American Journal of Pathology, Vol. 149, No. 1, July 1996

Retinoic Acid Induces Cells Cultured from Oral Squamous Cell Carcinomas to Become Anti-Angiogenic

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Retinoids have shown great promise as chemopreventive agents against the development of squamous cell carcinomas of the upper aerodigestive tract. However, the exact mechanism by which they block new tumors from arising is unknown. Here, we report that 13-cis- and all-trans-retinoic acid, used at clinically achievable doses of 10^{-6} mol/L or less, can directly and specifically affect cell lines cultured from oral squamous cell carcinomas, inducing them to switch from an angiogenic to an anti-angiogenic phenotype. Although retinoic-acid-treated and untreated tumor cells make the same amount of interleukin-8, the major inducer of neovascularization produced by such tumor lines, they vary in production of inhibitory activity. Only the retinoic-acid-treated cells produce a potent angio-inhibitory activity that is able to block in vitro migration of endothelial cells toward tumor cell conditioned media and to halt neovascularization induced by such media in the rat cornea. Anti-angiogenic activity is induced in the tumor cells by low doses of retinoids in the absence of toxicity with a kinetics that suggest that it could be contributing to the effectiveness of the retinoids as chemopreventive agents. (Am J Pathol 1996, 149:247–258)

Squamous cell carcinomas of the head and neck are particularly devastating due to a high frequency of recurrence after successful primary therapy. Not only does the original tumor often recur, but second primary tumors can also arise and are major contributors to treatment failure, especially in individuals who present with early-stage disease.[1] These secondary tumors are clearly independent from the initial primary lesion[2–4] and are thought to result from field cancerization,[5] a process by which multiple precancerous cells are induced throughout the oral mucosa as a result of exposure to carcinogens, especially those in alcohol and tobacco.[6] Retinoic acid is the only agent that has been able to halt the development of these second primary tumors in clinical trials.[7,8] Retinoic acid is also effective against oral leukoplakias,[9] some of which progress to malignancy, but it is seldom effective as a chemotherapeutic agent for the primary squamous cell carcinomas themselves.[9,10] There is not yet a clear understanding of how retinoic acid (RA) is acting to prevent additional primary oral squamous cell carcinomas from developing or why it is particularly effective against this tumor type.[11]

Current data suggest four possible mechanisms by which retinoids may be inhibiting oral squamous cell carcinomas. They may act by modulating tumor cell differentiation. Retinoids alter the expression of differentiation markers by cells cultured from tumors of the upper respiratory tract when they are treated *in vitro*[12,13] or *in vivo*.[14] Although retinoids increase the differentiation of hematological malignancies,[15] they consistently halt or decrease the differentiation of squamous cell carcinomas. Such a decrease might be expected to stimulate rather than limit tumor growth. In fact, one *in vivo* study has shown that the ability of retinoids to affect differentiation did not correlate with their ability to limit tumor growth.[14] Second, retinoids may directly inhibit tumor cell

Supported by National Institutes of Health grants K15 DE00313 (M. W. Lingen), RO1 HL 39926 (P. J. Polverini), RO1 CA52750 and R01 CA64239 (N. P. Bouck), and 1-P30 CA 60553 (R. H. Lurie Cancer Center).

Accepted for publication March 11, 1996.

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growth. Although they enhance the growth of normal mucosal cells, retinoids inhibit the proliferation of many cultured human tumor lines derived from oral squamous cell carcinomas growing on plastic, in agar, or as spheroids.[16] However, these effects are variable and somewhat modest, averaging approximately a 50% reduction in growth over 7 days. Third, retinoids may stimulate the apoptotic cell death of tumor cells. Indirect evidence for this phenomenon comes from a variety of different human lines from head and neck cancers that, when treated with RA while growing as spheroids, slow their growth significantly without a decrease in [^{3}H]thymidine labeling or in S phase fraction.[17] The hypothesis that apoptosis is responsible for the observed cell loss has yet to be tested directly.

Finally, retinoids may inhibit tumor angiogenesis. All tumors depend on new vessels to grow progressively to a size of clinical significance.[18] The inhibition of new vessels is especially effective at halting the development of microscopic nests of tumor cells[19,20] that are similar to the preclinical lesions that are presumably the targets of chemopreventive agents. Retinoids are effective anti-angiogenic agents. They have been shown to inhibit embryonic neovascularization on the chick chorioallantoic membrane,[21,22] to decrease the vascular response to a mouse carcinoma implanted in the avascular rabbit cornea,[23] and to lower the number of vessels growing toward a variety of different carcinoma cell lines implanted into the mouse skin.[24–27]

We have been investigating the mechanisms by which retinoids may be influencing angiogenesis induced by human squamous cell carcinomas of the oral cavity. The present paper examines the direct effect of retinoids on the angiogenic phenotype of the tumor cells themselves. Like all solid tumors, oral squamous cell carcinomas are well vascularized *in situ* where high vessel counts are correlated with poor outcome.[28,29] Tumor xenografts are potently angiogenic.[30] When injected intradermally into the flanks of nude mice, tumor cell lines derived from other epidermal tissues rapidly attract new vessels. Intradermal vascularity can be inhibited by prior treatment of the tumor cells with RA.[25,26] This result has been interpreted as indicating that the drug reduces the production of inducers of angiogenesis by the tumor cells. Here we report on the direct effects of RA on the angiogenic phenotype of tumor cell lines derived specifically from oral squamous cell carcinomas. We show that the major inducers of neovascularization elaborated by these lines are interleukin (IL)-8 and transforming growth factor (TGF)-β. RA treatment caused these oral squamous cell carcinoma lines to lose their angiogenic activity. Yet, in this cell type, no loss of inducers of angiogenesis was detected. Rather, RA induced the tumor cells to secrete inhibitor(s) of angiogenesis. The kinetics of the appearance of this inhibitory activity suggested that it may be contributing to the chemopreventive activity of the retinoids against oral squamous cell carcinomas.

Materials and Methods

Cell Culture

Human squamous cell carcinoma cell lines SCC-4, SCC-9, SCC-15, and SCC-25 were purchased from the American Type Culture Collection (Rockville, MD). The OSCC-3 cell line was cultured using standard techniques[31] at Northwestern University from a squamous cell carcinoma of the ventral tongue from a tumor specimen kindly provided by Harold J. Pelzer, Northwestern University. These cells were grown in Dulbecco's modified Eagle's medium (DME)/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum, hydrocortisone (0.4 μg/ml), penicillin (100 U/ml), and streptomycin (50 μg/ml). HaCat, an immortal human keratinocyte cell line[32] (kindly provided by Brian Nickoloff, University of Michigan), was grown in DME supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (50 μg/ml). The normal human oral keratinocyte cell strain NHOK-1 was cultured for these studies as described[31] from a biopsy of normal human buccal mucosa provided by Harold J. Pelzer. Cells were grown in K-STIM media (Collaborative Biomedical Products, Bedford, MA) and used before passage 3. All keratinocytes were cultured at 37°C in a 5% CO_2/95% air environment in humidified incubators.

Bovine adrenal microvascular endothelial cells, BP10T8, a kind gift of Judah Folkman, Harvard University, were grown in DME with 10% donor calf serum, endothelial cell mitogen (100 μg/ml; Biomedical Technologies, Stoughton, MA), glutamine (200 mmol/L), penicillin (100 U/ml), and streptomycin (50 μg/ml). Endothelial cells were cultured at 37°C in an 8% CO_2/92% air environment in humidified incubators.

The human fibrosarcoma (HT1080), human colon adenocarcinoma (HT29), and the human breast carcinoma (MDA-MB468) cell lines were purchased from American Type Culture Collection. These cell lines were grown in DME supplemented with 10% fetal calf serum, penicillin (100 U/ml), and strepto-

mycin (50 μg/ml) and cultured at 37°C in a 5% CO_2/92% air environment in humidified incubators.

For treatment of cells with RA, stocks of 10^{-1} mol/L all-*trans*-RA or, where indicated, 13-*cis*-RA (Sigma Pharmaceuticals, St. Louis, MO) were prepared in dimethylsulfoxide and stored at −20°C. The desired dilutions were made directly into the culture medium, keeping the final concentration of dimethylsulfoxide at less than 0.1%. Cells were treated with RA for the times and concentrations indicated for each individual experiment and refed with fresh media containing RA every second day. Control cultures were treated and refed with 0.1% dimethylsulfoxide alone. Viability of the RA-treated cells was determined in two ways. To assess plating efficiency in the drug, cells were plated in triplicate at 200 cells/plate in media containing varying concentrations of RA, and colonies were counted after 6 days. At high doses, the size of the clones was decreased as well as their number, but this effect was seen only at concentrations greater than 10^{-5} mol/L. In addition, cells exposed to RA for 6 days in a monolayer were harvested, and viability was determined by trypan blue exclusion. The percent survival for each concentration of RA was similar for both experiments. Data from the plating efficiencies are reported in Figure 4.

Serum-free CM was generated by rinsing the cells with DME three times, incubating them in DME/F-12 or DME for 4 hours, refeeding them with DME/F-12 or DME, and collecting the CM 24 hours later. The CM were concentrated and dialyzed using Centriprep-3 concentrators (Amicon, Beverly, MA). Protein concentrations were determined using the Coomassie protein assay reagent 23200 (Pierce Biomedical Co., St. Louis, MO). Media were tested at 1 μg total protein/well in the migration assay. For cells treated with RA, the drug was present throughout. Add-back experiments were performed to ensure that the concentration and dialysis of CM from RA-treated cells resulted in the complete removal of RA. CM from untreated SCC-9, RA-treated SCC-9, and untreated SCC-9 to which 1 μmol/L all-*trans*-RA was added back after collection were concentrated, dialyzed, and assayed for their ability to stimulate endothelial cell migration. The SCC-9 CM and the SCC-9 CM with the added back RA induced migration of 61 cells per 10 high power fields ± 7 and 57 cells per 10 high power fields ± 5, respectively, whereas the CM from the RA-treated SCC-9 cells induced migration to 11 cells per 10 high power fields ± 6.

Endothelial Cell Migration Assay

The endothelial cell migration assay was performed as previously described.[33] BP10T8 cells were starved overnight in DME containing 0.1% bovine serum albumin (BSA). Cells were then harvested with trypsin and resuspended in DME with 0.1% BSA at a concentration of 1.5×10^6 cells/ml. Cells were added to the bottom of a 48-well modified Boyden chamber (Nucleopore Corp., Cabin John, MD). The chamber was assembled and inverted, and cells were allowed to attach for 2 hours at 37°C to polycarbonate chemotaxis membranes (5-μm pore size) that had been soaked in 0.1% gelatin overnight and dried. The chamber was then reinverted, and test substances, tested in quadruplicate (1 μg total protein per test compound in a volume of 50 μl) were added to the wells of the upper chamber and the apparatus was incubated for 4 hours at 37°C. Membranes were recovered, fixed, and stained, and the number of cells that had migrated to the upper chamber per 10 high power fields were counted. Background migration to DME plus 0.1% BSA was subtracted, and the data were reported as the number of cells migrated per 10 high power fields (×400). The results of migration assays that are expressed as cells migrated per 10 high power fields represent data obtained from a single experiment. When data from experiments done on different days were combined for display purposes, data were expressed as the percentage of maximal migration to a positive control. In all cases, bars indicate standard errors based on an n of 4 derived from quadruplicate testing of each sample. All experiments were repeated one or more times with similar results. Growth factors were used where indicated at the following concentrations: acidic fibroblast growth factor (aFGF; 50 ng/ml), basic (b)FGF (15 ng/ml), IL-8 (40 ng/ml), TGF-β (1 pg/ml), vascular endothelial growth factor (VEGF; 100 pg/ml), and platelet-derived growth factor (PDGF; 250 pg/ml). Neutralizing antibodies were used at the following concentrations: anti-aFGF (25 μg/ml), anti-bFGF (20 μg/ml), anti-IL-8 (20 μg/ml), anti-TGF-β (10 μg/ml), anti-VEGF (20 μg/ml), and anti-PDGF (15 μg/ml). Optimal concentrations for growth factors and antibodies were determined previously by dose-response experiments (data not shown). All growth factors and neutralizing antibodies were obtained from R&D Systems, Minneapolis, MN. The antibodies were added to the CM just before testing. All antibodies were tested alone and found to be neutral in that they neither stimulated nor reduced basal levels of migration.

Corneal Neovascularization Assay

In vivo angiogenic activity was assayed in the avascular cornea of F344 female rats (Harlan Labs, Madison, WI) as previously described.[33] Briefly, test substances were combined with sterile Hydron casting solution (Interferon Sciences, New Brunswick, NJ), and the solution was pipetted onto the surface of Teflon rods (DuPont Corp., Wilmington, DE). The pellets were air dried in a laminar flow hood for 1 hour and refrigerated overnight. The following day, pellets were rehydrated with phosphate-buffered saline (PBS) and inserted into a surgically created corneal pocket within 1.5 mm of the limbus. Corneas were observed every other day until day 7 when the animals were anesthetized and perfused with PBS followed by colloidal carbon to stain the vessels. Responses were scored as positive when vigorous sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were detected. Negative responses were recorded when no growth was detected or when there was only an occasional sprout or hairpin loop with no evidence of sustained growth. Negative controls consisted of Hydron pellets containing DME/F-12 alone. Media were incorporated into pellets at a total concentration of 1 μg of total protein per cornea. Anti-IL-8 was used at a concentration of 50 ng/5-μl pellet.

Determination of IL-8 Content in Conditioned Media

The amount of IL-8 present in the CM of the untreated and RA-treated tumor cells was determined using a Quantikine immunoassay kit (R&D Systems). Briefly, 1 μg of total protein from each CM sample was assayed in duplicate for the presence of IL-8 using the sandwich enzyme-linked immunosorbent assay technique. The average of the two assays is presented. Individual assays of the same sample varied from one another by less than 10%.

Results

Normal and Immortal Keratinocytes Are Anti-Angiogenic

Whether or not angiogenesis occurs depends on the relative levels of inducers and inhibitors of neovascularization present in the environment around endothelial cells.[34] Like most normal healthy tissues, there is usually no neovascularization occurring within the oral mucosa and its underlying stroma. This lack of angiogenesis is presumably due in part to the predominance of inhibitors. To determine whether the normal epithelial cells that give rise to squamous cell carcinomas secrete molecules that inhibit angiogenesis, serum-free CM were collected from a strain of normal oral keratinocytes, NHOK-1, and from the immortal keratinocyte line HaCat. When tested alone, these media did not induce the migration of capillary endothelial cells (Figure 1, left hand bars) or stimulate corneal neovascularization (Table 1). Both cells were producing high levels of one or more molecules that inhibited angiogenesis. When their media was mixed 1:1 with media derived from

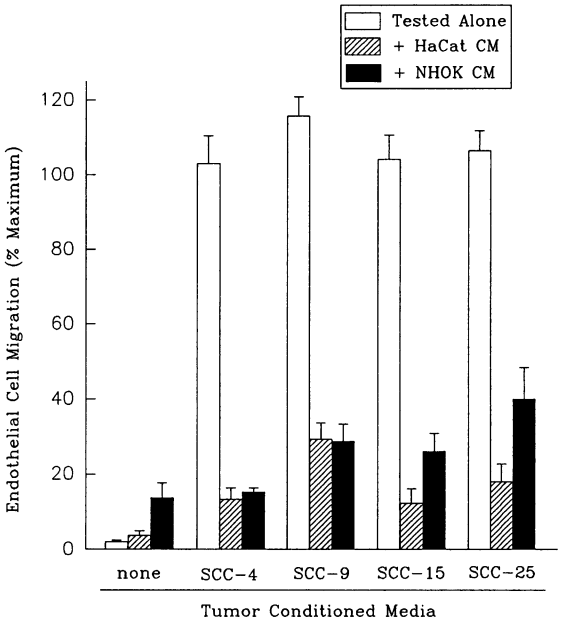

Figure 1. *Normal and immortal keratinocytes produced an inhibitor of angiogenesis. CM from normal (NHOK) or immortal (HaCat) keratinocytes were mixed in a 1:1 ratio with CM from the indicated squamous cell carcinoma cell lines and assayed for ability to stimulate or inhibit endothelial cell migration. Values are reported as a percentage of maximal migration to bFGF ± SEM.*

Table 1. *Corneal Neovascularization in Response to Tumor Cell Conditioned Media*

Media source	Positive corneas/number of implants (%)	
	Tested alone	Tested + CM from nontumorigenic cells*
Control		
DME	0/3 (0)	0/5 (0)
Tumor CM		
SCC-4	5/5 (100)	0/4 (0)
SCC-9	10/10 (100)	0/4 (0)
SCC-15	6/6 (100)	0/3 (0)
SCC-25	6/6 (100)	0/3 (0)
OSCC-3	7/7 (100)	1/5 (20)

*HaCat.

tumor cell lines, it was able to block angiogenesis whether measured by endothelial cell migration *in vitro* (Figure 1) or corneal neovascularization *in vivo* (Table 1). Although the HaCat cells can produce several potentially angiogenic factors including TGF-β,[35] IL-8,[36] and VEGF,[37] their CM was not angiogenic. This is likely due to the fact that 1) the cells are secreting high levels of an inhibitor, 2) levels of IL-8 were low in these unstimulated cells (see Table 4) as has been seen by others,[36] and 3) the levels of TGF-β secreted by this cell line is primarily in an inactive form.[37]

Human Oral Squamous Cell Carcinoma Lines Are Angiogenic Due to the Secretion of IL-8 and TGF-β

Media conditioned by cell lines derived from oral squamous cell carcinomas were potently angiogenic *in vitro* (Figure 1) and *in vivo* (Table 1). Neutralizing antibodies to a series of known angiogenic factors were tested to determine the molecules primarily responsible for this activity. Neutralizing antibodies to the angiogenic factor IL-8[38] resulted in a dramatic reduction in ability of media conditioned by three different tumor lines to induce endothelial cell migration (Figure 2A) and almost completely suppressed the ability of these media to induce corneal neovascularization (Table 2). In the quantitative *in vitro* assay, neutralizing IL-8 resulted in a reduction in activity of between 69 and 73%. The activity of these media could also be modestly reduced by antibodies that neutralized TGF-β1 (Figure 2A). When both IL-8 and TGF-β1 were neutralized, activity fell to background levels in all cases (Figure 2A). Neutralizing antibodies to other common angiogenic factors, including aFGF, bFGF, VEGF, and PDGF, had no effect on the inducing activity of any of the tumor cell CM (Figure 2, B and C). Pan-TGF-β antisera that recognizes all isoforms of TGF-β was no more effective than anti-TGF-β1 antibodies in reducing inducing activity (data not shown). The weak inducer of angiogenesis, TGF-α,[39] can also be produced by oral squamous cell carcinomas. However, an enzyme-linked immunosorbent assay indicated that it was present at a concentration of less than 15 ng/ml in the CM of the SCC-4, SCC-9, and SCC-25 (data not shown). This concentration is too low to induce either endothelial cell migration or *in vivo* neovascularization in our assays.

Retinoic Acid Induces an Anti-Angiogenic Phenotype in Oral Squamous Carcinoma Lines

When tumor cell lines were treated with RA, they lost their ability to induce angiogenesis in a dose-dependent manner (Figure 3). Cells were grown in various concentrations of RA for 7 days, and their secretions collected as serum-free CM. The ability of these media to induce endothelial cell migration was reduced to background levels after cells had been treated with concentrations of RA as low as 10^{-7} mol/L, one log less than the maximal achievable clinical dose of 10^{-6} mol/L.[40] Viability did not fall off significantly until the RA concentration exceeded 10^{-5} mol/L. Media conditioned by RA-treated tumor cells also demonstrated decreased angiogenic activity when tested *in vivo*. Cells treated with 10^{-8} mol/L RA produced media with reduced angiogenic activity. Such activity was eliminated when the concentration was increased to 10^{-6} mol/L (Table 3). The clinically active isomer 13-*cis*- RA was also as active as all-*trans*-RA in blocking tumor angiogenic activity (Figure 4).

RA treatment of tumor cells did not result in any change in secreted IL-8, the major inducer of angiogenesis made by these cells (Table 4). The reason tumor lines treated with retinoids were not angiogenic was because they were secreting high levels of a molecule or molecules that inhibit angiogenesis. This was demonstrated by showing that the media conditioned by drug-treated cells could block angiogenesis induced by media conditioned by untreated tumor cells, whether the mixture was tested *in vitro* (Figure 5) or *in vivo* (Table 3; Figure 6).

RA treatment did not elicit an anti-angiogenic phenotype from tumor cell lines derived from other tumor types (Figure 7). Drug treatment caused no change in the potently angiogenic phenotype of the human fibrosarcoma line HT1080. RA treatment caused the human colon carcinoma line HT-29 and the human breast carcinoma cell line MDA-MB468 to lose their ability to induce angiogenesis. However, this effect was not due to the induction of significant inhibitory activity because the mixture of RA-treated tumor cell CM with CM from untreated cells showed no inhibitory activity (Figure 7).

To determine how quickly RA could alter the angiogenic phenotype of the tumor cells and to find out how long this alteration persisted after withdrawal of the drug, tumor cells were treated with 10^{-6} mol/L RA for 7 days and then refed with media lacking drug for an additional 7 days. CM

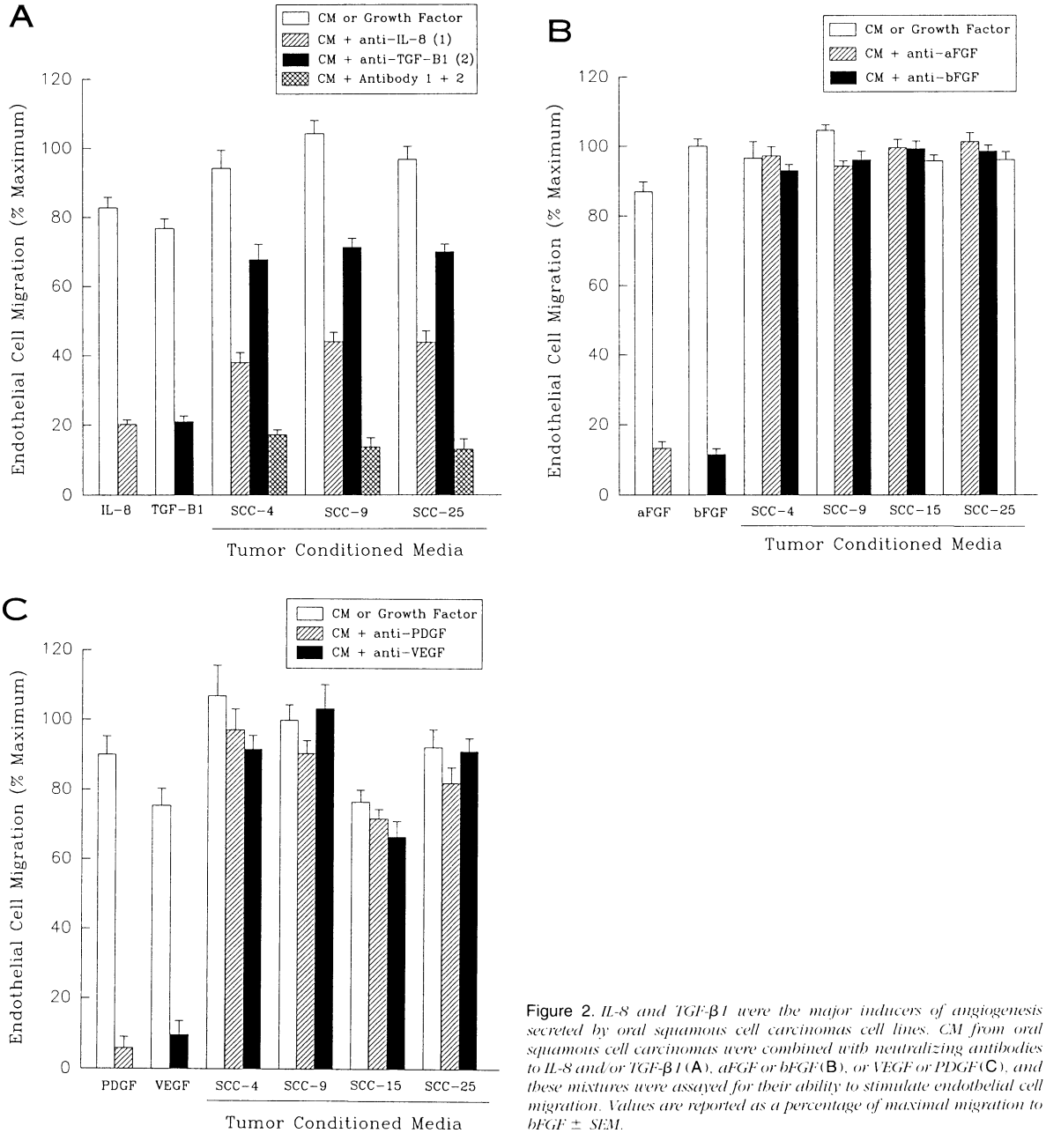

Figure 2. *IL-8 and TGF-β1 were the major inducers of angiogenesis secreted by oral squamous cell carcinomas cell lines. CM from oral squamous cell carcinomas were combined with neutralizing antibodies to IL-8 and/or TGF-β1* (A), *aFGF or bFGF* (B), *or VEGF or PDGF* (C), *and these mixtures were assayed for their ability to stimulate endothelial cell migration. Values are reported as a percentage of maximal migration to bFGF ± SEM.*

were collected at various time points and assayed for ability to stimulate endothelial cell migration (Figure 8). It required 4 days of RA treatment before the tumor cells completely lost their angiogenic activity. It required an additional 4 days without drug for the tumor cells to become angiogenic again. The inhibitory activity secreted by the tumor cells into their CM followed a reciprocal curve, reaching a peak of activity after 7 days of treatment. Inhibitory activity persisted for 2 days after drug removal and was gone by 5 days.

Discussion

RA treatment of tumor cell lines derived from oral squamous cell carcinomas caused the cells to switch from a potently angiogenic to an anti-angiogenic phenotype. This switch took place without the loss of viability at concentrations of retinoids as much as one log below those achievable clinically. The anti-angiogenic activity was significant in the context of tumor angiogenesis because it was potent enough to block neovascularization induced by tu-

Table 2. *Neutralizing Antibody to IL-8 Markedly Reduced Corneal Neovascularization Induced by Tumor Cell Conditioned Media*

Media source	Positive corneas/number of implants (%)	
	Tested alone	Tested + anti-IL-8
Control		
DME	0/3 (0)	ND
IL-8	3/3 (100)	0/3 (0)
Tumor CM		
SCC-4	5/5 (100)	1/5 (20)
SCC-9	4/4 (100)	0/4 (0)
SCC-15	3/3 (100)	0/3 (0)
SCC-25	3/3 (100)	1/4 (25)

ND, not done.

Table 3. *Corneal Neovascularization in Response to Conditioned Media from Retinoic-Acid-Treated Tumor Cells*

Media source	Positive corneas/number of implants (%)	
	Tested alone	Tested + CM from untreated tumor cells
Control		
DME	0/3 (100)	ND
RA-treated tumor cells*		
SCC-9 (10^{-10} mol/L RA)	4/4 (100)	ND
SCC-9 (10^{-8} mol/L RA)	2/4 (50)	ND
SCC-9 (10^{-6} mol/L RA)	1/4 (25)	1/3 (33)
SCC-15 (10^{-6} mol/L RA)	0/3 (0)	1/3 (33)
SCC-25 (10^{-6} mol/L RA)	0/4 (0)	1/4 (25)

*Cells were treated with indicated concentrations of all-*trans*-RA for 8 days, and CM were collected during the final 24 hours.
ND, not done.

mor cells. The media secreted by the treated cells were effective against media from tumor cells at a 1:1 mix, suggesting that if this switch were to take place in only one-half of the tumor cells that make up a small carcinoma *in vivo*, it would severely limit its ability to attract the vessels needed for further growth. Inhibitory activity was not induced by RA in tumor lines derived from two other tumor types, suggesting that this phenomenon may have some tissue specificity that could contribute to the particular success of retinoids in oral cancer.

IL-8 and TGF-β were the major angiogenic factors secreted by oral squamous cell carcinoma lines growing *in vitro*, with IL-8 predominating. This *in vitro* finding seems likely to reflect the *in vivo* situation, as IL-8 has also been found by immunohistochemistry to be associated with tumor cells in fresh samples of head and neck human squamous cell carcinomas[41] and in the closely related bronchogenic carcinomas.[42] In fresh tumor homogenates of the bronchial carcinomas, IL-8 was the primary mediator of angiogenesis. In addition, CM from psoriatic keratinocytes grown in cell culture are markedly angiogenic due to a 10- to 20-fold increase in their secretion of IL-8.[43]

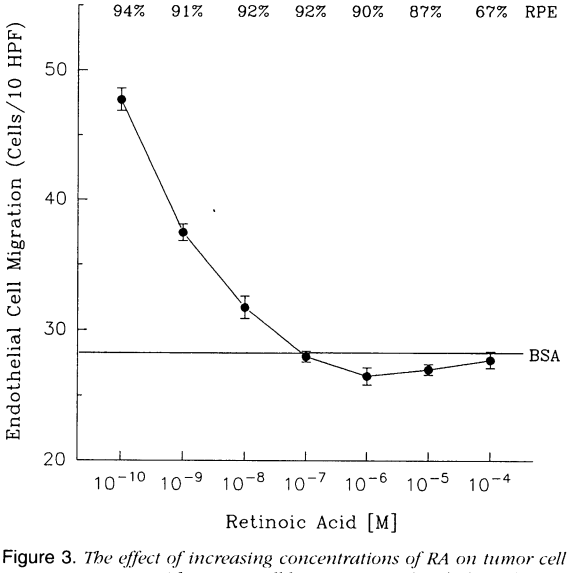

Figure 3. *The effect of increasing concentrations of RA on tumor cell angiogenic activity. The SCC-9 cell line was treated with the indicated concentrations of all-*trans*-RA for 7 days, and CM were collected during the final 24 hours and assayed for the ability to stimulate endothelial cell migration. Values are reported as the number of migrated cells per 10 high power fields ± SEM. Viability is reported across the top of the figure as a percentage of untreated controls. BSA indicates the background of random migration seen in the presence of media containing 0.1% bovine serum albumin alone.*

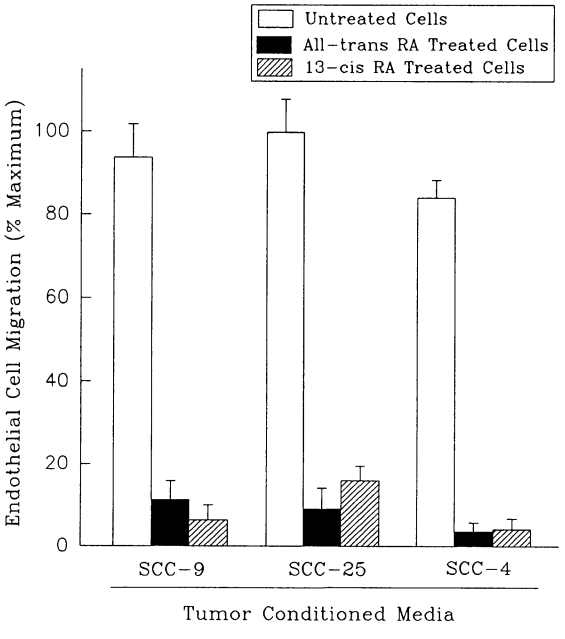

Figure 4. *Either all-*trans*- or 13-*cis*-RA switched tumor cells from an angiogenic to a non-angiogenic phenotype. Tumor cell lines were treated with either all-*trans*- or 13-*cis*-RA at 10^{-6} mol/L for 7 days, and CM were collected and assayed for the ability to stimulate endothelial cell migration. Values are reported as the percentage of maximal migration to SCC-25 CM ± SEM.*

Table 4. *Secretion of IL-8 by Untreated and Retinoic-Acid-Treated Cells*

Media source	Treatment	IL-8 (pg/μg total protein)
NHOK		
	None	75
	13-*cis*-RA	110
	All-*trans*-RA	90
HaCat		
	None	200
	13-*cis*-RA	125
	All-*trans*-RA	150
SCC-4		
	None	800
	13-*cis*-RA	900
	All-*trans*-RA	750
SCC-9		
	None	1600
	13-*cis*-RA	1700
	All-*trans*-RA	1300
SCC-15		
	None	1900
	13-*cis*-RA	1700
	All-*trans*-RA	2000
SCC-25		
	None	2400
	13-*cis*-RA	2300
	All-*trans*-RA	2250

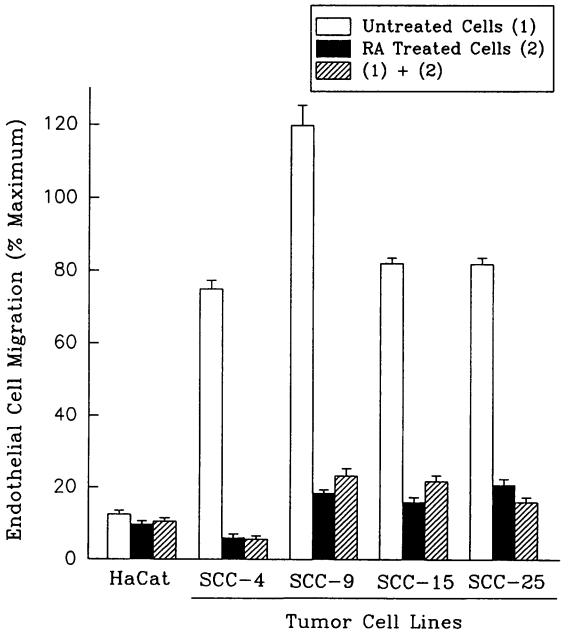

Figure 5. *CM from RA-treated squamous cell carcinomas contained angio-inhibitory activity. CM from untreated and all-*trans*-RA-treated cells were assayed alone or mixed together in a 1:1 ratio to determine their ability to stimulate or inhibit endothelial cell migration. Values are reported as a percentage of maximal migration to bFGF ± SEM.*

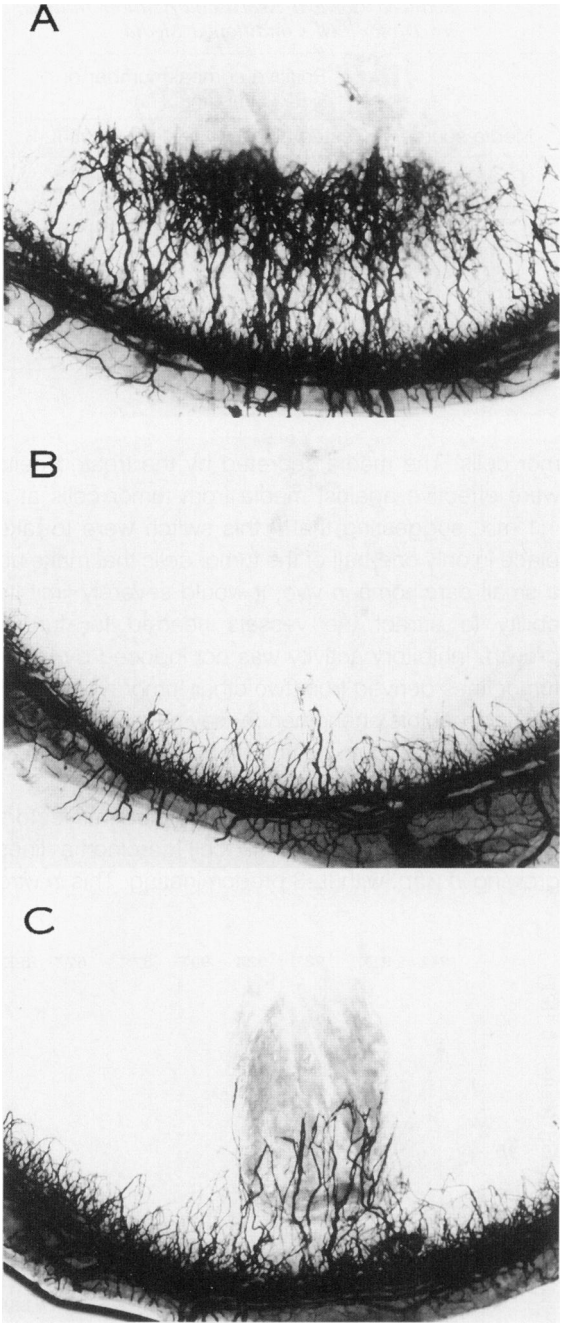

Figure 6. *Suppression of* in vivo *neovascularization by media conditioned by RA-treated tumor cells. Representative colloidal carbon perfused corneal whole mounts, demonstrating neovascular response to hydron pellets containing CM from untreated human squamous cell carcinoma cell line SCC-15* (**A**), *SCC-15 cells treated with* 10^{-6} *mol/L all-*trans*-RA* (**B**), *or a 1:1 mixture of CM from RA-treated and untreated SCC-15 cells* (**C**). *A similar marked suppression of neovascularization was also seen in the mixtures of CM from treated and untreated SCC-9 and SCC-25 cells.*

TGF-β is an angiogenic factor that consistently induces neovascularization *in vivo*[44,45] but has biphasic effects on endothelial cells growing *in vitro*, stimulating them at low (picograms per milliliter) concentrations and inhibiting them at higher (nanograms per milliliter) amounts.[46–49] It was secreted by oral carcinoma cell lines at low stimulatory levels. In addition to its angiogenic activity, TGF-β is also a

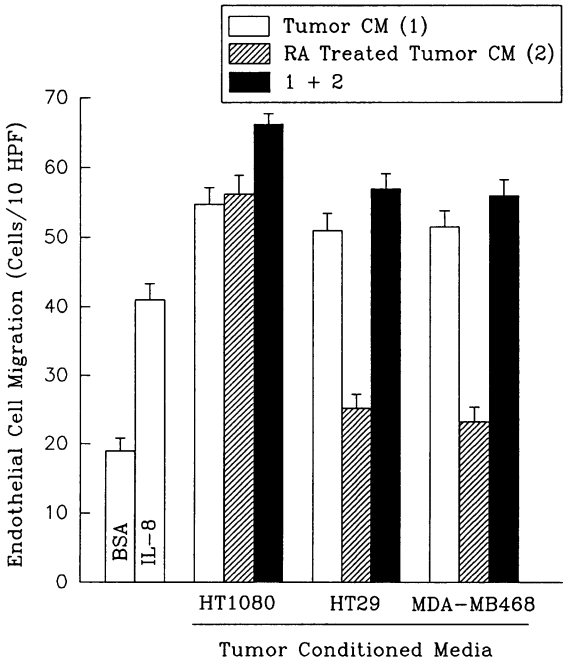

Figure 7. *Effect of RA on the angiogenic phenotype of other human tumor cell lines. CM from untreated and RA-treated human fibrosarcoma (HT1080), human colon carcinoma (HT 29), and human breast carcinoma (MDA- MB468) cell lines were assayed alone or mixed together in a 1:1 ratio to determine their ability to stimulate or inhibit endothelial cell migration. Values are reported as the number of cells migrated per 10 high power fields to IL-8 ± SEM.*

potent inhibitor of the growth of epithelial cells. However, most successful oral squamous cell carcinomas have lost this sensitivity.[50–52] Although the angiogenic factor bFGF has been reported to be

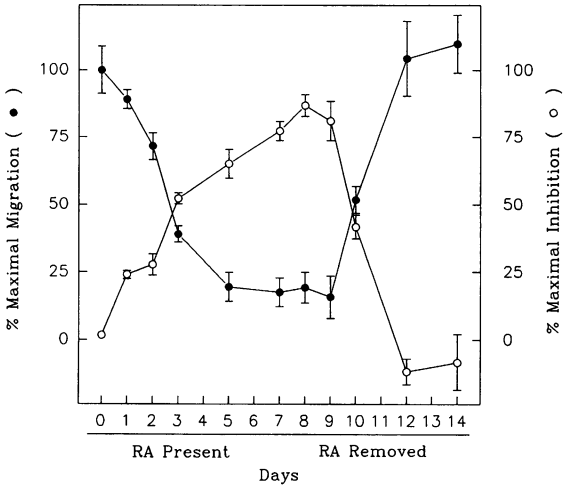

Figure 8. *Time course of induction and decay of the anti-angiogenic phenotype in tumor cells treated with RA. SCC-9 cells were treated with 10^{-6} mol/L all-trans-RA for 7 days, refed with media lacking RA, and then cultured for another 7 days. CM were collected at indicated times and tested alone for their ability to stimulate capillary endothelial cell migration (●) or assayed with CM from untreated SCC-9 cells for their ability to inhibit endothelial cell migration (○). Values are reported as a percentage of maximal migration to SCC-9 CM ± SEM.*

produced by squamous cell carcinomas of the head and neck,[53] it was not a major contributor to the angiogenic activity secreted by the cell lines tested here. It may also play a minor role *in vivo*, where it is expressed at levels equal to or less than those in adjacent normal mucosa and its level of expression fails to correlate with tumor vessel counts.[54]

Normal keratinocytes cultured from the oral cavity secreted low levels of IL-8 and high levels of inhibitory activity capable of suppressing angiogenesis induced by media from tumor cells. If these cells are typical of the cells that give rise to oral squamous cell carcinomas, then during tumor progression, they must lose their inhibitory activity and increase their secretion of the inducer of angiogenesis, IL-8. Normal human fibroblasts also develop an angiogenic phenotype by losing inhibitory activity, in this case known to be due to thrombospondin,[55] and by increasing the concentration of inducers of inducers of angiogenesis.[56,57] Loss of inhibitory activity in fibroblasts is due to loss of the p53 tumor suppressor gene. A similar suppressor-based mechanism may be operating in oral keratinocytes from the hamster buccal pouch, which also becomes angiogenic as the result of a genetic loss that triggers loss of angioinhibitory activity[58] (Lingen and Polverini, unpublished data). However, the inhibitor in this system has not been identified.

The inhibitor or inhibitors induced in tumor cells by treatment with RA have not been identified to date. Therefore, it is not known whether they are the same or different from those produced by normal keratinocytes. Preliminary experiments have determined that the retinoid-induced activity is due to a heparin-binding protein or proteins, which are likely to be novel inhibitors. Although retinoids can induce thrombospondin in squamous cell carcinomas,[59] the inhibitory activity was not due to thrombospondin (data not shown). It also was not due to interferons or IL-1 because the inhibitory activity was not neutralized by antibodies against these molecules (data not shown). Nor is it due to tissue inhibitors of metalloproteinases (TIMPs), for no change in the amount of TIMP-1 or TIMP-2 on Western blot or in the activity of TIMPs by zymogram analysis could be detected after retinoid treatment (data not shown). Physical and biochemical characteristics of the inhibitory activity are not consistent with it being identical to any of the other inhibitors of angiogenesis known to be produced by mammalian cells.[34] The novel RA-induced inhibitor(s) is currently being purified.

Until the molecules responsible for the inhibitory activity are identified, it will not be possible to say whether they are induced *in vivo* by clinical treat-

ments with RA and whether they play a key role in the ability of these drugs to prevent new primary oral cancers. However, the observation that initial high doses followed by low maintenance doses were the most effective regimen in clinical chemoprevention trials[8] does correspond with our observation that inhibitory activity is slow to arise, taking up to 5 days to reach maximal effectiveness. Once achieved, these levels may be maintained for up to 2 days in the absence of drug. Thus, the inhibitory phenotype may be maintained despite the fluctuations in retinoid plasma levels that are unavoidable in clinical trials. Our observation that the effects of retinoids on the inhibition of angiogenesis are completely reversible is also consistent with the clinical data showing that the chemopreventive activity of these agents ceased when patients went off of the trial.[7] It is likely that the angio-inhibitory effect of retinoids on tumor cells that is documented here acts in combination with RA-induced inhibition of endothelial cell responsiveness[60] and of tumor-associated macrophage-mediated angiogenesis (Lingen, Bouck, and Polverini, unpublished data). These three anti-angiogenic activities of RA could account for a significant portion of RA's activity as a chemopreventive agent against oral cancers.

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