiment was concluded. Values obtained with cells from the same cuvette were then used to determine the effects of retinoic acid on intracellular free Ca^{2+} levels. The results of this experiment (Figure 5) supported the data presented in Table 2. That is, the intracellular free Ca^{2+} level decreased in the UM-SCC-1 cells after addition of retinoic acid. Further, it can be seen that the reduction in intracellular Ca^{2+} occurred within 10 to 15 minutes after treatment.

Discussion

In this study we have examined the effects of retinoic acid on cell–substrate adhesion in a human squamous carcinoma cell line. This cell line, UM-SCC-1, behaves in many respects in a manner similar to that of normal keratinocytes. Both the normal keratinocytes and the UM-SCC-1 cells grow as undifferentiated cells in serum-free culture medium when the Ca^{2+} concentration is 0.15 mmol/l. When the extracellular Ca^{2+} concentration is raised to 1.4 mmol/l, the cells continue to proliferate but express morphologic features of differentiated cells. Retinoic acid stimulates proliferation of both cell types in se-

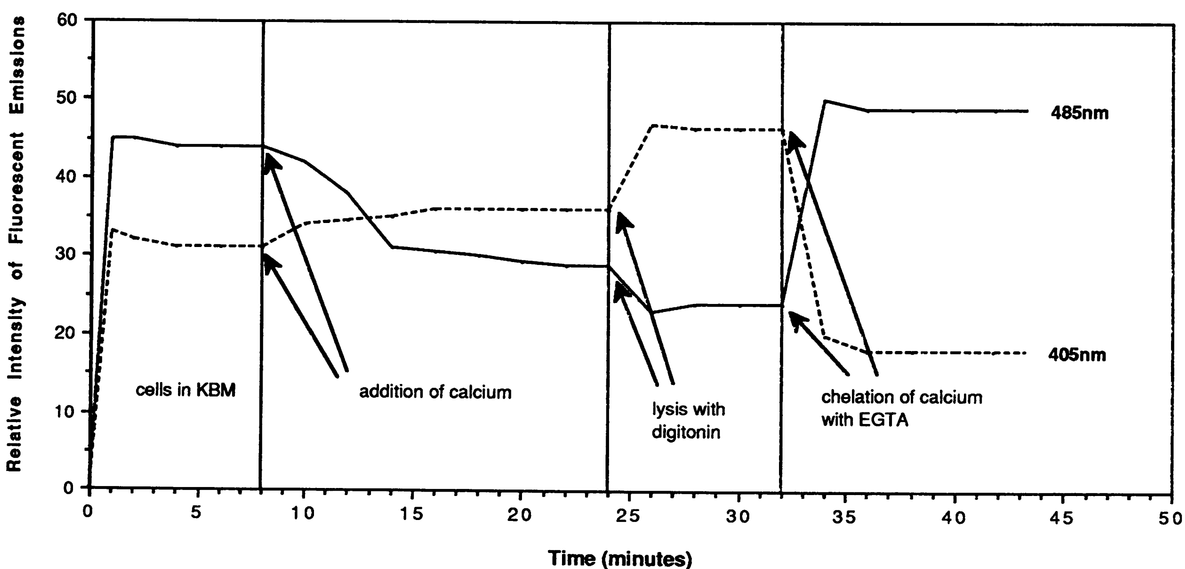

Figure 4. *Chart-strip showing changes in fluorescent emissions at 405 and 485 nm of Indo-1–loaded UM-SCC-1 cells on alteration of the extracellular Ca^{2+} concentration. 405:485 ratios were determined for cells preincubated for 1 day in KBM and examined sequentially in KBM* (A), *after addition of Ca^{2+} to the cuvette to bring the extracellular Ca^{2+} to 1.4 mmol/l* (B), *after addition of digitonin to lyse cells and determine R_{max}* (C) *and after addition of EGTA to chelate Ca^{2+} and determine R_{min}* (D). *Based on the formula provided in Materials and Methods, the intracellular Ca^{2+} concentration was estimated to be 42 nmol/l for cells in KBM alone and 173 nmol/l after addition of 1.4 mmol/l Ca^{2+}.*

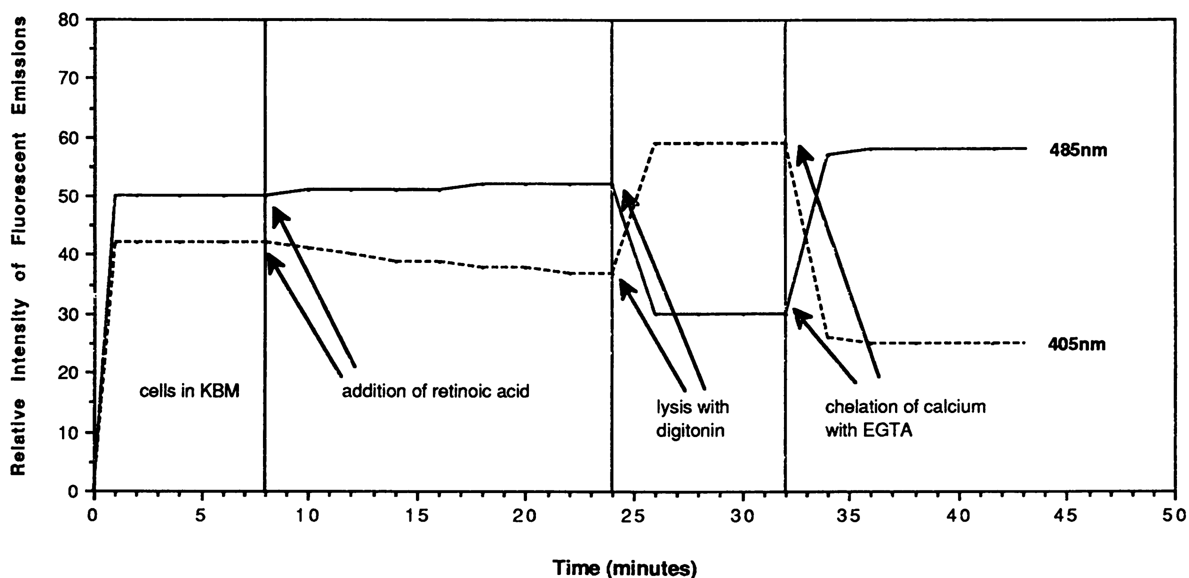

Figure 5. *Chart-strip showing changes in fluorescent emission at 405 and 485 nm of Indo-1–loaded UM-SCC-1 cells on addition of 0.5 μg/ml retinoic acid. 405:485 ratios were determined for cells preincubated for 1 day in KBM and examined sequentially in KBM* (A), *after addition of 0.5 μg/ml of retinoic acid to the cuvette* (B), *after addition of digitonin to lyse cells and determine R_{max}* (C), *and after addition of EGTA to chelate Ca^{2+} and determine R_{min}* (D). *Based on the formula provided in the Materials and Methods section, the intracellular Ca^{2+} concentration was estimated to be 77 nmol/l for cells in KBM and 51 nmol/l after addition of retinoic acid.*

rum-free, growth factor-deficient medium but does not reverse the morphologic features associated with elevation of the external Ca^{2+} level (see reference 10 for keratinocyte data and Figures 1 and 2 for data with UM-SCC-1 cells). In addition, it was shown recently that treatment with a combination of interferon-γ and tumor necrosis factor-α inhibits proliferation and induces differentiation in both normal keratinocytes and UM-SCC-1 cells.[19,20] Finally, the data from the present study indicate that the UM-SCC-1 cells are similar to normal keratinocytes in their reduced cell–substrate adhesion after exposure to retinoic acid. Thus we believe that the UM-SCC-1 cells may serve as a useful model for investigating the effects of retinoic acid on epithelial cell behavior and that the data obtained with these cells may be applicable to normal keratinocytes as well as to the UM-SCC-1 cells themselves. It should be noted that these responses to retinoic acid appear to be specific to epithelial cells. In fibroblasts, retinoic acid stimulates proliferation only in the presence of a suboptimal concentration of extracellular Ca^{2+}.[21,22] Furthermore fibroblast adhesion is stimulated rather than inhibited by retinoic acid.[23,24]

How retinoic acid modulates epithelial cell adhesion is not known. There are a number of possibilities. Both fibronectin and thrombospondin are potent epithelial cell adhesion factors,[13–15] and the decreased production of these matrix components could contribute to the decreased adhesion seen after retinoic acid treatment. It is interesting in this regard that decreased matrix production and decreased adhesion were observed within 4 hours of treatment with retinoic acid. Alternatively the decrease in adhesion seen after retinoic acid treatment could be a result of altered Ca^{2+} availability. There are a number of ways in which reduced Ca^{2+} could influence cell–substrate adhesion. Thrombospondin, for example, is known to undergo Ca^{2+}-dependent conformational changes[25] that alter its ability to support adhesion. Furthermore adhesion receptors have an absolute requirement for divalent cations,[26] and the reduction in Ca^{2+} levels after retinoic acid treatment could alter adhesive interactions mediated by these proteins. Changes in intracellular Ca^{2+} levels were observed within 10 to 20 minutes after treatment with retinoic acid and preceded detectable effects on adhesion. Perhaps a combination of effects is responsible for the alterations in adhesion seen after retinoic acid treatment.

Much additional work remains to be done. In addition to the obvious questions raised by these observations, it still needs to be determined if the alterations in sensitivity to trypsin/EDTA-mediated release from the substratum induced by retinoic acid are relevant to the mechanism by which retinoic acid modulates adhesion *in vivo*. It has been previously shown that systemic retinoid treatment for psoriasis leads to decreased permeability barrier function and decreased epidermal cohesion.[8,9,11,12] The changes noted here in culture are consistent with these previous *in vivo* observations, but whether the *in vivo* effects of retinoic acid are associated with reduced matrix production or a reduction in intracellular Ca^{2+} remain to be seen.

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