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SHORT COMMUNICATION

Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting *bax* and *p21*^{CIP} expression

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We investigated whether resveratrol (RV) affects azoxymethane (AOM)-induced colon carcinogenesis, by administering RV (200 μg/kg/day in drinking water) to male F344 rats for 100 days, beginning 10 days before carcinogen treatment (two weekly doses of 15 mg/kg AOM). Aberrant crypt foci (ACF) were isolated and proliferation, apoptosis and expression of the cell cycle genes *bax* and *p21* were determined. RV significantly reduced the number of ACF/colon [25.7 ± 3.6 (mean ± SEM) versus 39.4 ± 3.3 in controls; $P < 0.01$] and their multiplicity (2.7 ± 0.3 versus 4.9 ± 0.6 in controls; $P < 0.01$), and also abolished large ACF. In RV-treated rats, *bax* expression was enhanced in ACF but not in the surrounding mucosa. In both controls and RV-treated rats, proliferation was higher in ACF than in normal mucosa. *p21* was expressed in ACF of controls and of RV-treated rats and in normal mucosa of controls, but was lost in normal mucosa of RV-treated animals. In conclusion, the results suggest a protective role of RV in colon carcinogenesis with a mechanism involving changes in *bax* and *p21* expression.

Resveratrol (3,4',5-trihydroxystilbene; RV) is a natural phytoalexin found in grapes, red wines and other food products, the natural function of which appears to protect the plant from environmental stress and pathogenic attack (1). High concentrations of RV are present in some oriental herbal medicines used to treat fever, hyperlipidemia, inflammation and atherosclerosis (2). RV has been shown to be non-toxic by clinical test (3). Several epidemiological studies have indicated that, whereas an excess of alcohol results in tissue injury, moderate consumption of red wine may actually protect against coronary heart disease (4,5).

It has recently been reported that RV inhibits the development of preneoplastic lesions in mouse mammary organ culture and skin cancer promotion models in mice (6). RV has also been reported to induce apoptosis in human prostate cancer cell lines (7), to inhibit the growth of human breast cell lines (8) and to reduce ascites hepatoma growth in rats (9). Given these encouraging results, we aimed to investigate whether RV might also interfere with the development of colon cancer, one of the principal neoplastic diseases in Western countries (10,11). In the rat colon, we evaluated aberrant crypt foci (ACF), preneoplastic proliferative lesions that have been widely used as a surrogate end-point for carcinogenesis tests (12–15). We also studied the expression of two genes involved in the regulation of cell proliferation and apoptosis, *p21* and *bax,* as well as kinetic growth indices, in both ACF and the surrounding normal mucosa.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; RV, resveratrol.

Two-month-old male F344 rats (Charles River, Como, Italy), weighing ~150 g, were randomly allocated into two groups (10 rats/group). Ten days before carcinogenic treatment and until the end of the experiment, the control group received ethanol (0.3 ml/l in the drinking water); the study group received RV (Sigma, Milan, Italy) (200 μg/kg body wt/day) dissolved in ethanol to make it soluble and then diluted in the water (0.3:1000). Both groups were injected s.c. with two doses (1 week apart) of 15 mg/kg azoxymethane (AOM; Sigma); the rats were killed by carbon dioxide asphyxia 3 months after the first dose of AOM. Colon and rectum were processed for the determination of ACF (14,15), which were classified as small (one to four crypts/ACF), medium (five to seven crypts/ACF) or large (eight or more crypts/ACF) (14,15).

Growth parameters and gene expression were evaluated in histological sections of ACF and in the surrounding mucosa. Each section was observed under the microscope to identify ACF, which was recognized in the slide stained with H&E as having a large crypt diameter and often being irregularly shaped. For each rat, at least 10 fully longitudinal crypts/segment of the colorectum were scored to determine each of the following parameters: mitotic index, apoptotic index, BrdU-labeling index, *bax* and *p21* gene expressions.

Immunohistochemistry was performed with standard techniques using anti-BudR mouse monoclonal antibodies (Amersham, Milan, Italy) to determine proliferative activity with the labeling index; apoptosis was detected by the TUNEL method using the '*in situ* cell death detection, fluorescein' kit (Boehringer Mannheim, Germany). Labeling and mitotic and apoptotic indices (%) were calculated as the mean number of labeled, mitotic and apoptotic cells/number of cells in the crypt section×100, respectively.

Bax and *p21* expressions were measured using mouse monoclonal antibodies (Santa Cruz, CA) and calculated as the number of cells expressing the protein/number of cells in the crypt section×100. A sample was considered positive if showed >30% or >5% expression (for *bax* and *p21*, respectively). This background level of expression was subtracted from the values obtained for each sample.

No difference was observed between the two experimental groups in water or food intake or in body weight (data not

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Table I. Effect of RV on kinetic growth parameters

	Mitotic index		Labelling index		Apoptotic index	
	ACF	non-ACF	ACF	non-ACF	ACF	non-ACF
Control	1.78 ± 0.20[a]	0.63 ± 0.08[b]	21.33 ± 2.24[a]	8.90 ± 0.71[b]	1.14 ± 0.21[a]	0.35 ± 0.09[b]
RV	1.82 ± 0.19[a]	0.72 ± 0.08[b]	22.71 ± 1.97[a]	9.42 ± 0.97[b]	1.45 ± 0.16[a]	0.36 ± 0.04[b]

Values are means ± SEM ($n = 10$).
[a,b]Values not sharing the same superscript are significantly different ($P < 0.01$).

Table II. Effect of RV on the expression of bax and p21 protein levels

	bax				p21			
	ACF		non-ACF		ACF		non-ACF	
	n/tot	%[a]	n/tot	%[a]	n/tot	%[a]	n/tot	%[a]
Control	10/10	53 ± 1.3[b]	10/10	50 ± 2.2[b]	10/10	2.2 ± 0.1[b]	10/10	1.1 ± 0.2[c]
RV	10/10	57 ± 1.3[c]	5/10	45 ± 1.6[d]	10/10	1.5 ± 0.1[c]	0/10	0[d]

Data represent means ± SEM ($n = 10$); n/tot, number of positive samples/samples analyzed.
[a]Number of cells expressing the protein/number of cells in crypt section×100.
[b,c,d]Values not sharing the same letter are significantly different ($P < 0.05$ or less).

shown). In contrast, RV significantly reduced the number of ACF in the colorectal mucosa (25.7 ± 3.6 and 39.4 ± 3.3 in RV and control rats, respectively, $P < 0.01$). The inhibitory effect of RV was even more pronounced when crypt multiplicity was considered: the mean number of crypts/ACF was 2.7 ± 0.3 and 4.9 ± 0.6, in RV and control rats, respectively ($P < 0.01$). RV also reduced the number of small and medium ACF (small ACF were 10.11 ± 0.8 and 14.2 ± 0.8, and medium ACF were 11.5 ± 0.8 and 18.6 ± 0.9 in RV and control rats, respectively; $P < 0.01$). RV also halted the development of large ACF.

Mitotic and labeling indices were higher in the ACF than in the surrounding colorectal mucosa in all animals (Table I), no significant difference being found between RV-treated and control rats. Similarly, the apoptotic index in both RV-treated and control rats was significantly higher in ACF than in the surrounding mucosa (Table I). The cycling and apoptotic cells were mostly in the lower third of the crypt, in both RV and control groups (~75%).

In all the control rats, *bax* was expressed to a similar degree in both ACF and non-ACF colorectal mucosa (Table II). In contrast, in the RV-treated rats we found that only five of the 10 rats expressed *bax* in the surrounding mucosa, and to a significantly lower degree than controls. Moreover, in the ACF of the RV-treated rats, *bax* was expressed to a significantly higher degree than in the ACF of control rats.

In both ACF and non-ACF mucosa of the control rats, *p21* was expressed in all animals, the expression being significantly higher in ACF than in the surrounding mucosa. In contrast, in the RV-treated rats *p21* expression was virtually absent in the surrounding mucosa, while in the ACF *p21* was expressed, although to a lower degree than in the control rats.

The chief finding of this study is that RV inhibits the number of AOM-induced ACF, and their growth, assessed either as multiplicity or number of large ACF; this suggests that, in the rat colon, RV reduces both the initiation and the promotion phases of carcinogenesis. Our data on the chemopreventive activity of RV in colon carcinogenesis largely agree with the findings reported in the literature.

How might RV interfere with the development of ACF? RV has antioxidant activity (16) and prevents free radical production in HL-60 cells (17). Alternatively, the anti-initiating activity of RV might be related to its antimutagenic activity against dimethylbenz[*a*]anthracene (18). RV also induces phase II enzymes of the drug metabolizing system involved in carcinogen detoxification (19), is a potent antimitogen for hepatic stellate cells (20) and inhibits the lipopolysaccharide-induced production of NO and tumor necrosis factor by Kupffer cells. The anti-promotion function of RV may result from its ability to inhibit cyclooxygenase activity (6,21), since cyclooxygenase inhibitors reduce ACF and colon cancer in both animals and humans (22,23).

Any or all of these mechanisms may be responsible for the inhibitory effect of RV on the development of AOM-induced ACF. We found that RV caused an enhancement of *bax* expression in the ACF but not in the surrounding mucosa. It thus appears possible that the enhanced apoptosis in the ACF of the RV-treated rats contributes to the reduced growth of the foci. Accordingly, it has been suggested that several chemicals may protect against colon carcinogenesis by increasing apoptosis in tumour tissues/cells. (24). Our results are consistent with recent reports showing that RV induces apoptosis in HL-60 leukemia and T47D breast carcinoma cell lines, whereas it does not affect the survival of surrounding blood lymphocytes; this increases its chemotherapeutic potential (25). Moreover, Huang *et al.* (26) have reported that RV triggers apoptosis in mouse epidermal and lymphoblast cell lines by inducing p53 protein and activating p53-dependent transcription activity, as well as through the ceramide/SMase-independent pathway.

The effect of RV on proliferation is more difficult to explain. In agreement with other studies (27,28), we found that the proliferative activity assessed by mitotic and labeling indices, was higher in the ACF than in the surrounding mucosa in both

RV-treated and control rats, but there was no difference in these parameters between RV-treated and control rats. We also investigated whether RV affects expression of the *p21* gene, the prototype inhibitor of cyclin-dependent kinases, since loss of *p21* function has been implicated in colorectal carcinogenesis (29) and activity of this gene has been found to be modulated by drugs affecting the cell cycle progression (30). Surprisingly, we found that *p21* expression was entirely lost in the non-ACF mucosa of RV-treated animals, while it was present in the ACF of both control and RV-treated rats, as well as in the non-ACF mucosa of controls. Given the lower multiplicity of ACF in the RV-treated rats, these results suggest that loss of *p21* expression in the neighbourhood of these preneoplastic lesions might give them a growth disadvantage. In this respect, it is interesting to note that cells lacking *p21* have been reported to undergo apoptosis in response to DNA-damaging agents (31) and that, unlike *p21* proficient tumors, the irradiation of tumors lacking *p21* activity caused tumor shrinkage (32).

In vitro, RV has been reported to arrest HL-60 cells at the S/G_2 phase transition, resulting in increased numbers of cells in the G_1/S phase; this activity appears to be due to an increase in cyclins A and E and inactive cdc2, but no modulation of p21 expression was found (33). RV also induced a slight accumulation of AH-130 cells in the G_2/M cell cycle phase (9) and shifted the cell cycle distribution of U937 cells toward a dose-dependent arrest at the S phase checkpoint (L.Tessitore, unpublished data). The impairment of cell cycle progression observed with RV may, at least in *in vitro* models, be due to its ability to inhibit enzyme activities essential for DNA synthesis, such as DNA polymerase and ribonucleotide reductase (34,35).

Overall, the present study shows that prolonged daily administration of RV significantly decreases the number and crypt multiplicity of ACF in the colorectal mucosa, suggesting a protective role of RV in colon carcinogenesis. The results also showed that RV modulates *bax* and *p21* expressions differently in ACF and non-ACF perifocal mucosa. How the differential expression of these two genes is translated into a growth disadvantage for ACF in RV-treated animals is not fully understood at present. However, these results indicate that, in these *in vivo* models, it is important not to focus the attention only on control and treated animals, but also to study the differential kinetic growth response between the two cellular subpopulations: that of the emerging preneoplastic lesions and that of the non-preneoplastic tissue.

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