In Vivo Inhibition of Angiogenesis and Induction of Apoptosis by Retinoic Acid in Squamous Cell Carcinoma

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ABSTRACT
Retinoids inhibit the growth and reverse aberrant differentiation of squamous cell carcinoma (SCC) cells in vitro. To investigate the potential mechanisms of antitumor activity of retinoids in vivo, we used the cervical SCC cell line ME-180 as a s.c. tumor xenograft in athymic nude mice. After s.c. injection, tumor cells were allowed to form visible tumors and antitumor activity of all-trans-retinoic acid (tRA) was studied. tRA was administered daily for a 1-week or a 2-week period at 60 mg/kg/day. Tumor specimens were then analyzed using immunohistochemical staining for the number of blood vessels and apoptotic cells and for proliferating cell nuclear antigen expression. Furthermore, we studied the effect of the tRA treatment on the expression of a binding protein for fibroblast growth factors (BP; Gen-Bank accession no. M60047) that is a candidate angiogenesis modulator in SCC (F. Czubayko et al., J. Biol. Chem., 269: 28243–28248, 1994). We found that in vivo tRA treatment reduces BP expression in SCC xenografts, inhibits their angiogenesis, induces apoptosis of the tumor cells, and leads to a decrease of the tumor growth rate. We speculate that the tRA down-regulation of BP is responsible for the reduction of angiogenesis.

INTRODUCTION
Retinoids, a group of naturally occurring and synthetic analogues of vitamin A, are potent regulators of epithelial differentiation and growth within many neoplastic cell systems including several SCC cell lines (1–3). Most important, retinoids have been shown to suppress carcinogenesis in various epithelial tissues (oral cavity, skin, lung, prostate, and mammary gland) in animal model systems (4–9), and also to have clinical efficacy as chemotherapeutic agents against selected malignancies (10, 11). Since retinoids have been shown to have clinical efficacy, there is continuing interest in their therapeutic potential such as against SCCs in particular, since they arise from epithelial tissues whose development is strongly influenced by retinoids (1).

The mechanism of action of retinoids on tumors seems to be related to their effects on proliferation and differentiation of the tumor cells themselves (1–3). However, other studies also showed that retinoids markedly inhibited angiogenesis in the chorioallantoic membrane model (12, 13) and in an experimental in vivo model of cutaneous angiogenesis in the mouse (14, 15). In addition, retinoids have been linked to the induction of apoptosis (programmed cell death) in several in vitro and in vivo models of cell death (16–18).

In this article, we describe a model system with which we attempted to elucidate the mechanisms by which retinoids suppress growth of SCCs in vivo. As a tumor model, we chose a human cervical SCC cell line, ME-180, that forms tumors in athymic nude mice and that is growth inhibited by retinoids in vitro (19, 20). Because this SCC cell line recapitulates the main characteristics of keratinocyte squamous differentiation and responsiveness to retinoids (3), it can serve as a model to study the mechanism underlying the effects of retinoids on tumors in vivo. Furthermore, we tried to understand to what extent tumor angiogenesis is affected by retinoid treatment. In addition to a quantitation of microvessels in the tumors, we also studied the retinoid effects on a binding protein for FGFs (21) that can activate locally stored FGF, is highly expressed in SCC, and is a likely candidate molecule to modulate SCC angiogenesis as shown by us earlier (22).

MATERIALS AND METHODS
Cell Lines. Cervical squamous carcinoma cells (ME-180) were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in improved MEM (Biofluids, Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD).

Tumor Growth in Animals. Female athymic nude mice (NCr nu/nu; National Cancer Institute, Frederick, MD) were given s.c. injections of 1 × 10⁶ ME-180 cells (five mice per group and two sites per mouse). After tumors were established, mice were randomized and treated daily for 8 days or 15 days p.o. (by gavage) with aliquots of either vehicle alone (purified sesame oil; Croda, Parsippany, NJ) or tRA (Ligand Pharmaceuticals, Inc., San Diego, CA) suspended in 0.2 ml of vehicle. All animals were sacrificed at day 15 of treatment, and tumors samples were flash frozen or fixed in 10% buffered formalin solution overnight, embedded in paraffin, and sectioned.
Stainings. Histological slides stained with H&E were analyzed by a pathologist. Furthermore, formalin-fixed, paraffin-embedded sections were deparaffinized by xylene and rehydrated with graded alcohols. Endogenous peroxidases were inactivated with 0.3% hydrogen peroxide solution (Mallinckrodt, Paris, KY). For microvessel and PCNA stainings, slides were washed twice with PBS (Biofluids, Inc.), and nonspecific sites were blocked with a 1:6 dilution of normal horse serum. Slides were again washed twice in PBS and incubated with 1:20 anti-platelet-endothelial cell adhesion molecule rat anti-mouse monoclonal antibody, CD31, (PharMingen, San Diego, CA) and 1:200 anti-PCNA mouse anti-human monoclonal antibody (Signet, Dedham, MA), respectively, for 2 h. Slides were again washed twice in PBS and a 1:200 biotinylated rabbit anti-rat antibody for platelet-endothelial cell adhesion molecule staining and a 1:200 biotinylated horse anti-mouse antibody for PCNA staining (Vector Laboratories, Burlingame, CA) were applied as secondary antibodies for 30 min. Slides were washed twice in PBS and then treated according to the supplier’s instructions for the ABC kit (Bioclmada, Foster City, CA) and revealed with diaminobenzidine (Sigma Chemical, St. Louis, MO). For apoptosis staining, slides were incubated in buffer A [0.05 mM Tris (pH 7.5)-5 mM MgCl2-0.76 mM mercaptoethanol-0.005% BSA] for 5 min and then incubated for 1 h at 37°C in a humid chamber with 50 units/ml Klenow DNA polymerase 1 (Boehringer Mannheim, Indianapolis, IN), 5 mM biotinylated dUTP (Boehringer Mannheim), and 2 mM of dATP, dGTP, and dCTP (Promega, Madison, WI) in buffer A. Slides were rinsed for 15 min in buffer B (30 mM NaCl-30 mM sodium citrate) to stop the enzymatic reaction. Slides were then washed with PBS, treated with the ABC kit, and then revealed with diaminobenzidine. For all stainings, slides were counterstained with hematoxylin and then mounted in Permount Mounting Medium (Fisher Scientific Co., Pittsburgh, PA).

Detection of mRNA by Northern Blots. Total RNA was isolated with the RNA STAT-60 method using commercially available reagents and protocols (RNA STAT-60; Tel-Test, Inc., Friendswood, TX). Thirty mg of total RNA were separated by electrophoresis in a 1.2% formaldehyde agarose gel and then blotted onto nylon membranes (Schleicher & Schuell, Keene, NH). The blots were prehybridized in 6× SSC [0.9 mM sodium chloride, and 0.09 mM sodium citrate (pH 7.0)], 0.5% (w/v) SDS, 5× Denhardt’s solution [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, and 0.1% (w/v) BSA; Life Technologies, Inc.] for 4 h at 42°C and hybridized overnight at 42°C in hybridization solution (same composition as the prehybridization solution) containing 50 ng of a [α-32P]dCTP-labeled BP cDNA probe described earlier (22). This probe was prepared by random-primed DNA labeling (Boehringer Mannheim). The final concentration of the labeled probe was always greater than 106 cpm/ml of hybridization solution. After hybridization, blots were washed three times with 2× SSC and 0.1% SDS for 10 min at 42°C, and finally once with 1× SSC and 0.1% SDS for 20 min at 65°C. Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Methods. Statistical significance was assessed using Student’s t test. P values of <0.05 were considered to be statistically significant.

RESULTS

Relationship among Angiogenesis, Apoptosis, PCNA, and Tumor Growth of ME-180 Xenografts during tRA Treatment. Squamous carcinoma cells ME-180 were injected s.c. into athymic nude mice, and tumor growth was monitored twice per week. When the mean tumor size reached 20.8 ± 2.6 mm2 (n = 22) 13 days after injection, mice were randomized and given vehicle alone for another 15 days or tRA (60 mg/kg) for 15 days or with vehicle alone for 7 days and then tRA for 8 days. All animals were then sacrificed, and tumor samples were analyzed. H&E staining showed that ME-180 cells formed in vivo well-differentiated SCCs characterized by high levels of keratinized structures. tRA did not alter the morphology of the tumors (Fig. 1, a–c). However, tumor vascularity showed a strong inhibition by the 8- and 15-day treatment regimen with tRA (Fig. 1, d–f). In a random selection of three of the control tumors, we counted an average of 7.0 ± 0.2 (n = 3) microvessels/field. The 8- and 15-day treatment with tRA decreased the number of microvessels per field by 28.1 ± 0.2% (n = 3; P < 0.001) and by 54.3 ± 15.0% (n = 3; P < 0.001), respectively (Fig. 2a). In parallel, histological sections were stained for apoptosis by DNA end labeling (Fig. 1, g–i). Quantitative evaluation (Fig. 2b) showed that the 8- and 15-day tRA treatment increased apoptotic cells by 275 ± 25% (n = 3; P < 0.01) and 575 ± 38% (n = 3; P < 0.001), respectively. Finally, sections were stained for PCNA whose expression is associated with late G1 and S-phase (Fig. 1, j–l). As shown in (Fig. 2c), PCNA expression was not changed quantitatively by the 8-day tRA treatment regimen but decreased by 46.1 ± 6.4% (n = 3; P < 0.001) by the 15 days of tRA treatment. In parallel with this, only the 15-day treatment group of the ME-180 tumors showed a slowed growth rate of 31.3 ± 4.6% (n = 8; P < 0.05; Fig. 2d).

Inhibition of BP mRNA Expression by tRA in ME-180 Xenografts. Since we saw an effect on tumor angiogenesis, but not on tumor cell proliferation, with the 8-day treatment (Fig. 2, a and d), we suspected that down-regulation of angiogenic factors in the SCC cells at that point may precede direct effects of retinoids on the growth and differentiation phenotypes of the cells. However, classical angiogenic growth factor such as FGFs or VEGF or their receptors do not appear to be downregulated by retinoids as far as current literature shows. On the other hand, we found recently that in vitro treatment with retinoids down-regulates in ME-180 cells mRNA coding for a BP for FGFs (23). This BP is highly expressed in clinical SCCs but not in normal tissues and appears to function as a positive modulator of angiogenesis through locally stored FGFs as shown by us recently (22). BP mRNA was decreased by 50.3 ± 8.7% (n = 6; P < 0.001) by the 8-day treatment with tRA and remained decreased by the same amount in the 15-day treatment group (Fig. 3). In conjunction with the high levels of expression of basic FGF in SCC as well as in normal skin (24), we propose that this down-modulation of an activator of locally stored basic FGF could in fact explain the reduction of angiogenesis as well as its early initiation described above.

DISCUSSION

Retinoids have long been known to be potent modulators of differentiation and growth in a variety of tissues (1–3), including...
Fig. 1  Immunohistology studies of tumor specimens from ME-180 tumor xenografts. Tumor samples from control animals (a, d, g, and j) and from animals treated with tRA for 8 days (b, e, h, and k) and 15 days (c, f, i, and l) are shown. H&E staining (a–c), anti-CD31 staining for microvessels (d–f), end labeling of free DNA to detect apoptotic cells (g–i), and anti-PCNA staining for proliferating cells (j–l) are depicted. X400. A brown stain indicates apoptotic cells in g–i and proliferating cells in j–l.
human papilloma virus-transformed human cervical keratinocytes (25). Retinoids maintain normal differentiation of epithelial tissues by preventing aberrant squamous differentiation of cells in nonkeratinizing epithelia, and they can also reverse squamous metaplasia, which develops during vitamin A deficiency (1). In vitro models have usually examined retinoid-induced effects using markers of differentiation such as transglutaminase type I, loricrin, involucrin, filaggrin, and keratin K1 (26). Clinical studies evaluating the efficacy of retinoids against squamous cell cancers have been limited to those of the skin and cervix (10, 11, 27). Furthermore, tRA exhibited some effectiveness in chemoprevention trials of cervical dysplasia (28, 29), and additional reports described that tRA transcriptionally decreased epidermal growth factor receptor expression in ME-180 cells (32). However, to our knowledge, there is no report of retinoids down-regulating any angiogenic growth factor such as FGF or VEGF or their receptors. However, as mentioned earlier, a potential target gene for retinoids could be the binding protein for FGF (BP), which has been shown by us to play an important role in angiogenesis (22). Moreover, we suggest that induction of apoptosis is the result of the inhibition of angiogenesis by tRA.

We report that a 1-week treatment with tRA inhibits in vivo SCC angiogenesis and concomitantly increases apoptosis in the tumors. A longer treatment of 2 weeks also inhibits proliferation of the tumor cells assessed by staining for PCNA and reduces the rate of increase of tumor growth. We propose that the tRA mechanisms of action are linked to both an inhibition of stromal cell induced-angiogenesis (Figs. 1, d-f, and 2a) and a stimulation of tumor apoptosis (Figs. 1, g-i, and 2b). This later leads to an inhibition of proliferation characterized by PCNA expression and a decrease of the tumor growth rate (Figs. 1, j-l, and 2, c and d). Moreover, we suggest that induction of apoptosis is the result of the inhibition of angiogenesis by tRA.

The results presented here show an antiangiogenic activity of retinoids in a SCC tumor model. Angiogenesis, consisting of new vessel formation and subsequent development of microcirculation within tumor tissue, is an essential requirement for the growth of solid neoplasms (30). Moreover, it has been suggested that angiogenesis precedes or accompanies malignant growth in experimental models (31) as well as in the natural progression of human tumors of the cervix, skin, and breast (30). The mechanism of antiangiogenic action of tRA in other models (12-15) is not known, but it could be due to its inhibitory effect on tumor or endothelial cell proliferation as well as on the production or release of angiogenic factors by tumors cells. It has been described that tRA transcriptionally decreased epidermal growth factor receptor expression in ME-180 cells (32). However, to our knowledge, there is no report of retinoids down-regulating any angiogenic growth factor such as FGF or VEGF or their receptors. However, as mentioned earlier, a potential target gene for retinoids could be the binding protein for FGF (BP), which has been shown by us to play an important role in angiogenesis (22). Moreover, we showed that BP is expressed at a high level in SCC tissues from patients and in SCC cell lines of different origins, in particular in ME-180 cells. Recently, we found that BP mRNA is down-regulated by in vitro treatment of ME-180 cells with retinoids (23). Here, we have expanded this finding.

**Fig. 2** Relationship between angiogenesis (a), apoptosis (b), PCNA expression (c), and tumor growth (d). Quantitations were carried out by two independent blinded observers. Twenty high-power fields (×400) in a random selection of three tumor samples for each of the groups were assessed. Necrotic areas were excluded from this analysis. The fold increase in tumor size is shown for control and for animals treated with tRA for 8 and 15 days. Tumor size was calculated from the product of the perpendicular diameters of the tumors. Columns, means; bars, SE. **, P < 0.001 versus control; *, P < 0.05 versus control (Student’s t test).

**Fig. 3** Levels of BP mRNA in ME-180 tumors after treatment of animals with tRA. RNA was prepared from tumor samples from control animals (n = 7) and animals treated for 8 (n = 6) and 15 (n = 6) days. BP expression was quantified by Northern blot with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a loading control. Signal intensities were quantified by phosphorimaging. Hybridization signals with two representative samples for each group are shown in A and quantitative data in B. Columns, means; bars, SE. **, P < 0.001 versus control (Student’s t test).
into the in vivo situation and we show that the effects of tRA are apparent at the end of the 8-day treatment period (Fig. 3). Interestingly, a pilot study showed a small, but detectable in vivo down-regulation of BP mRNA as early as 3 days after initiation of the treatment protocol. Taking the biological role of BP into account, we propose that the down-regulation of BP mRNA by the tRA treatment is responsible in large part for the reduction of tumor angiogenesis in this SCC model system. A subsequent “bystander effect” on the tumor cells themselves, i.e., induction of apoptosis due to this reduced angiogenesis as described in studies blocking an angiogenesis-induced integrin (33) is possible.

In addition to the effects of the tRA treatment on angiogenesis, our study also demonstrates that tRA induces apoptosis in vivo in a SSC model system. Another study previously reported that retinoids induced apoptosis in SCCs and this was done in vitro in a multicellular tumor spheroid model for SCCs (34). Apoptosis, the regulated or programmed death of cells, is an important process controlling the growth of both normal and neoplastic tissues (35). In squamous differentiating tissues, a balance exists between the rate of proliferation and differentiation. It has been suggested that retinoids may control the rate at which cells undergo squamous cell differentiation, leading to an inhibition of the proliferation. The regulation of apoptosis may be another mechanism by which retinoids control homeostasis in these tissues.

We propose that tRA treatment in vivo inhibits stromal cell induced-angiogenesis and induces tumor apoptosis. It is even conceivable that the reduction of the angiogenic stimulus from the tumor cells via a reduction of the angiogenesis modulator BP could lead to apoptosis of the tumor cells as a bystander effect.

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REFERENCES


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