Suppression of 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinogenesis in Rats by Resveratrol: Role of Nuclear Factor-κB, Cyclooxygenase 2, and Matrix Metalloprotease 9

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ABSTRACT

We have recently reported that resveratrol (trans-3,4′,5-trihydroxystilbene), a polyphenolic phytoalexin found in grapes, fruits, and root extracts of the weed Polygonum cuspidatum, is a potent inhibitor of nuclear factor (NF)-κB activation. Because NF-κB suppression has been linked with chemoprevention, this prompted us to investigate the chemopreventive potential of resveratrol by testing it against mammary carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA) in female Sprague Dawley rats. Dietary administration of resveratrol (10 ppm) had no effect on body weight gain and tumor volume but produced striking reductions in the incidence (45%; P < 0.05), multiplicity (55%; P < 0.001), and extended latency period of tumor development relative to DMBA-treated animals. Histopathological analysis of the tumors revealed that DMBA induced ductal carcinomas and focal microinvasion in situ (7 of 7), whereas treatment with resveratrol suppressed DMBA-induced ductal carcinoma. Immunohistochemistry and Western blot analysis revealed that resveratrol suppressed the DMBA-induced cyclooxygenase-2 and matrix metalloprotease-9 expression in the breast tumor. Gel shift analysis showed suppression of DMBA-induced NF-κB activation by resveratrol. Treatment of human breast cancer MCF-7 cells with resveratrol also suppressed the NF-κB activation and inhibited proliferation at S-G2-M phase. Overall, our results suggest that resveratrol suppresses DMBA-induced mammary carcinogenesis, which correlates with down-regulation of NF-κB, cyclooxygenase-2, and matrix metalloprotease-9 expression.

INTRODUCTION

Almost 600,000 new cases of breast cancer are identified each year worldwide. In North America, breast cancer accounts for over one quarter of all cancers in women and is second only to lung cancer as a cause of cancer-related deaths (1). Despite abundant information about its etiopathogenesis and early detection, effective therapeutic modalities for patients with advanced stages of the disease are still needed. Adjunct therapy after ablative surgery is effective only when the tumor is detected early. Accumulating evidence derived from laboratory studies and study cohorts drawn from the general population have led to the search for “chemoprotection” agents to attenuate the risk of breast cancer based on observation that most human cancers are associated with a long period of latency (2, 3). Several nonnutritive phytochemicals found in natural products and associated with pharmacological attributes reveal evidence that they inhibit, delay, and/or reverse cancer evoked by either environmental insults and/or lifestyle (4, 5). Several of these chemopreventive agents act at the initiation, promotion, and/or progression stages conceptually associated with the ontogeny of multistage carcinogenesis.

Resveratrol (3,4′-trihydroxystilbene), a natural phytoalexin present in grapes and many other natural sources, has been suggested to play a role in reducing the risk of coronary heart disease and cancer (6–8). In addition, resveratrol intake has been reported to have anti-inflammatory and anti-atherosclerosis functions and to modulate hepatic aliprotein and lipid synthesis, platelet aggregation, and production of antithromogenic eicosanoids by human platelets and neutrophils (7, 9–11). Resveratrol has also been reported to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary organ cultures and the promotional stage of mouse skin carcinogenesis (7). Additionally, it mediates reduced aberrant colonic crypt foci formation and inhibits of the growth of a wide variety of human-derived tumor cells, including leukemic, prostate, breast, and endothelial cells (12–19). Other targeted cellular effects belonging to resveratrol involve inhibition of the enzymes protein kinase C, ribonucleotide reductase, cyclooxygenase, and nitric oxide synthase and inhibition of aryl hydrocarbon-induced cytochrome P-450 IA1 (20–25).

A close structural similarity exists between synthetic estrogen (4,4′-dihydroxy-trans-α,β-diethylstilbene) and resveratrol. It is unclear, however, whether resveratrol is an estrogen receptor agonist or antagonist. Estrogen agonists have been reported to exert protective action against estrogen-dependent cancers, such as cancer of the breast and endometrium; presumably, resveratrol interacts with estrogen receptor to inhibit its activation (26). Lu and Serrano (26) reported that resveratrol acts as an estrogen receptor antagonist in the presence of estrogen, leading to inhibition of growth of human breast cancer cells.

In recent years, the importance of the transcription factor NF-κB in promoting tumorigenesis has been well recognized. NF-κB binds to consensus elements within the promoter regions of a variety of targeted genes (27). Further investigations have revealed that the expression of a multitude of critical genes are regulated by NF-κB, including immunoreceptors, transcription factor-associated proteins (c-myc and p53), cell adhesion molecules (intracellular adhesion molecule, vascular cell adhesion molecule 1, and endothelial leukocyte adhesion molecule 1), and enzymes involved in tumor metastasis (COX-2, inducible nitric oxide synthase, and MMP-9). Under normal conditions, NF-κB is retained in the cytoplasm of cells, where it is bound by inhibitory proteins known as IκBs. It has also been documented that during carcinogenesis, NF-κB has the potentiality to mediate several of the events associated with multistep processes including acquisition of features such as promotion of cell survival and dysregulation of normal control of proliferation, metastasis, and angiogenesis (27–29). The constitutive activity of NF-κB has been shown to be essential for proliferation of several cell types, e.g., smooth muscle cells and hepatocytes during liver regeneration after partial hepatectomy or toxic damage (30, 31).

Previous reports from our laboratory and another have demonstrated the ability of resveratrol to down-regulate NF-κB expression in vitro (32, 33), leading to speculation that it would in turn inhibit...
cellular genes regulated by NF-κB and those involved in multistage tumorigenesis. In the present study, we evaluated the effect of resveratrol in inhibiting chemically induced mammary carcinogenesis in a rat model. This is the first report to indicate that resveratrol has a chemopreventive effect on breast tumorigenesis in vivo. Given the progressive aberrant expression of constitutive NF-κB factor with progression of the disease (34), we hypothesize that resveratrol interferes with cognate signaling by inhibiting of NF-κB activity during the mammary tumorigenesis cascade. We therefore used immunohistochemical and Western blot methods to examine the expression of two enzymes, COX-2 and MMP-9, both of whose promoter sequences contain binding sites for NF-κB. Additionally, we investigated the effect of resveratrol on NF-κB activation and cell growth in MCF-7 breast adenocarcinoma cells.

MATERIALS AND METHODS

Chemicals

Penicillin, streptomycin, RPMI 1640, FCS, and trypsin were obtained from Life Technologies, Inc. (Grand Island, NY). BSA, MTI, leupeptin, aprotinin, and DMBA of highest purity were purchased from Sigma Chemical Co. (St. Louis, MO). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 10^{6} units/mg, was kindly provided by Genentech (South San Francisco, CA). The radioisotope [5- methyl-^3^H] thymidine was obtained from Amersham Pharmacia Biotech, and [γ-^3^P]ATP (5 mCi) was purchased from ICN Radiochemicals (Costa Mesa, CA). Antibodies used were as follows: anti-p50, against the epitope corresponding to amino acids mapping within the NH2-terminal domain of human NF-κB p65; anti-p50, against a peptide 15 amino acids long mapping at the NLS region of NF-κB p50; and COX-2, against the epitope corresponding to amino acids 50–111 mapping near the COOH terminus of COX-2 of human origin. These were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For immunohistochemistry, COX-2 antibody (murine polyclonal) was purchased from Cayman Laboratories (Ann Arbor, MI), whereas rabbit antirat polyclonal antibody for MMP-9 was procured from Cell Sciences, Inc. (Norwood, MA). For Western blot analysis, antibodies used were as follows: anti-p65, against the epitope corresponding to amino acids mapping near the COOH terminus of COX-2 of human origin. These were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For immunochemistry, COX-2 antibody (murine polyclonal) was purchased from Cayman Laboratories (Ann Arbor, MI), whereas rabbit antirat polyclonal antibody for MMP-9 was procured from Cell Sciences, Inc. (Norwood, MA). Resveratrol was obtained from several sources; for animal experimentation, resveratrol (>98% pure) was purchased from Alexis Cooperation, San Diego, CA. For in vitro studies, resveratrol was procured from Sigma Chemical Co. A stock solution of resveratrol was made in DMSO at a concentration of 10 mM. The NF-κB oligonucleotide from the HIV long terminal repeat, 5′-TGTTACAAGGGACCTTCTGCTGGGACTTTCCAGGGAGGCTGG-3′ and a mutated double-stranded oligonucleotide, 5′-TGTTACACTACCATTTTCGAGGGGCGCTGG-3′ were from Life Technologies, Inc. (Grand Island, NY; underlined regions represent a consensus NF-κB binding sequence). All other chemicals were purchased from authentic sources and were of highest grade and purity.

Chemoprevention Studies

Animals. Female Sprague Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats arrived at 40 days of age and were placed on a common pellet diet and quarantined for 2 days. The animals were housed three/cage in standard rat Plexiglass cages in a room maintained at constant temperature and humidity under 12-h light and darkness. A complete health status was determined. None of the rats exhibited major lesions, and all were pathogen free. Before initiating the experiment, we acclimatized all rats on a common pellet diet and quarantined for 2 days. The animals were placed on a common pellet diet and quarantined for 2 days. Before initiating the experiment, we acclimatized all rats to the laboratory environment and then divided the rats into three experimental groups based on body weight:

- Group I (n = 7) received DMBA and pulverized diet; group II (n = 12), designated as a negative control, received DMBA and pulverized diet; and group III (n = 12) received DMBA and pulverized experimental diet containing resveratrol.

Animals were allowed free access to the basal diet or diet containing the chemopreventive agent and drinking water throughout the experiment. Our experimental protocol was reviewed and approved by M. D. Anderson Cancer Center Animal Care and Use Committee.

Treatment with Chemopreventive Agent. Animals (groups I and II) were given a normal diet containing vehicle control. Starting at 45 days of age, rats belonging to group III were treated with resveratrol (100 μg/rat) in the diet. The dose of resveratrol was determined to be 12–15 g/rat/day. Resveratrol was dissolved in 70% ethanol and then added into the diet 1 day in advance and left at room temperature for 1 day. After ethanol evaporation, food was given to the rats. Food cups were changed two times a week.

Chemoprevention Study Design. Rats belonging to the appropriate experimental group were given the chemopreventive agent beginning at day 45 of age. One week later (day 0), rats belonging to groups II and III were given 10 mg of DMBA by gavage in sesame oil. This dose of DMBA is suboptimal to produce sufficient tumors to allow evaluation of both reduction and increase in the end point of carcinogenicity. Rats were weighed weekly, palpated for mammary tumors once/week (starting 4 weeks after DMBA treatment), and monitored daily for signs of toxicity. The study was terminated at 120 days after DMBA administration. All surviving animals, including those that did not seem to develop mammary tumors as well as the control group (group I), were killed by CO2 asphyxiation and completely necropsied to evaluate possible signs of toxicity. Tumors were removed and fixed in 10% buffered formalin. Thirteen DMBA-induced mammary tumors from resveratrol (group III) or control (group II) were evaluated with a blind method for histopathology. The tumors were subjectively graded either as carcinomas or fibroadenomas. The end point for data analysis included: (a) the number of animals with tumors (tumor incidence); (b) the number of tumors/animal (tumor multiplicity); (c) latency to tumor appearance; and (d) tumor volume. Estimates of tumor volume were determined using the formula V = (r^3 × π)/3 × m, where r is half of the average diameter (in millimeters) measured with a vernier caliper at two different planes.

Histological Sections

Formalin-fixed tissue was paraffin-embedded, sectioned at 3–5 μm, and stained with H&E. Sections were evaluated for tumor cell cytology, mitotic rate, growth pattern, necrosis, cystic change, and associated inflammatory cellular response.

Immunohistochemistry

Immunohistochemical studies were performed using paraffin-embedded material, heat-induced antigen retrieval (citrate buffer, pH 6.0), and polyclonal antirat antibodies specific for MMP-9 (Cayman Laboratories, Inc.; Ann Arbor, MI; COX-2 (1:500; Cayman Chemical Co., Ann Arbor, MI). The detection system used was the LSAB2 detection kit (DAKO). Negative controls also were run.

Preparation of Nuclear Extract from Tissue Samples

Normal mammary epithelium and tumor samples (200–250 mg) randomly selected from untreated control and experimental groups were minced and incubated on ice for 30 min in 0.5 mL of cold buffer A, composed of 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.1% IGEPAL CA-630, and 0.5 mM phenylmethylsulfonyl fluoride. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 14,000 rpm at 4°C for 10 min. The nuclear pellet obtained was suspended in 0.2 mL of buffer B [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 4 μm leupeptin] and incubated on ice for 2 h with intermittent mixing. The suspension was then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at −70°C until use. The protein concentration was measured by the method of Bradford with BSA as the standard. EMSA was performed by incubating 12 μg of nuclear protein extract by procedure as described (36).

Western Blot Analysis

Mammary epithelium and/or tumor specimens from each of the above-mentioned experimental groups were thawed on ice and homogenized at 4°C using a Dounce homogenizer in RIPA lysis buffer for extracting total cell protein. Sixty μg of whole-cell protein was resolved on 10% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibodies against MMP-9 (1:3000) or COX-2 (1:1000), separately. The blots were washed, exposed to horseradish...
Cell Line

MCF-7 cells were a kind gift of Dr. Kapil Mehta (M. D. Anderson Cancer Center, Houston, TX). Cells were maintained in culture in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in an atmosphere of 5% CO₂ at 37°C.

EMSA

MCF-7 cells (1 × 10⁶/ml) were preincubated with different concentrations of resveratrol (0, 10, 25, and 50 μM) for 4 h and then treated with TNF (0.1 nM) for 30 min at 37°C. Nuclear extracts were then prepared according to the method described by Chaturvedi et al. (35). The protein content was measured by the method of Bradford (36). EMSA was performed by incubating 8 μg of nuclear extract with 16 fmol of ³²P-end-labeled, double-stranded 45-mer NF-κB oligonucleotide. The incubation mixture included 2 μg of poly(deoxyinosinic-deoxycytidylic acid) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gel, and then the gel was dried. The radioactive bands from dried gels were visualized by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Image Quant software.

The composition and specificity of binding was examined by competition with 100-fold excess of unlabeled oligonucleotide and with a mutated oligonucleotide. For the supershift assays, nuclear extract were incubated with the antibodies against either p50 or p65 subunits of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA (36).

Evaluation of Cell Viability by MTT

MCF-7 cells were plated (in triplicate) in 96-well plates (5000 cells/well), 24 h before addition of resveratrol. Medium was then aspirated, and cells were exposed to 2-fold serial dilutions of resveratrol in 0.2 ml of fresh medium and incubated at 37°C in an atmosphere of 5% CO₂ for 72 h. Thereafter, cell viability was measured by the MTT method using a multiscanner autoreader (Dynatech MR 5000, Chantilly, VA; Ref. 32). To examine the antiproliferative effects of resveratrol, 2000 cells in 0.1 ml were plated overnight and then treated with 10 or 50 μM resveratrol in 0.2 ml for 2, 4, and 6 days. Thereafter, the cell viability was determined by the MTT method.

Evaluation of Cell Viability by Thymidine Incorporation

The sensitivity of MCF-7 cells to resveratrol was also determined by [³H]thymidine incorporation. Briefly, MCF-7 cells (5000 cells/well) were plated in 0.1 ml of medium (RPMI 1640 plus 10% fetal bovine serum) in a 96-well plate. After overnight incubation in a CO₂ incubator at 37°C, the medium was removed, and different concentrations of resveratrol (twofold serial dilutions starting from 100 μM concentration) were added in 0.2 ml of fresh medium for 72 h. During the last 6 h of incubation before harvesting, 0.1 μCi [³H]thymidine was added to each well. Thereafter, the medium was aspirated, wells were washed with PBS, and cells were detached by the addition of a solution containing trypsin (0.5%) plus EDTA (5.3 mM). The cell suspension was harvested on a Filter Mate 196 cell harvester (Packard Instruments, Meriden, CT) and lysed by washing with distilled water. Radioactivity bound to the filter was measured by Direct Beta Counter (Matrix 9600; Packard Instruments, Meriden, CT).

FACS Analysis

Cells (2 × 10⁶) in 1 ml of culture medium were plated in 6-well plates overnight and then treated with 50 μM resveratrol or medium for 6, 12, 24, or 48 h. Thereafter, the cells were harvested by trypsinization, washed twice with PBS, and fix in 5 ml of 95% ethanol. After 12 h incubation at −20°C, cells were washed and resuspended in PBS. The cellular DNA was then stained with 1 ml of propidium iodide (50 μg/ml) containing 0.1 ml of RNase (1 mg/ml). After incubation at 37°C for 30 min, cells were analyzed by FACS analysis.

RESULTS

Resveratrol Suppresses DMBA-induced Rat Mammary Tumorigenesis. NF-κB inhibitors have been shown to exhibit chemopreventive properties (37–45). To determine whether resveratrol affects DMBA-induced rat mammary carcinogenesis, experiments were designed to examine chemoprevention after continuous dietary administration of resveratrol beginning 1 week before carcinogen administration and lasting until termination of the experiment (Fig. 1). The rationale for using this specific protocol relates to identifying agents that intervene in initiation and progression of cancer. No adverse effect on body weight gain during the observation period was detectable, and the average body weight gain was similar in all of the groups throughout the experiment (267 ± 2.8, 267 ± 4.5, and 268 ± 2.7 for groups I, II, and III, respectively; P = not significant; Fig. 2). Moreover, no evidence of development of any spontaneous tumor, including tumors of the breast, occurred in animals randomized to the negative control group (group I) during the span of the study. Administration of resveratrol and/or DMBA did not reveal any gross changes in the liver, lung, kidneys, stomach, or intestinal tract of animals. In group II, 75% of the animals treated with DMBA (positive control) developed palpable breast tumors at 11 weeks after DMBA treatment (Fig. 3), and the average number of tumors/tumor-bearing animal was 2.41 ± 0.5 (mean ± SE; Fig. 4). Rats of group III, which received resveratrol-supplemented diet in addition to DMBA, had a tumor incidence of only 41% (P < 0.05), and the average number of tumors/tumor-bearing animal was reduced to 1.08 ± 0.5 (P < 0.001). Furthermore, differences between the groups (group II versus group III) in cancer latency was observed; in the resveratrol-treated animals, the latency was 98 days after DMBA treatment compared with 77 days for DMBA-only treatment. No significant difference in the tumor volume was noted between groups II and III (1.58 ± 0.36 versus

Statistical Analysis

Body weight, tumor incidence, tumor multiplicity, and tumor volume were determined for the animals fed control diet and for those fed on the modified diet containing resveratrol. The data were analyzed and compared by χ² test using Sigma-Stat software (Jandel Scientific, San Rafael, CA).

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1.26 ± 0.70, respectively). In our study, two animals were removed, one each from group II and group III before termination schedule because of the presence of necrotizing tumor.

**Resveratrol Suppresses DMBA-induced Ductal Carcinoma.** Histopathological analysis was performed on tumor samples randomly selected from untreated (group I), DMBA-treated (group II), and DMBA + resveratrol-treated (group III) animals (Fig. 5). Normal mammary epithelium from control animals showed no pathological abnormality (Fig. 5A). Tumors obtained from DMBA-treated animals had ductal carcinomas and focal microinvasion *in situ* (7 of 7; Fig. 5, B–D). The surrounding breast tissue showed papillomatosis, atypical ductal epithelial hyperplasia, and no fibroadenomas or infarcts. In contrast, tumors harvested from DMBA- and resveratrol-treated animals exhibited evidence of fibroadenoma (2 of 4) and partially infarcted ductal carcinoma *in situ* with microinvasion (4 of 4; Fig. 5, E and F).

**Resveratrol Suppresses DMBA-induced Intracellular Expression of COX-2 and MMP-9 Proteins.** Immunohistochemistry revealed the absence of COX-2 activity in normal mammary epithelium (Fig. 5G), high expression in tissue from DMBA-treated rats (7 of 7; Fig. 5H), and low expression (3 of 4; Fig. 5I) or no expression (1 of 4) in DMBA + resveratrol-treated rats. When examined for MMP-9 expression by immunohistochemistry, staining in the normal mammary epithelium (Fig. 5J) was consistently absent, the breast tissue from the DMBA-treated group showed uniformly positive staining (7 of 7; Fig. 5K), and the tissue from the DMBA + resveratrol-treated group showed low expression (4 of 4; Fig. 5L).

To further confirm the effect of resveratrol on DMBA-induced COX-2 and MMP-9 expression, we examined protein expression by Western blot analysis. Because both COX2 and MMP-9 are regulated by NF-κB activation, we also measured the nuclear levels of NF-κB by EMSA in the mammary tissue. As shown in Fig. 6, none of the untreated tissues samples expressed NF-κB DNA-binding activity; tissues derived from 8 of 9 DMBA-treated animals expressed NF-κB DNA-binding activity, whereas only 3 animals of 9 from the DMBA + resveratrol-treated group expressed NF-κB. These results suggest that DMBA induces NF-κB DNA-binding activity in most cases, and resveratrol suppresses it. When examined for COX-2 and MMP-9 expression, tissues from all of the DMBA-treated group expressed these proteins (none in the untreated group), but only low levels were detected in tissues from animals treated with DMBA together with resveratrol. These results are consistent with that obtained from immunohistochemistry.

**Resveratrol Inhibits TNF-induced NF-κB Activation in Human Breast Cancer Cells.** Our results show that resveratrol suppressed DMBA-induced mammary carcinogenesis. NF-κB has been implicated in carcinogenesis. Previously, we and others have shown that
Resveratrol does suppress NF-\(\kappa\)B activation in various cell types (32, 33). Whether resveratrol inhibits NF-\(\kappa\)B activation in breast cancer cells was investigated. MCF-7 cells were pretreated for 4 h with different concentrations of resveratrol and then stimulated with 0.1 nM TNF for 30 min. As assessed by trypan blue and MTT assay, resveratrol did not affect cell viability at this concentration and time point. Nuclear extracts were made and assayed for NF-\(\kappa\)B by EMSA. As shown in Fig. 7A, TNF induced DNA-binding activity of NF-\(\kappa\)B, and resveratrol inhibited this activation in a dose-dependent manner; full inhibition occurred at 50 \(\mu\)M resveratrol. Resveratrol alone at this concentration did not activate NF-\(\kappa\)B.

Various combinations of Rel/NF-\(\kappa\)B proteins can constitute an active NF-\(\kappa\)B heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA was indeed NF-\(\kappa\)B, we incubated the nuclear extract from TNF-activated cells with antibodies to either p50 (NF-\(\kappa\)B) or the p65 (relA) subunit and then conducted EMSA. Antibodies to either subunit of NF-\(\kappa\)B shifted the bands to a higher molecular weight (Fig. 7B), thus suggesting that the TNF-activated complex consisted of both the p50 and p65 units. Preimmune serum had no effect on the mobility of NF-\(\kappa\)B. Excess unlabelled NF-\(\kappa\)B almost completely eradicated the band, indicating the specificity of NF-\(\kappa\)B. Further specificity is indicated by the observation that the oligonucleotide probe with labeled mutated NF-\(\kappa\)B binding site failed to bind the NF-\(\kappa\)B protein.

Fig. 5. Effect of resveratrol on the DMBA-induced pathology and on COX-2 and MMP-9 expression. Representative H&E-stained sections (A–F) and immunohistochemical staining (G–L) for COX-2 and MMP-9 of breast lesions are shown. Breast tissue from untreated control (A, normal), from DMBA-treated animals (B, papillomatosis; C, ductal carcinoma in situ/atypical ductal hyperplasia; and D, invasive ductal carcinoma), and from DMBA- and resveratrol-treated animals (E, fibroadenoma; and F, basic carcinoma with extensive necrosis) are shown. G–I, immunohistochemical staining for COX2 protein demonstrates COX-2 negativity in breast tissue from untreated rat (G), strong positivity in ductal carcinoma from DMBA-treated rat (H), and faint positivity in the resveratrol plus DMBA-treated tumor with necrosis (I). J–L, immunohistochemical staining for MMP-9. Lack of immunoreactivity in the untreated breast tissue (J), strong cytoplasmic staining in the tumor from DMBA-treated rat (K), and weak immunopositivity in DMBA plus resveratrol-treated tumor (L) are shown.
Resveratrol Inhibits the Growth of Human Breast Cancer Cells. Whether resveratrol suppresses the proliferation of human breast cancer cells was also investigated. MCF-7 cells were treated with 2-fold serial dilution of resveratrol for 72 h, either in the presence or absence of resveratrol, and then examined for growth by MTT and cell proliferation by thymidine incorporation. Results in Fig. 8A indicate that resveratrol inhibited the growth of human breast cancer cells in a dose-dependent manner, with almost 60% suppression of cell viability at 100 μM concentration. When examined for cell proliferation by DNA synthesis, 90% inhibition of thymidine incorporation occurred with 100 μM resveratrol (Fig. 8B). Thus, breast cancer cells were more sensitive to resveratrol in the DNA synthesis assay than that in the assay of mitochondrial activity. We also examined the effect of resveratrol on the proliferation of MCF-7 cells. Fifty μM resveratrol was sufficient to completely suppress the proliferation of breast cancer cells (Fig. 8C).

We also examined the effect of resveratrol on the cell cycle analysis of MCF-7 cells. Results shown in Fig. 8D clearly show resveratrol induced the accumulation of cells in S-phase of the cell cycle. In the resveratrol-treated samples at 24 h, 48% of the cells were in S-phase as compared with 11% in the control. A complete suppression of the cells in the G2-M phase of the cycle could be noted as early as 12 h after resveratrol treatment.

**DISCUSSION**

The present study demonstrates the chemopreventive action of resveratrol in a well-established, chemically induced animal protocol of breast cancer and its relation to NF-κB expression. In recent years, “cancer chemoprevention” by biologically active dietary or nondietary supplements has generated immense interest in view of their putative role in attenuating the risk of developing cancer. Against this background, resveratrol is a promising agent in affording chemoprotection against several major human epithelial and nonepithelial cancers. Resveratrol was first evaluated in vitro and reported to inhibit mammary lesions induced by DMBA, leading to speculation of a specific effect on the mammary gland independent of systemic drug metabolism. Additionally, divergent beneficial modulatory effects of resveratrol has been reported in the literature (46). We report in this study that resveratrol fed to Sprague Dawley female rats inhibited tumor formation in comparison to vehicle treatment in DMBA-initiated mammary carcinogenesis. Tumor incidence calculated as the percentage of animals with one or more palpable tumors/treatment group was reduced by 45% after resveratrol supplementation. Other indices of chemopreventive response-tumor multiplicity, expressed as average number of tumors that developed per animal/week in each treatment group was reduced from 2.41 to 1.04 tumors/animal by resveratrol feeding at the termination of the experiment. Furthermore, relative to control animals, the latency to onset of tumor development was prolonged by 3 weeks by resveratrol.

Precisely how resveratrol inhibits breast cancer is not certain, although several possible modes of action have been proposed and studied at the cellular and molecular level. Resveratrol is able to mimic the activity and effects of endogenous 17β-estradiol. According to a proposed hypothesis, estrogen has a dual affect on breast cancer risk (47). Evidence indicates that estrogens promote the growth of existing malignancies in the breast. In contrast under certain circumstances such as pregnancy, during the prepubertal period and childhood, estrogen actually reduces breast cancer risk through estrogen-induced activation of certain tumor suppressor genes including BRCA1 and p53 (47). It may thus be speculated that resveratrol, as a putative estrogen agonist, augments mammary tissue differentiation and maturation beyond the optimum period of carcinogen sensitivity, thus conferring a protective effect. It has been reported that complete morphological differentiation of the mammary gland protects against mammary carcinogenesis in Wistar Furth rats. Pretreatment of rats with estradiol and progesterone or completion of pregnancy and lactation before carcinogen exposure markedly reduces the susceptibility of the gland to chemical carcinogenesis (48).

In our study design, resveratrol treatment was initiated before onset of puberty. Because resveratrol is known to induce differentiation (49), it is likely that resveratrol treatment accelerated mammary tissue differentiation, leading to refractory cell phenotypes during the period.
RESVERATROL BLOCKS MAMMARY CARCINOGENESIS

Fig. 7. A, resveratrol suppresses TNF-induced NF-κB activation in human breast MCF-7 cells. A total of 1 × 10⁶ cells/ml were preincubated at 37°C for 4 h with different concentrations of resveratrol (0–50 μM), followed by a 30-min incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described in “Materials and Methods.” B, supershift and specificity of NF-κB activation. Nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated MCF-7 cells (1 × 10⁶ cells/ml), incubated for 30 min with different antibodies as indicated, preimmune sera, unlabeled wild-type oligo or mutant oligo, and then assayed for NF-κB, as described in “Materials and Methods.”

of carcinogen sensitivity. The mammary gland of the rat undergoes extensive development after 32–35 days of age, first appearing as terminal end buds that subsequently evolve into alveolar buds and eventually into terminal ducts; this occurs at 40–60 days of age. Under normal conditions, DMBA is most effective in producing tumors during the most active transition period of terminal end bud evolution into alveolar buds. The greater incidence and tumor yield in the DMBA-treated animals occurred because transition in the mammary tissue was occurring in the normal window of DMBA sensitivity. Such a mechanistic proposition has been put forth by Anderson et al. (50), who studied the effect of constant light on DMBA-induced mammary tumorigenesis in rats. Neonatal genistein treatment exerts its chemopreventive action by directly enhancing maturation of the terminal ductal structures and by altering the endocrine system to reduce cell proliferation in the mammary gland (51, 52).

Several other related mechanisms relevant to the observed chemopreventive effects of resveratrol may be cited. As a corollary to an earlier report from this laboratory, resveratrol substantially inhibited TNF-induced NF-κB in MCF-7 cells, further strengthening the idea that inhibition is not cell type specific. Moreover, no general transcription suppression to resveratrol occurs under our experimental conditions (32).

How resveratrol blocks TNF-induced NF-κB in MCF-7 cells is not clear. Most inhibitors of NF-κB mediate their effect through suppression of phosphorylation and degradation of IκBα. Resveratrol has been shown to block the phosphorylation and the degradation of IκBα (33). We have shown that resveratrol blocks the phosphorylation of p65 required for its transactivation function (32). Reports of another study by Kim et al. (53) revealed aberrant expression of NF-κB in mammary tumors induced by DMBA treatment of Sprague Dawley rats compared with normal mammary gland of age-matched, vehicle-treated control animals. Moreover, they reported that NF-κB/Rel activation occurs before malignant transformation and is not present normally in the mammary gland, suggestive of a significant association between activation of NF-κB expression and the progression of epithelial cells to a malignant phenotype. Moreover, aberrant expression of NF-κB in human breast cancer specimens has also been reported (34). Thus, our previous findings that resveratrol mediated down-regulation of NF-κB factor indicate that resveratrol may attenuate the early critical steps involved in carcinogen-driven transformation of mammary epithelial cells, including dysregulation of normal control of proliferation and protection from apoptosis.

Antioxidants such as N-acetylcysteine and pentoxifylline, which are already in clinical use, repress NF-κB activity and concurrently exhibit significant inhibitory effects on proliferation of breast cancer cells in culture (54, 55). Resveratrol has been reported recently to mediate phosphorylation of p53 at serine 15 through extracellular signal-regulated kinase and p38 kinase activities, leading to induction of apoptosis (56). The observed dose-dependent effect of resveratrol on the proliferation of MCF-7 cells noted in our present study implies that resveratrol-mediated chemoprotection may occur through the up-regulation of apoptosis. Several in vitro studies have documented an antiproliferative effect of resveratrol on many cell types (57–63), including those derived from the human breast (14, 26). Resveratrol has been shown to inhibit the transition of cells from the S-to-G2 phase of the cell cycle (15–18, 59, 60). Our results are in agreement with these reports.

It is now well established that once breast cancer initiation has taken place, estrogen promotes the growth of transformed cells, leading to the development of detectable breast cancer. Resveratrol behaves like the partial estrogen receptor agonist tamoxifen, which blocks the action of estrogen in the breast and effectively prevents primary and recurring breast tumor development (64). Another relevant important mechanism of action of many chemopreventive agents is through their ability to modulate the xenobiotic-metabolizing enzymes, e.g., by inhibiting metabolic activation of a procarcinogen or by increasing detoxification of reactive metabolites. In polycyclic aromatic hydrocarbon tumorigenicity, oxidative phase I biotransformation results in highly reactive diol epoxides that form covalent adducts with DNA. Reports indicate that the level of polycyclic aromatic hydrocarbon-DNA adducts is related to the level of CYP1A1 expression (65). Interestingly, resveratrol has been reported to inhibit constitutive and inductive expression of oxidative phase I biotransformation-related CYP1A1 in human bronchial epithelial and breast cancer cells (23, 24, 66). Furthermore, Jang et al. (8) have shown that resveratrol induces quinone reductase activity, a phase II enzyme, in cultured mouse hepatoma cells. Thus, one may also interpret the observed chemopreventive action of resveratrol primarily at the level of inhibition of procarcinogen activation, leading to reduced bioacti-
vated DMBA metabolites as well as increased expression of phase II detoxification enzymes. Several presumptive chemopreventive agents reveal the potential to induce and enhance detoxification activities in host target organs. Moreover, resveratrol-mediated down-regulation of CYPIAI also implies the 2-hydroxylation of 17β-estradiol and estradiol, which may further attenuate the effect of estrogen on the development of breast cancer.

Consistent with the hypothesis that COX-2 is not expressed in normal tissue, no evidence of its expression was detectable by immunohistochemistry performed on normal mammary tissue. However, resveratrol has been reported to inhibit COX-2 transcription and activity in phorbol ester-treated human mammary epithelial cells (20). Qualitative immunohistochemical analysis for COX-2 revealed immunoreactivity in fibroadenoma as well as in tumor specimens derived from resveratrol-pretreated animals. It can thus be inferred from our findings that resveratrol-mediated down-regulation of COX-2...
may be effective in attenuating the early stages of carcinogenesis, thus reducing tumor incidence and multiplicity. COX-2 inhibitors demonstrate strong chemoprotective potential. Besides COX-2, MMP-9 is another gene regulated by activation of NF-κB. Our results show clearly that treatment of animals with DMBA also induced MMP-9, and this induction was suppressed by treatment with resveratrol. Both COX-2 and MMP-9 have been implicated in tumor invasion and metastasis. Whether suppression of tumor growth by resveratrol in the animals is attributable to inhibition of DMBA-induced NF-κB activation is not clear. Because NF-κB activation was suppressed in most of the resveratrol-treated animals, it suggests that NF-κB may play an important role. However, NF-κB-independent mechanisms cannot be excluded based on our studies.

In conclusion, the present study demonstrates that resveratrol inhibits rat mammary tumor development and illustrates an emerging concept of chemoprevention through inhibition of transcription factor NF-κB. In addition, resveratrol functions as an inhibitor of breast cancer cells in vitro mediated through modulation of cell proliferation and apoptosis. There is no study reporting on the pharmacokinetics of resveratrol metabolism in humans subjects, and thus additional studies are warranted to determine the optimum effective dose of this phytochemical compound in inhibiting cancers in humans.

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REFERENCES

RESVERATROL BLOCKS MAMMARY CARCINOGENESIS


Suppression of 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinogenesis in Rats by Resveratrol: Role of Nuclear Factor-κB, Cyclooxygenase 2, and Matrix Metalloprotease 9

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