Intake of grape-derived polyphenols reduces C26 tumor growth by inhibiting angiogenesis and inducing apoptosis

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ABSTRACT This study evaluated the in vivo antitumor activity of grape-derived polyphenols. BALB/c mice were subcutaneously implanted with C26 colon carcinoma cells, and 2 d later they received either solvent or red wine polyphenols (RWPs) (100 mg/kg/d, human equivalent dose ≈500 mg/d) in the drinking water for 25 d. Wistar rats received either solvent or RWPs (100 mg/kg/d, human equivalent dose ≈1000 mg/d) in the drinking water 1 wk before injection of azoxymethane and were studied 10 wk later. In mice, RWPs inhibited tumor growth by 31%, reduced tumor vascularization and the number of lung metastases, decreased proliferation as indicated by down-regulation of Ki67, cyclin D1, and UHRF1, and increased apoptosis as indicated by TUNEL staining and active caspase-3 levels in tumor cells. RWPs reduced expression of VEGF, matrix metalloproteinase (MMP)-2, MMP-9, and cyclooxygenase-2 and increased expression of tumor suppressor genes p16INK4A, p53, and p73 in tumor cells. In rats, RWPs reduced by 49% the number of azoxymethane-induced aberrant crypt foci (preneoplastic lesions) in colon. Thus, RWPs effectively reduced the development of colon carcinoma tumors in vivo by blunting tumor vascularization and by inhibiting proliferation and promoting apoptosis of tumor cells subsequent to an up-regulation of tumor suppressor genes.—Walter, A., Etienne-Selloum, N., Brasse, D., Khallouf, H., Bronner, C., Rio, M.-C., Beretz, A., Schini-Kerth, V. B. Intake of grape-derived polyphenols reduces C26 tumor growth by inhibiting angiogenesis and inducing apoptosis. FASEB J. 24, 3360–3369 (2010). www.fasebj.org

Key Words: colon cancer • red wine polyphenols • neovascularization • tumor suppressor gene • metastasis • aberrant crypt foci

Colorectal cancer is the fourth most common form of cancer occurring worldwide (1). It accounts for ~1 million new cancer cases each year (2). Colorectal cancers develop as a result of the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium to colon adenocarcinoma. Such aberrant transformations have been suggested to involve alterations in TGF-β and Wnt signaling pathways such as adenomatous polyposis coli and β-catenin gene mutations as well as mutations of K-RAS and p53 (3–5). Epidemiological and experimental studies have suggested that the risk of developing colon cancer may be attributable, besides genetic and epigenetic alterations, to environmental factors (6). Indeed, consumption of high levels of red meat and fat together with low levels of fruits, vegetables, and fibers has been suggested to increase the risk of colorectal cancer (6, 7). Exaggerated cell proliferation, resistance to apoptosis, and tumor angiogenesis are associated with poor prognosis of colorectal cancer (8–11). Therefore, novel treatments to control these crucial events are critically warranted to inhibit tumor growth and metastasis.

It has been suggested that nutritional prevention could reduce the occurrence of colon cancer by ~60% (12). Several epidemiological studies indicate that regular and moderate consumption of red wine is associated with a 22% decreased risk of cancer (13). In addition, regular intake of a flavonoid mixture, containing apigenin and (−)-epigallocatechin gallate, reduced the recurrence rate of colon neoplasia in patients with resected colon cancer (14). Many plant polyphenolic compounds have been shown to have cancer preventive properties. For example, (−)-epigallocatechin gallate, a major constituent of green tea has been shown to have antitumor activity in animal models of colon (15) and skin (16) cancer. Red wine polyphe-
nols (RWPs) inhibited colon carcinogenesis induced by azoxymethane in rats (17) and resveratrol, a compound found in red wine, reduced glioma growth in rats (18). In addition, RWPs have antiangiogenic properties by preventing the expression of matrix metalloproteinase (MMP)-2 and VEGF, two major proangiogenic factors, both in vitro (19, 20) and in vivo (21). Furthermore, RWPs have been shown to prevent the potential effect of low doses of angiotensin II on ischemia-induced neovascularization in the rat hind limb (22). Therefore, the aim of the present study was to evaluate the in vivo anticancer properties of RWPs in both the syngeneic C26 tumorigenesis mouse model and in the azoxymethane-induced colon cancer rat model, and, if such properties were found, to determine the underlying mechanism.

MATERIALS AND METHODS

Preparation of red wine polyphenolic extract

Red wine polyphenolic dry powder was obtained from French red wine (Corbières A.O.C.) and prepared by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Professor P.-L. Teissedre (Département d’Oenologie, Université Bordeaux, Bordeaux, France) as described previously (21). One liter of wine provided 2.9 g of phenolic extract, which contained 471 mg/g of total phenolic compounds, expressed as gallic acid. Phenolic levels in the red wine extract were measured by HPLC. The extract contained 8.6 mg/g catechin, dimers (B1, 6.9 mg/g; B2, 8.0 mg/g; B3, 20.7 mg/g; and B4, 0.7 mg/g), anthocyanins (malvidin-3-glucoside, 11.7 mg/g; peonidin-3-glucoside, 0.06 mg/g; and cyanidin-3-glucoside, 0.06 mg/g), phenolic acids (gallic acid, 5.0 mg/g; caffeic acid, 2.5 mg/g; and caftaric acid, 12.0 mg/g), and stilbenes (trans-resveratrol, 0.4 mg/g and trans-piceid, 0.9 mg/g).

Syngeneic C26 tumorigenesis mouse model

C26 cells were derived from colon carcinomas induced in BALB/c mice by repeated intrarectal instillations of N-nitroso-N-methylurethane and exhibited an in vitro doubling time of 1.7 d (23). Cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, and 40 µg/ml gentamicin at 37°C. At 70–80% confluency, cells were trypsinized and portioned into aliquots for injections.

Nine-week-old BALB/c female mice (Charles River Laboratories, Saint Germain sur l’Arbresle, France) were used in the syngeneic C26 tumorigenesis mouse model. C26 cells (n=25×10⁶; 0.1 ml of PBS) were subcutaneously injected on both shaved flanks of mice. Forty mice were randomly divided into two groups: a control group receiving the vehicle of RWPs (3% ethanol, pH=3.3) and an RWP-treated group receiving 100 mg/kg/d of RWPs in the drinking water, corresponding to a human equivalent dose of 500 mg/d (24). The administration of RWPs started 2 d after C26 cells were injected to avoid any effect during the early phase of tumor cell implantation into the host tissue. Mice were checked every 2 d for the detection of palpable tumors, and thereafter the tumor volume (larger diameter × smaller diameter² × 0.4) was determined using a caliper every 2–3 d. Mice were sacrificed 27 d after injection of C26 cells.

The tumors were collected and then either dry frozen in liquid nitrogen or embedded in paraffin or Tissue-Tek OCT.

Microangiography of tumors

Tumor vessel density was evaluated by a high-definition micro-computed tomography system (25), 27 d after injection of C26 cells. Mice were anesthetized with pentobarbital (50 mg/kg i.p.), and, thereafter, the vascular system was flushed by infusion of 15 ml of heparinized saline solution into the left vertebral apex before being fixed with 4% paraformaldehyde. Then, the vascular system was filled with a radio-opaque silicon rubber (Microfil; Flow Tech Inc., Carver, MA, USA), which polymerized within 20 min. Tumors were excised and placed under an X-ray source. Vessel density of tumors was expressed as the number of pixels corresponding to the silicon rubber in each tumor.

Histology and immunohistochemistry

Capillary density was assessed using cryosections (10 µm thick) of tumors embedded in Tissue-Tek OCT, which were incubated with rat monoclonal anti-CD31 (1:50 dilution; BD Pharmingen, San Diego, CA, USA), a marker of endothelial cells. Paraffin-embedded tumor sections (5 µm thick) were first subjected to antigen retrieval and blocking of endogenous peroxidase activity (3% hydrogen peroxide in PBS). Then, sections were incubated with either rabbit polyclonal anti-VEGF (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-MMP-2 or MMP-9 (1:100 and 1:200 dilution, respectively; Chemicon-Millipore, Billerica, MA, USA), rabbit polyclonal anti-tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2 (1:50 and 1:25 dilution, respectively; Santa Cruz Biotechnology), rabbit polyclonal anti-cylooxygenase (COX)-2 (1:100 dilution; Cayman Chemical, Ann Arbor, MI, USA), rabbit monoclonal anti- active caspase-3 (1:100 dilution; Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-p16INK4A (1:50 dilution; Delat Biolabs, Gilroy, CA, USA), mouse monoclonal anti-p53 (1:50 dilution; Santa Cruz Biotechnology), rabbit monoclonal anti-p73 (1:100 dilution; Abcam, Cambridge, UK), mouse monoclonal anti-p21 (1:20 dilution; BD Pharmingen), rabbit polyclonal anti-Ki67 (1:300 dilution; Novocasta, Newcastle-on-Tyne, UK), mouse monoclonal anti-cyclin D1 (1:50 dilution; Santa Cruz Biotechnology), or mouse monoclonal anti-UHRF1 (1:75 dilution; Proteogenix, Oberhausbergen, France). Sections were then incubated with the appropriate biotinylated secondary antibody followed by conjugated horseradish peroxidase-streptavadin before the incubation with 3,3’-diaminobenzidine working solution at room temperature and counterstained with diluted Harris hematoxylin. Capillary density was quantified on three different sections for each tumor distant of 100 mm, and 30 fields for each section were analyzed. Capillary density is expressed as the percentage of brown pixels per field. For the quantification of VEGF, MMP-2, MMP-9, TIMP-1, and TIMP-2 immunostaining, the percentage of brown pixels was quantified on 4 different fields. For the quantification of COX-2, Ki67, cyclin D1, UHRF1, p16INK4A, p21, p53, and p73, and active caspase-3 immunostaining in tumors, the number of positive nuclei was counted as well as the total number of cells in 5 arbitrarily selected fields from each tumor at ×400. The index for each marker was determined as the number of positive nuclei × 100/total number of cells.

In situ zymography

Unfixed tumor cryosections (7 µm thick) were incubated for 6 h at 37°C with 0.1 mg/ml of Oregon green-conjugated
gelatin (Molecular Probes, Leiden, The Netherlands) in developing buffer (Invitrogen, Cergy Pontoise, France). The gelatinase activity, visualized by the appearance of green fluorescence, was observed with a microscope at ×200 and quantified by the average of fluorescence surface on 6 different fields.

In situ apoptosis detection by TUNEL staining

Paraffin-embedded tumor sections (5 μm thick) were analyzed by TUNEL staining using an in situ cell death detection kit (Fluorescein; Roche Diagnostics, Indianapolis, IN, USA). Apoptotic cells, indicated as green-positive cells, and the total number of cells, indicated by DAPI staining, were counted in 5 arbitrarily selected fields at ×400, and then the apoptotic index was calculated as the number of apoptotic cells × 100/total number of cells.

Azoxyemethane-induced colon cancer in rats

Six-week-old male Wistar rats (Charles River Laboratories) were randomly assigned into 2 groups (4 rats/group) receiving either vehicle (3% ethanol, pH = 3.3) or RWPs (100 mg/kg/d, human equivalent dose =1000 mg/d) in the drinking water until the end of the experiment. One week after starting with the RWP treatment, all rats received an intraperitoneal injection of azoxymethane (15 mg/kg), which was repeated 1 wk later. Eight weeks later, rats were sacrificed, and the colon was excised and carefully flushed with 0.9% NaCl at 4°C. The mucosa was pinned flat on a paraffin wax block in a Petri dish, mucosal side up, stained with 0.2% methylene blue for 5 min, and examined under an inverse magnifying glass. Aberrant crypts were enlarged and protruding when compared with surrounding normal crypts. The number of aberrant crypt foci (ACF) per colon (between the cecum and the rectum) and the number of aberrant crypts per focus were determined.

Statistical analysis

Results are shown as means ± se. Statistical comparisons between the control group and RWP-treated group were performed using an unpaired Student’s t test. Tumor incidence is defined as the percentage of tumors of ≤40 mm³. The incidence data were analyzed with the log-rank test. P < 0.05 was considered statistically significant.

RESULTS

RWP treatment reduces C26 tumor growth

Injection of C26 cells into the flank of mice was associated with the detection of palpable tumors after d 4, whereas they were only detectable after d 6 in the RWP group. Administration of RWPs in the drinking water starting d 2 after injection of C26 cells reduced tumor incidence from 85 to 42% at d 17 (Fig. 1A). Tumor incidence reached 100% in the control group at d 22 but only at d 27 in the RWP group, indicating that RWP treatment delayed the tumor incidence (Fig. 1A). Tumor volume increased steadily in both groups (Fig. 1B). However, this response was significantly reduced in the RWP group compared with that in the control group (Fig. 1B). At d 27, tumor volume was 970 ± 66 mm³ in the control group compared with 670 ± 49 mm³ in the RWP group, indicating that the RWP treatment reduced tumor volume by 31%. Monitoring of the body weight indicated that the RWP treatment did not affect the weight evolution over the 27-d investigation period (body weight was 19.0 ± 0.9 and 19.0 ± 0.7 g in the control group and RWP group, respectively; n=11).

RWP treatment inhibits tumor angiogenesis and the expression of major proangiogenic factors

Tumor growth is closely dependent on the formation of new blood vessels, which is under the control of major proangiogenic factors including VEGF, MMP-2, and MMP-9 (26). In addition, COX-2 overexpression has also been linked to tumor angiogenesis (27). Therefore, experiments were done to determine whether the RWP treatment retards tumor growth by preventing its vascularization. Tumor vascularization was assessed by microangiography, which allows evaluation of blood vessels with diameters ≥10 μm. As shown in Fig. 2A, the RWP treatment reduced by 40% the volume of blood vessels in tumors. In addition, immunohisto-

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Figure 1. RWP treatment reduces C26 tumor growth in mice. A) Kinetics of the incidence of palpable tumors (volume>40 mm³). B) Kinetics of the development of tumor volume. RWPs (100 mg/kg/d) were added to the drinking water 2 d after injection of C26 cells until d 27. Results are expressed as means ± se from 40 tumors in each group (20 mice/group). *P < 0.05.
chemical staining of CD31, an endothelial cell marker, in tumors was performed to evaluate all blood vessels including those with a diameter < 10 μm. As indicated in Fig. 2B, the RWP treatment reduced by 53% the density of microvessels in tumors. Next, the effect of the RWP treatment on the expression of several major proangiogenic factors in tumors was determined using an immunohistochemical approach. As expected, pronounced staining for VEGF, MMP-2, MMP-9, and COX-2 was observed in tumors from the control group, whereas only slight staining was observed in those from the RWP-treated group (Fig. 3A). Indeed, the RWP treatment reduced significantly specific staining for VEGF (−43%), MMP-2 (−27%), MMP-9 (−26%), and COX-2 (−55%). Moreover, the reduced expression of both gelatinases, MMP-2 and MMP-9, was associated with significantly decreased (−20%) gelatinolytic activity in tumors from RWP-treated mice compared with control mice as assessed by in situ zymography (Fig. 3B). MMP-2 and MMP-9 gelatinolytic activities are regulated extracellularly, mainly based on the balance between proenzyme activation and inhibition by their respective tissue inhibitor of MMPs, TIMP-2 and TIMP-1 (28). The expression level of both TIMP-1 and TIMP-2 was similar in tumors of control mice and RWP-treated mice (Supplemental Fig. 1).

RWP treatment inhibits tumor cell proliferation

The effect of the RWP treatment on tumor cell proliferation was assessed by analyzing the expression of Ki67, a marker of the G1, S, M, and G2 phases of the cell cycle (29), cyclin D1, which promotes proliferation and is mostly expressed during the G1-S phase transition (30), and UHRF1, a protein required for cell proliferation, particularly for the G1-S transition, in part, by repressing tumor suppressor gene expression (31). As expected, strong immunostaining for Ki67, cyclin D1, and UHRF1 was observed in tumors from the control group, whereas low-level staining was found in those from the RWP-treated group (Fig. 4A). Quantification of Ki67-, cyclin D1-, and UHRF1-positive cells indicated that the RWP treatment reduced the number of positive cells in tumors by 60, 68, and 51%, respectively (Fig. 4A). Moreover, the RWP treatment was associated with a 3.4-fold increased expression of p21, an inhibitor of cell cycle progression, in tumor cells (Fig. 5).

RWP treatment induces tumor cell apoptosis and the expression of tumor suppressor genes

The effect of RWPs on tumor cell apoptosis was assessed by TUNEL and immunohistochemical staining of the active form of caspase-3. A low number of TUNEL-positive cells and of active caspase-3-positive cells was observed in tumors from the control group (Fig. 4B). In contrast, staining for TUNEL and active caspase-3 markedly increased in the RWP-treated group (Fig. 4B). Indeed, the RWP treatment induced a 5.5- and 14.0-fold increase in the number of positive cells for TUNEL and active caspase-3, respectively (Fig. 4B).

Tumor suppressor genes such as p16INK4A, p53, and p73 have a major role in the control of the cell cycle (32–34). Therefore, the possibility that the RWP treatment up-regulates the expression of tumor suppressor
genes, leading subsequently to cell cycle arrest and induction of apoptosis, was examined. Low immunohistochemical staining of p16INK4A, p53, and p73 was observed in tumors from the control group, whereas pronounced staining was found in those from the RWP-treated group (Fig. 5). The RWP treatment induced a 6-, 27-, and 40-fold increase in the number of positive cells for p16INK4A, p53, and p73, respectively (Fig. 5).

RWP treatment reduces the number of lung metastases

Because the RWP treatment strongly reduced tumor angiogenesis, which plays a critical role in tumor metastasis, we determined whether the antiangiogenic effect is associated with reduced development of C26 metastases in lungs. The number of metastases in the control group was 30 ± 3 metastases/lung (n=6), whereas it was markedly reduced to 13 ± 2 metastases/lung (n=6) in the RWP-treated group.

RWP treatment reduces the number of ACF per colon in rats

Azoxymethane injection in rats was associated with the appearance of 219 ± 10 ACF/colon in the control group and 112 ± 20 ACF/colon in the RWP-treated group (Fig. 6A, B). Thus, intake of RWPs reduced the development of ACF in the colon by 49%. A more detailed analysis of the ACF indicated that the RWP treatment significantly reduced the number of ACF containing 2 aberrant crypts by 39% and those containing 3 aberrant crypts by 47%, whereas those containing...
either 1, 4, or more aberrant crypts were also reduced, but these effects did not reach statistical significance (Fig. 6C).

DISCUSSION

The present findings demonstrate that regular intake of RWPs in the drinking water markedly delayed the incidence and reduced the growth of C26 tumors in BALB/c mice. The antitumor effect of RWPs involves reduced tumor vascularization as a consequence of decreased expression of major proangiogenic factors including VEGF, MMP-2, MMP-9, and COX-2 and an effect on tumor cells causing inhibition of cell proliferation and induction of apoptosis subsequent to the expression of p21, an inhibitor of the cell cycle, and the expression of tumor suppressor genes, including p16INK4A, p53, and p73. Moreover, the RWP treatment strongly inhibited the development of premalignant lesions induced by azoxymethane in the rat colon. Taken together, the present findings highlight the potential of grape-derived polyphenols as chemopreventive agents against colorectal cancer.

The growth and progression of solid tumors is critically dependent on the formation of new blood vessels and on the autonomous and uncontrolled proliferation and reduced apoptosis of cancer cells. As a consequence, targeting those key events in tumorigenesis is an interesting strategy to effectively counteract the development of tumors. The present findings indicate that the anticancer activity of grape-derived polyphenols involves their dual ability to prevent angiogenesis and to inhibit cancer cell function. Indeed, the RWP treatment markedly reduced the vascular network in C26 tumors as assessed by microangiography and immunohistochemical staining of CD31. The possibility that the reduced vascular density is associated with decreased blood flow and, hence, a limited supply of oxygen and nutrients to cancer cells, remains to be determined. The development of new blood vessels is controlled predominantly by major proangiogenic factors including VEGF, which promotes endothelial cell migration and proliferation and the formation of new blood vessels, and the gelatinases MMP-2 and MMP-9, which degrade the extracellular matrix, thereby promoting endothelial cell migration (35, 36). Indeed, strategies aiming to decrease VEGF production or to inhibit VEGF receptor and MMP activity effectively reduced tumor vascularization and growth (37, 38). The present findings indicate that the RWP treatment markedly reduced the expression level of VEGF, MMP-2, and MMP-9 and the gelatinase activity in tumors. Although the mechanism underlying the inhibitory effect of RWPs on VEGF, MMP-2, and MMP-9 expression is unclear, previous studies have shown that RWPs inhibited growth factor-induced VEGF expression by preventing the activation of the p38 mitogen-activated protein kinase pathway (20). Furthermore, RWPs inhibited thrombin-induced MMP-2 expression and activity via direct inhibition of membrane type 1 MMP in vascular smooth muscle cells (19). In addition, the reduced tumor gelatinase activity in the RWP-treated group cannot be explained by changes in TIMP-1 and TIMP-2 expression. Besides VEGF and MMPs, COX-2 has also been suggested to contribute to tumor growth and angiogenesis.
Indeed, an increased expression level of COX-2 has been observed in several types of human tumors such as colorectal cancers (39). Moreover, alterations in COX-2 expression and the abundance of its enzymatic product, prostaglandin E2 (PGE2), have been involved in colorectal tumorigenesis by promoting tumor maintenance and progression, encouraging metastatic spread, and possibly also participating in tumor initiation (40). The COX-2/PGE2 pathway might promote tumor growth by stimulating its vascularization (27) possibly via increased expression of VEGF (41) and MMPs (42). COX-2 may also contribute to the development of tumors by converting procarcinogens into carcinogens (43) and by inhibiting apoptosis because exposure of a human colon cancer cell line to a selective COX-2 inhibitor increased apoptosis and, conversely, pre-exposure of these cells to PGE2 increased the level of the antiapoptotic protein Bcl-2 (44). Thus, the present observation that the RWP treatment markedly reduced COX-2 expression in C26 tumors may help to explain their anticancer activity. The inhibitory effect of RWPs on COX-2 expression is most likely due to their ability to prevent NF-κB activation (45, 46).

Figure 5. RWP treatment induces the expression of p21, an inhibitor of cell cycle progression and of p16INK4A, p53, and p73, tumor suppressor genes, in C26 tumors. Representative immunohistochemical staining for p16INK4A, p21, p53, and p73 in tumor sections from the control group and the RWP group; view ×400. Corresponding cumulative data are also shown. Results are expressed as means ± se from 8 mice in each group. *P < 0.05.

Besides affecting vascularization, the tumor growth inhibitory effect of the RWPs may also be due to their ability to inhibit proliferation of cancer cells and to promote apoptosis. Indeed, the RWP treatment caused a marked reduction of the cell proliferation marker Ki67 and of cyclin D1, a protein involved in the G1-S transition of the cell cycle. In addition, the present findings indicate that UHRF1, a protein required for cell proliferation, particularly for the G1-S transition, is markedly expressed in C26 tumors and that this effect is also reduced by the RWP treatment. Increased UHRF1 expression levels have also been found previously in several cancer cells (47). Because down-regulation of UHRF1 markedly reduced the expression of VEGF in Jurkat cancer cells (48), UHRF1 is likely to be an upstream target of RWPs to prevent VEGF expression and possibly also tumor vascularization. Taken together, the present findings suggest that RWPs prevent tumor growth by arresting tumor cells in the G0/G1 phase of the cell cycle. Indeed, treatment of Jurkat cells with RWPs caused their accumulation in the G0/G1 phase (49). The present findings further suggest that the inhibitory effect of RWPs on the cell cycle is most likely the consequence of the up-regulation of the cyclin-dependent kinase inhibitor p21, which negatively regulates the G1-S transition. Consistent with this concept, overexpression of p21 inhibited growth in the human colon cancer cell line HT-29 (50). Furthermore, the present findings indicate that the ability of RWPs to inhibit tumor cell proliferation and induce apoptosis may also be due to their ability...
to up-regulate the expression of several tumor suppressor genes including p16
\(^{\text{INK4A}}\), p53, and p73. Indeed, up-regulation of p16
\(^{\text{INK4A}}\) expression has been shown to induce G1 phase arrest and to promote apoptosis in pancreatic cancer cells (51). In addition, introduction of the wild-type p53 gene, using an adenoviral vector in pancreatic cancer cells with p53 mutations, induced apoptosis and inhibited cell growth (52). Moreover, overexpression of p73 in HeLa cells enhanced cisplatin-induced apoptosis (53). However, the mechanism underlying the stimulatory effect of RWPs on the expression of tumor suppressor genes is presently unclear. Recently, it has been shown that apple polyphenols restored expression of p16
\(^{\text{INK4A}}\) in colon cancer cell lines by reducing expression of DNA methyltransferase 1, which induces DNA methylation (54). The demethylating activity of polyphenols could contribute to explaining their ability to reactivate silenced tumor suppressor genes.

In addition, we have also assessed the effect of RWPs in an azoxymethane-induced colon cancer model of tumorigenesis. Azoxy methane-induced colon tumors share many histopathological characteristics with human colon tumors such as mutation on K-ras and β-catenin (55). ACF, preneoplastic lesions, are often used to determine the anticancer properties of chemopreventive agents. The present findings indicate that the RWP treatment very effectively reduced the number of ACF induced by azoxymethane. Previous studies have also shown that green tea, apple polyphenols, and resveratrol reduced the azoxymethane-induced formation of ACF (56–58). It will be of interest to further characterize the mechanisms underlying the inhibitory effect of RWPs on the formation of ACF and to determine whether polyphenols are able to prevent the progression of ACF into tumors by reducing the number and the size of tumors as well as their vascularization.

In summary, the present findings provide in vivo evidence for the antiangiogenic, the antiproliferative, and the proapoptotic effects of grape-derived polyphenols, which are associated with an effective inhibition of colon carcinoma tumor growth in mice. In addition, RWPs also effectively prevented preneoplastic lesions induced by a carcinogen at the colon mucosa in rats. Thus, grape-derived polyphenols display attractive anticancer properties by targeting several key processes for tumorigenesis, supporting their role as potential chemopreventive agents against cancer.

The authors thank Isabelle Stoll and Sara Zgheib for providing valuable technical help. This work was supported by Ligue Nationale Française contre le Cancer, Comité du Haut-Rhin de la Ligue contre le Cancer, Association pour la Recherche contre le Cancer, and VINIFLHOR (Ministry of Agriculture, France).

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Received for publication November 9, 2009. Accepted for publication April 15, 2010.