Curcumin delays development of medroxyprogesterone acetate–accelerated 7,12-dimethylbenz[a]anthracene–induced mammary tumors

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Abstract

Objective: Combined hormone therapy (HT) containing estrogen and progestin (medroxyprogesterone acetate [MPA]) leads to increased risk of breast cancer in postmenopausal women, compared with HT regimens containing estrogen alone or placebo. We previously reported that in animal models, progestins can accelerate the development of mammary tumors by increasing vascular endothelial growth factor (VEGF) levels. We furthermore showed that curcumin, an Indian spice derived from the turmeric root, specifically inhibits MPA-induced VEGF secretion from breast cancer cells in vitro. In the present study, we investigated whether curcumin inhibits 7,12-dimethylbenz[a]anthracene (DMBA)–induced, MPA-accelerated tumors in Sprague-Dawley rats.

Methods: On day 0, virgin female Sprague-Dawley rats (age, 55 d) were given DMBA (20 mg/rat). Sixty-day timed-release pellets containing 25 mg MPA were implanted into the rats on day 30. Curcumin was administered daily at a rate of 200 mg kg−1 day−1 from days 26 to 50, and animals were killed on day 52 (n = 15-19 per group).

Results: Treatment with curcumin delayed the first appearance of MPA-accelerated tumors by 7 days, decreased tumor incidence by the end of the experiment, and reduced tumor multiplicity in DMBA-induced MPA-accelerated tumors. Curcumin also prevented many of the gross histological changes seen in the MPA-treated mammary gland. Immunohistochemical analyses of mammary tumors showed that curcumin decreased MPA-induced VEGF induction in hyperplastic lesions, although it did not affect the levels of estrogen and progestrone receptors.

Conclusions: We suggest that curcumin be tested as a dietary chemopreventive agent in women already exposed to MPA, in an effort to decrease or delay the risk of breast cancer associated with combined HT.

Key Words: Breast cancer – Progestins – Vascular endothelial growth factor – Curcumin – (7, 12-Dimethylbenz[a]anthracene).

Received February 24, 2009; revised and accepted April 27, 2009.

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Funding/support: This research was supported by National Institutes of Health Grant CA-86916 and in part by R56CA-86916 from the National Cancer Institute; P01060723 from the Susan Komen for Cure grants; Funds from Research Diagnostic Lab and a COR grant, both from the University of Missouri College of Veterinary Medicine; a National Institutes of Health-funded Minority Biomedical Research Training Initiative grant from the Department of Veteran Pathobiology; and a Phi Zeta, Pi Chapter grant.

Financial disclosure/conflicts of interest: S.M.H. is the Zalk Missouri Professor of Tumor Angiogenesis.

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A number of clinical studies have shown that the use of combined estrogen/progestin hormone therapy (HT) by postmenopausal women increases the incidence of breast cancer and elevates the risk of recurrence of breast tumors compared with treatment with estrogen alone or placebo.1 Because of the time frame within which tumors are detected after estrogen/progestin combination therapy, we and others have suggested that combined HT may cause existing but undetectable tumors or preneoplastic lesions to progress to frank tumors.2,3 The molecular basis by which progestin consumption accelerates the growth of mammary tumors is not known. We recently showed that progestins accelerate the development of 7,12-dimethylbenz[a]anthracene (DMBA)–induced mammary tumors as well as human tumor xenografts in nude mice by increasing production of the potent angiogenic molecule vascular endothelial growth factor (VEGF). By promoting angiogenesis in this manner, progestins create a favorable milieu for tumor expansion.4 Consequently, by blocking progestin-induced expression of VEGF or neutralizing VEGF activity by blocking its receptor, VEGFR2, we can potentially reduce the proliferation of breast cancer cells, both in vivo and in vitro.5,7

Approximately 6 million women in the United States use HT to treat the symptoms of menopause8; such extensive exposure to progestin will therefore predispose a large number of postmenopausal women to future development of breast cancer.1,3,8 Consumption of an antiangiogenic compound along with combined HT could prove beneficial by significantly
reducing the risk of estrogen and progesterone-receptor (PR)–positive breast cancer associated with HT regimens that include a progestin component. Curcumin is an Indian spice that has been shown to have antiangiogenic properties in multiple types of cancer.\textsuperscript{9,10} This phytoestrogen has been reported to have low affinity for both estrogen receptors (ERs) and PRs\textsuperscript{11} and reduces hormone-induced cell proliferation.\textsuperscript{12} Consideration of curcumin as a chemopreventive agent is gaining popularity\textsuperscript{13}; however, its usefulness as a chemopreventive agent for progesterin-dependent breast cancer remains unexplored. We previously reported that curcumin inhibits the progesterin-induced elaboration of VEGF from T47-D human breast cancer cells\textsuperscript{14} and suggested that curcumin should be considered for use in clinical trials, either as a cotreatment with combined HT to reduce the risk of breast cancer in postmenopausal women or perhaps as a chemopreventive additive after HT to decrease the development of breast cancer in HT-exposed women.

Here, we have tested our proposal in a well-established animal model of DMBA-induced breast cancer, in which VEGF is produced and tumors develop rapidly after exposure to progestins.\textsuperscript{2} We selected medroxyprogesterone acetate (MPA) as the progestin of choice because it is used worldwide in HT and because we have previously shown that curcumin acts specifically to inhibit MPA-induced VEGF secretion in breast cancer cells in vitro.\textsuperscript{14} Before undertaking this study, we hypothesized that curcumin would delay the formation of MPA-driven, DMBA-induced mammary tumors, as well as prevent the elevation of VEGF levels in the mammary gland. We report that curcumin does indeed delay the appearance of MPA-accelerated, DMBA-induced mammary tumors and also blocks the production of VEGF in breast cancer cells. Importantly, curcumin prevents the appearance of gross morphological abnormalities in the mammary glands of rats with DMBA-induced tumors after MPA exposure.

**METHODS**

**Animals**

Intact virgin female Sprague-Dawley rats (Harlan Breeder, Indianapolis, IN), 40 to 45 days old, were housed according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care under the conditions of 12-hour light/dark cycles and ad libitum access to food and water. All surgical and experimental procedures were in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23).

Following the protocol previously established by our laboratory,\textsuperscript{2} animals were given a single dose of DMBA (20 mg/rat; Sigma-Aldrich, St. Louis, MO) in peanut oil via gavage on day 0. From days 26 to 50, 200 mg/kg curcumin (Acros Organics, Morris Plains, NJ; made daily to a concentration of 200 mg/mL in peanut oil\textsuperscript{13}) was injected daily IP; control animals were given peanut oil alone. On day 30, animals were anesthetized, and 60-day release pellets containing 25 mg MPA (Innovative Research, Sarasota, FL) or placebo pellets were implanted subcutaneously on the dorsal side (Fig. 1A) (n = 15-19). Animals were palpated two to three times weekly throughout the study. The appearance of the first tumor was recorded in each group (the MPA group developed tumors more rapidly than other groups); this determined the delay in tumor appearance in all other groups compared with the MPA group.\textsuperscript{2} On day 52, after DMBA administration, the animals were killed and mammary tissues were collected.

**Histology and immunohistochemical analysis**

The effect of curcumin on mammary tumorigenesis was assessed by measuring the levels of VEGF, estrogen receptors (ER-\textalpha and ER-\beta), and PRs. Both auxiliary and abdominal mammary glands were used for analysis.

Tissues were fixed overnight in 10% neutral buffered formalin for microscopy and 4% paraformaldehyde for immunohistochemistry. Tissues were processed for paraffin infiltration and embedding. Sections (5 \mu m) were mounted on ProbeOn Plus microscope slides (Fischer Scientific, Inc., Pittsburgh, PA). Light microscopic examination of serial hematoxylin and eosin–stained sections representative of a given tissue was performed for classification purposes.\textsuperscript{16} Before immunohistochemistry, sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, rinsed in distilled water, and stored in phosphate-buffered saline (PBS) at 4°C until use. Sections were heated in 10 mmol/L citrate buffer (pH 6.0) to induce epitope retrieval for PR, ER-\alpha, and VEGF immunolabeling. Slides were treated with 3% H\textsubscript{2}O\textsubscript{2} in absolute methanol (to inactivate endogenous peroxidase activity) before being washed three times in PBS and immersed in 10% bovine serum albumin for 20 minutes. Sections were incubated for 60 minutes at room temperature with each of the following polyclonal antibodies: anti-PR antibody (1:50 dilution of a rabbit antihuman PR polyclonal antibody [A0098] that reacts with the DNA-binding domain [amino acids 533-547]; DAKO, Carpinteria, CA), anti–ER-\alpha (1:300 dilution of a rabbit anti–ER-\alpha polyclonal antibody [sc-542] raised against an ER-\alpha peptide of mouse origin; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and an anti–VEGF antibody (1:100 dilution of a rabbit anti–VEGF polyclonal antibody [sc-152]; Santa Cruz, Biotechnology). Sections were then washed and sequentially incubated with a secondary antibody (biotinylated swine antirabbit immunoglobulin G [DAKO]) and a streptavidin–linked horseradish peroxidase product (BD PharMingen, San Diego, CA) for 30 minutes at room temperature. Alternatively, some sections were incubated with EnVision+, a horseradish peroxidase–labeled polymer conjugated to antirabbit antibodies (DAKO). Bound antibodies were visualized after incubation with 3,3'-diaminobenzidine solution (0.05% with 0.015% H\textsubscript{2}O\textsubscript{2} in PBS; Zymed Corp., San Francisco, CA) for 3 to 5 minutes. Sections were counterstained with Meyer hematoxylin, dehydrated, cleared, and cover-slipped for microscopic examination.
Statistics

Groups were compared with respect to tumor latency and multiplicity at the conclusion of the study. Latency period differences were compared using a general linear model (PROC GENMOD in SAS) in which the link function was logit and the distribution was binomial. MPA-treated, DMBA-induced animals were 2.2 times more likely to develop tumors than animals given DMBA alone and 4.4 times more likely than animals given curcumin alone or placebo (n = 15-19/group). MPA-treated, DMBA-induced animals were 2.2 times more likely to develop tumors than animals given DMBA alone and 4.4 times more likely than animals given curcumin alone or placebo (n = 15-19/group).

RESULTS

Curcumin delays MPA-accelerated tumors in DMBA-treated rats

In our previously established model, we showed that animals treated with MPA 4 weeks after the administration of DMBA develop well-vascularized mammary tumors earlier than those receiving placebo pellets and concluded that this
was due to increased expression of VEGF. Here, we used this model to determine whether curcumin inhibits VEGF expression and might therefore prevent the development of MPA-accelerated tumors. After DMBA administration, MPA pellets were implanted subcutaneously into the rats to accelerate the development of mammary tumors. Two groups of animals (n = 15/group) were given curcumin at a rate of 200 mg kg$^{-1}$ day$^{-1}$ starting 4 days before implantation of the 25 mg MPA pellet, a dose comparable to that of women prescribed MPA during HT. Treatment was continued and was terminated 50 days after the initial DMBA treatment. Curcumin delayed the appearance of MPA-accelerated tumors (Fig. 1A): tumors were first detected in MPA-treated animals on day 35 after DMBA treatment, whereas curcumin delayed the appearance of tumors until day 42. Comparison of latency data from treatments of MPA and MPA + curcumin did not show a significant difference when analyzed using a $\chi^2$ test. However, when compared using a general linear model (PROC GENMOD in SAS) in which the link function was logit and the distribution was binomial, according to the calculated odds ratio, the odds of cancer were 2.2 times greater with MPA than with MPA + curcumin and 3.05 and 4.4 times greater than with curcumin alone or placebo. Curcumin was unable to delay natural tumor development that occurs in response to DMBA (Fig. 1A). Thus, curcumin delays DMBA-induced tumors whose development is accelerated by MPA.

Curcumin also reduced the overall incidence of MPA-accelerated tumors (Fig. 1A). When the first tumor was detected in the MPA + curcumin group (day 42; 1/15, 6%), the incidence of tumors had reached 21% (4/19) in the group receiving MPA alone. However, these data also did not reach statistical significance.

**Curcumin inhibits multiplicity in MPA-accelerated tumors in DMBA-treated rats**

At the conclusion of the study (day 50; Fig. 1A), we found that curcumin significantly reduced the number of tumors per tumor-bearing animal in the DMBA-induced, MPA-accelerated group from 2 to 1 (Fig. 1B).

**Curcumin inhibits MPA-induced morphological changes in mammary glands**

To determine whether curcumin affects MPA-induced changes in mammary gland morphology, non–tumor-bearing mammary tissue was collected at the end of the experiment (day 52). Mammary gland tissue was also analyzed from DMBA-treated rats that were administered curcumin without MPA. Mammary tissue from animals given MPA exhibited extensive proliferation of the mammary epithelium, resulting in a filling in of the ducts and the formation of hyperplastic alveolar nodules (Fig. 2). However, hyperplastic ductules and nodule formation were minimal when curcumin was administered before MPA, and mammary tissue resembled that of normal mammary glands.

**FIG. 2.** Curcumin prevents MPA-induced morphological changes in the mammary gland. Mammary gland tissue was collected at the conclusion of the study on day 52, sectioned, and stained with H&E. One representative section is shown for each group. Scale bars = 500 μm. MPA, medroxyprogesterone acetate; H&E, hematoxylin and eosin.
DMBA-treated controls or lesions found in the group treated with DMBA + curcumin (Fig. 2).

**Curcumin blocks MPA-driven VEGF induction in hyperplastic lesions in the mammary gland**

Based on our previous in vitro studies, we hypothesized that curcumin would reduce levels of MPA-induced VEGF and that this might explain both the delayed onset of tumor formation and the reduction in tumor cell proliferation. Consequently, we measured VEGF expression by immunohistochemistry in sections of mammary gland obtained from each of the experimental groups described in the previous section. Hyperplastic lesions of DMBA-treated, MPA-exposed mammary glands were strongly stained for VEGF (Fig. 3A, right panels), whereas curcumin administration reduced the levels of VEGF staining. Although VEGF levels within the

![VEGF Staining in Ductal Epithelial Cells](image)

**FIG. 3.** A: ER and PR expressions are unaffected by curcumin, and VEGF levels are decreased by curcumin in MPA + curcumin-treated DMBA-induced mammary tumors. Mammary gland tissues were collected at the conclusion of the study on day 52, sectioned, and immunostained for ER-α, ER-β, PR, and VEGF as described in “Methods.” No significant differences were observed in the intensity of the staining between the treatment groups for ER-α, ER-β, and PR; however, curcumin blocked MPA-driven increases in VEGF levels in hyperplastic lesions (red arrows). Insets represent negative controls with no primary antibody staining for each antibody. B: Photographs of slides were analyzed using FoveaPro 3.0 analysis software. Positive VEGF staining was quantified as the number of VEGF-positive pixels in three different fields. Although not statistically different, the amount of VEGF was reduced in the group treated with MPA + curcumin compared with the group treated with MPA alone. No significance was determined when analyzed using analysis of variance. ER, estrogen receptor; PR, progesterone receptor; VEGF, vascular endothelial growth factor; MPA, medroxyprogesterone acetate.
mammary glands of MPA-treated animals were reduced by 34% when curcumin was coadministered, because of the small sample size, there was no statistical significance as analyzed by ANOVA (Fig. 3B).

**Estrogen and PR levels are not affected by curcumin treatment**

Signaling through ERs is critical for PR expression, and PR activity is essential for VEGF induction. Although curcumin does not bind to either ERs or PRs with high affinity, if curcumin were to affect the levels of these receptors, it could provide a mechanism for the observed reduction in VEGF in tumor cells and the subsequent delay in the appearance of tumors after MPA exposure. However, immunostaining for ER (ER-α and ER-β) and PR did not differ between the mammary tissues from any of the experimental groups (Fig. 3A), demonstrating that curcumin does not affect the expression of either receptor type.

**DISCUSSION**

In this report, we provide evidence that the dietary compound curcumin, used as a spice mainly in Asian countries, delays the appearance of progestin-accelerated breast tumors in a DMBA-induced tumor model and reduces the risk of tumor development. This effect is exerted through inhibition of VEGF production by tumor cells (Fig. 3A), a finding that is concordant with the results of our in vitro studies using cultured breast cancer cells. Importantly, we also observed that curcumin reduced the multiplicity of tumors generally observed after acceleration of tumor development with progestins and that it preserved the morphology of mammary glands in MPA-treated animals; the abnormal proliferation of intraductal tumor cells leading to hyperplastic lesions in MPA-treated animals was absent in curcumin-treated animals. There were more proliferating lobules in the MPA-treated group than in controls, whereas curcumin-treated animals were histologically similar to the placebo group, suggesting that the turmeric root derivative helps maintain normal morphology. Singletary et al. showed that curcumin, when administered before DMBA, delays DMBA-induced mammary tumorigenesis; however, in the current study, we are the first to show that MPA-accelerated, DMBA-induced mammary tumors can be delayed by curcumin and that the morphology of the mammary gland can be protected. A limitation of our study is the relatively small sample size used for comparative purposes, with tumor incidence, latency, and VEGF staining in the hyperplastic regions of the mammary gland consequently not attaining significance. However, the ability of curcumin to protect against increased multiplicity of MPA-induced tumors and to preserve the normal cellular structure of the mammary gland provides a strong rationale for considering its use as a chemopreventive agent against progestin-dependent mammary tumors in women who have already been exposed to combined HT containing MPA.

The specific mechanism by which curcumin inhibits angiogenesis remains to be elucidated; however, there are studies suggesting that cyclin D and p21 are involved in its anticancer properties. To date, though, no studies that would shed light on how curcumin affects progestin-dependent mammary cancers have been published. A number of studies in other models report that nuclear factor (NF)κB is a target for curcumin and that in breast and ovarian cancer cells, curcumin inhibits NF-κB activation and thereby decreases VEGF mRNA expression. Curcumin has also been reported to reduce the expression of matrix metalloproteinases as a consequence of decreased NF-κB activity and transcriptional down-regulation of activator protein 1, indicating that it may prevent the inflammatory response usually associated with tumor progression. It may be that in our studies, curcumin inhibits NF-κB activity, although another possible mechanism of action is the specific induction of cancer cell apoptosis and cell cycle arrest at the G2 phase, a possibility that remains to be tested. Curcumin has been shown to exhibit antigenotoxic activity against DMBA-induced mammary tumors. In the earlier study, it was demonstrated that at the same doses used in our studies, curcumin inhibited DMBA-induced mammary tumorigenesis, as well as DMBA-DNA adduct formation in rats when administered before DMBA. However, in our studies, we focus on the inhibitory activity of curcumin on MPA-accelerated tumors using the DMBA model, in which the turmeric root derivative is administered well after DMBA.

ERs are major regulators of PRs in breast cells and ER-β has been associated with inhibition of angiogenesis and growth of human breast cancer xenografts. We therefore sought to determine whether curcumin directly suppresses levels of PR, thereby inhibiting the production of MPA-induced VEGF, or blocks ER signaling, which would indirectly suppress PR levels. However, we detected no differences in the levels of either type of ER (ER-α or ER-β) or PRs in the mammary glands of animals receiving DMBA and subsequently treated with curcumin relative to controls, indicating that curcumin exerts its anticancer properties directly on the mammary gland with little or no effect on ovarian hormone receptors. Thus, combination treatments with agents targeting ER and PR in addition to curcumin could be a viable option.

**CONCLUSIONS**

In conclusion, in this preclinical study, we have shown that curcumin has the ability to inhibit MPA-driven mammary tumorigenesis. Although we did not elucidate the specific mechanism by which curcumin exerts its suppressive effects, we did show that down-regulation of either PR or ER was not involved. We also showed that curcumin attenuates MPA-induced increases in VEGF levels in the mammary gland. Finally, we demonstrated that curcumin blocks pathological changes in mammary gland morphology arising as a consequence of the tumorigenesis brought about by exposure to MPA. The dose of curcumin administered to animals in our studies elicited no toxic effects as judged by lack of any loss in animal weight, demonstrating its safety even at high doses. Furthermore, a phase 1 clinical trial with curcumin
reports that no toxicity was observed when participants were given up to 8,000 mg/day. We propose therefore that curcumin be considered an excellent candidate for use as a chemopreventive agent in clinical trials involving postmenopausal women taking combined HT containing both estrogens and progestins.

REFERENCES