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QUERCETIN INHIBITS p21-RAS EXPRESSION IN HUMAN COLON CANCER CELL LINES AND IN PRIMARY COLORECTAL TUMORS

Franco O. RANELLETTI[1]*, Nicola MAGGIANO[2], Fabio G. SERRA[2], Riccardo RICCI[2], Luigi M. LAROCCA[2], Paola LANZA[2], Giovanni SCAMBIA[3], Andrea FATTOROSSI[3], Arnaldo CAPELLI[2] and Mauro PIANTELLI[4]

[1]*Institute of Histology, Università Cattolica del S. Cuore, Roma, Italy*
[2]*Institute of Pathology Università Cattolica del S. Cuore, Roma, Italy*
[3]*Institute of Gynecology, Università Cattolica del S. Cuore, Roma, Italy*
[4]*Department of Human Pathology, Università G. D'Annunzio, Chieti, Italy*

Immunocytochemical studies have revealed that 10 μM quercetin reduced the steady state levels of p21-ras proteins in both colon cancer cell lines and primary colorectal tumors. These findings were confirmed by Western blot and flow cytometric analysis showing that the inhibition of p21-ras expression by quercetin was time- and concentration-dependent. Twenty-four-hour treatment with 10 μM quercetin reduced p21-ras levels to about 50% of control values. Quercetin was similarly effective in inhibiting the expression of K-, H-, and N-ras proteins. Moreover, the effect of quercetin on *ras* oncogene expression was not dependent on the cell cycle position of colon cancer cells and appeared to be specific and not merely a consequence of overall inhibition of protein synthesis. Northern blot analysis revealed that quercetin produced in colon cancer cells an early (30 min) reduction of the steady state levels of K-, H-, and N-ras mRNAs. This reduction was also present after 6 hr of flavonoid treatment. These effects of quercetin suggest a possible chemopreventive role for this compound in colorectal carcinogenesis. *Int. J. Cancer* 85:438–445, 2000.
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Protooncogenes may be activated and lead to neoplastic transformation through various genetic mechanisms, including point mutations and amplification. Many different human tumors, such as pancreatic, ovarian, leukemias as well as colorectal carcinomas, contain *ras* genes showing point mutations at codons 12, 13, or 61 (Bos, 1989). Resulting mutated ras oncoproteins are preferentially in a GTP-bound state as they have a decreased GTPase activity and/or changed GTP/GDP binding capacity. As a consequence, the increases in active GTP-bound ras proteins produce strong downstream signals and alter normal cellular functions. In some tumors, overexpression of normal ras proteins may constitute an alternative mechanism for *ras* activation able to change from non-invasive to invasive the phenotype of cells within the neoplasia (Theodorescu *et al.*, 1990).

In human colorectal cancers, point mutation is considered the primary mechanism of *ras* activation. In large adenomas and carcinomas, *ras* gene mutations, mainly at codons 12 or 13 in K-*ras*, have been recognized in 40–50% of cases (Bos, 1989). Moreover, K-*ras* codon 12 mutation has been reported to determine the polypoid growth of colorectal cancer (Chiang *et al.*, 1998). N-*ras* mutations are also present but in a small percentage of colorectal tumors (Bos, 1989); although frequent quantitative and qualitative changes in N-ras protein have been described (Kim *et al.*, 1997).

The plant flavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone) inhibits cell growth of human colon carcinoma cell lines (Hosokawa *et al.*, 1990; Ranelletti *et al.*, 1992) and of primary colorectal tumors (Ranelletti *et al.*, 1992). Prompted by observations that, in other cell types, quercetin and related flavonoids are able to target the function of *ras* gene products presumed to be the primary determinants of the neoplastic properties of the cells (Kuo and Yang, 1995; Lü *et al.*, 1996; Avila *et al.*, 1996; Csokay *et al.*, 1997), we investigated whether quercetin can modulate *ras* oncogene expression in colon cancer cell lines and in primary colorectal tumors.

MATERIAL AND METHODS

Cell cultures

Colo-205, Colo-320HSR, Colo-201, LS-174 and WiDr human colon cancer cell lines, kindly provided by Dr. S.D. Showalter (NCI, Frederick, MD), were cultured as previously reported (Ranelletti *et al.*, 1992). Before each experiment cells were routinely analyzed for the presence of Mycoplasma (Mycoplasma detection kit; Boehringer, Mannheim, Germany).

Immunocytochemical analysis

WiDr cells were plated at $10^4/cm^2$; after 24 hr, the medium was replaced with fresh medium and 10 μM quercetin (3,3′,4′,5,7-pentahydroxy-flavone; Aldrich, Steinhein, Germany) or vehicle alone (0.5% ethanol) were added to the cultures for 48 hr. After the first 24 hr of treatment, the same quercetin and vehicle alone concentrations were added again to treated and control cultures, respectively. Cells were removed by tripsinization and fixed with 3% paraformaldehyde in PBS for 10 min and permeabilized by exposure to 0.3% Triton X-100 in PBS for 10 min. Following careful washes with PBS containing 1% (w/v) BSA (PBS-BSA), immunocytochemical staining for p21-ras was performed by incubating cells overnight at 4°C with 10 μg/ml of monoclonal antibody (Mab) anti pan-ras Ab1 (clone F132–62; Oncogene Science/Calbiochem, Cambridge, MA). Cells were washed in PBS and incubated with 1:100 goat anti-mouse gold conjugated (GAM-Auroprobe one; Amersham, Ailesbury, UK) for 4 hr at room temperature. After repeated washing in PBS, the cells were further fixed in 1% glutaraldehyde in PBS for 30 min, extensively washed in PBS and then dehydrated in a graded ethanol series and embedded in LR-White (London Resin Company Ltd, London, UK). Negative controls were performed omitting the primary antibody. Semi-thin sections were treated by silver enhancing kit (Amersham), and observed without counterstaining.

Immunohistochemical analysis on primary colonic tumors

Colorectal carcinomas were obtained from 2 male and 2 female patients who did not receive chemoterapy prior to resection. According to site, Dukes' classification and differentiation state, individual colorectal carcinomas were: case 1. sigmoid, B, well differentiated; case 2. sigmoid, D, poorly differentiated; case 3. cecum, C, moderately differentiated; case 4. rectum, A, well differentiated. Tumor fragments were cultured at 37°C for 24 hr in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD) containing 20 mM N-2-ethanesulfonic acid, 10% FCS, and penicil-

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*Correspondence to: Institute of Histology, Università Cattolica del S. Cuore, Largo F. Vito 1, I-00168 Rome, Italy. Fax: +39 06 3051157. E-mail: ibiap@rm.unicatt.it

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lin-streptomycin solution (100 IU/ml and 100 µg/ml, respectively), in the presence of 10 µM quercetin or vehicle alone. At the end of treatment, the tissue fragments were fixed in 4% neutral buffered paraformaldehyde for 2 hr and then embedded in paraffin. Sections of embedded tumors were stained for p21-ras using anti-pan-ras Ab1 mouse MAb (Oncogene Science) or anti-cytokeratin MAb (clone AE1/AE3; Dako, Glostrup, Sweden) at 1:200 dilution. Bound antibodies were visualized with the ABC immunoperoxidase system (Vector, Burlingame, CA). Negative controls were performed omitting the primary antibody.

Western blot analysis

Cells were plated at a density of 4×10^4 cells/ml; after 24 hr, cells were treated with vehicle alone or 10 µM quercetin for the indicated times. Lysates were prepared by homogenizing cell pellets in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Some of the tumor fragments utilized for the immunohistochemical analysis (see above) were homogenized in 2 volumes of disruption buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 0.5% sodium deoxycholate, 2 U/ml kallicrein inhibitor aprotinin) and left for 30 min on ice. The lysates were cleared by centrifugation, and the protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Munich, Germany), using bovine serum albumin (BSA) as standard. Equivalent amounts of proteins were separated by 12.5% SDS PAGE and electrophoretically transferred to a 0.45-µm nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) which was incubated overnight at 4°C with 5% milk powder in TBST buffer (20 mMTris, pH 8.0, 125 mM NaCl, 0.5% Tween 20) to block non-specific binding. The p21-ras was detected by incubating overnight, at 4°C, the membranes with anti-pan-ras Ab3 mouse MAb (clone: RAS-10, Oncogene Science) or anti-pan-ras Ab1 mouse MAb (Oncogene Science) in 2.5% non-fat dried milk solution in TBST buffer. Blots were extensively washed in TBST buffer. Indirect immunostaining was achieved using peroxidase ABC (Vector). Peroxidase was developed with the DAB substrate kit (Vector). The membranes were reprobed with anti-ß-tubulin MAb (clone 2.28.33; Sigma, Deisenhofen, Germany) or with an anti-cytokeratin MAb (clone AE1/AE3) to normalize for protein loading. AE1/AE3 showed a broad reactivity in the Western blot analysis of case 4 tumor specimen, revealing 2 intense bands and many other faint bands, as they are a mixture of 2 monoclonal antibodies recognizing most of the acidic (type I) and all known basic (type II) cytokeratins. To normalize for protein loading, we measured the most intense bands. For measurement of band intensity, digital images of immunoblots were analyzed by using the Scan analysis software system (Biosoft, Elsevier, Amsterdam, The Netherlands).

RNA hybridization analyses

Cells were plated at 4×10^4/ml; after 24 hr, cells were treated for 30 or 360 min with 10 µM quercetin or vehicle alone. At the end of treatment, cells were washed 3 times with cold PBS. RNA prepared from both vehicle and quercetin treated cells by Rna-fast (Molecular Systems, San Diego, CA), following manufacturer's instructions, was separated by electrophoresis in 1.5% agarose-6% formaldehyde gels. RNA blotting onto nylon membranes, positively charged (Boehringer Mannheim), hybridization with radiolabeled DNA probes, and autoradiography were performed by standard procedures (Maniatis *et al.*, 1982). ^{32}P-labeled H-, K- and N-ras cDNA probes (Oncogene Science), were prepared by using a random-primed DNA labeling kit (Boehringer Mannheim). To control for lane loading, membranes were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using ^{32}P-labeled 40-mer human GAPDH DNA probe (Oncogene Science). For measurement of band intensity, digital images of Northern blots were analyzed by using the Scan analysis software system (Biosoft; Elsevier).

Cytofluorimetric analysis

Cells exposed to both vehicle alone or quercetin, were fixed in 1% paraformaldehyde at room temperature for 5 min followed by exposure to cold (−20°C) absolute methanol for 10 min. After extensive washing in PBS, cells were resuspended in PBS-BSA to reduce non-specific binding of MAbs. Incubations with 10 µg/ml anti pan-ras Ab1 or anti Ha-ras OP23 or anti N-ras OP25 or anti Ki-ras OP24, MAbs (Oncogene Science) were carried out for 30 min at room temperature. Cells were then washed extensively in PBS-BSA and reacted with the $F(ab')_2$ fragment of a FITC-conjugated anti-mouse immunoglobulin goat antiserum (Coulter-Immunotech, Milan, Italy). Normal mouse immunoglobulin served

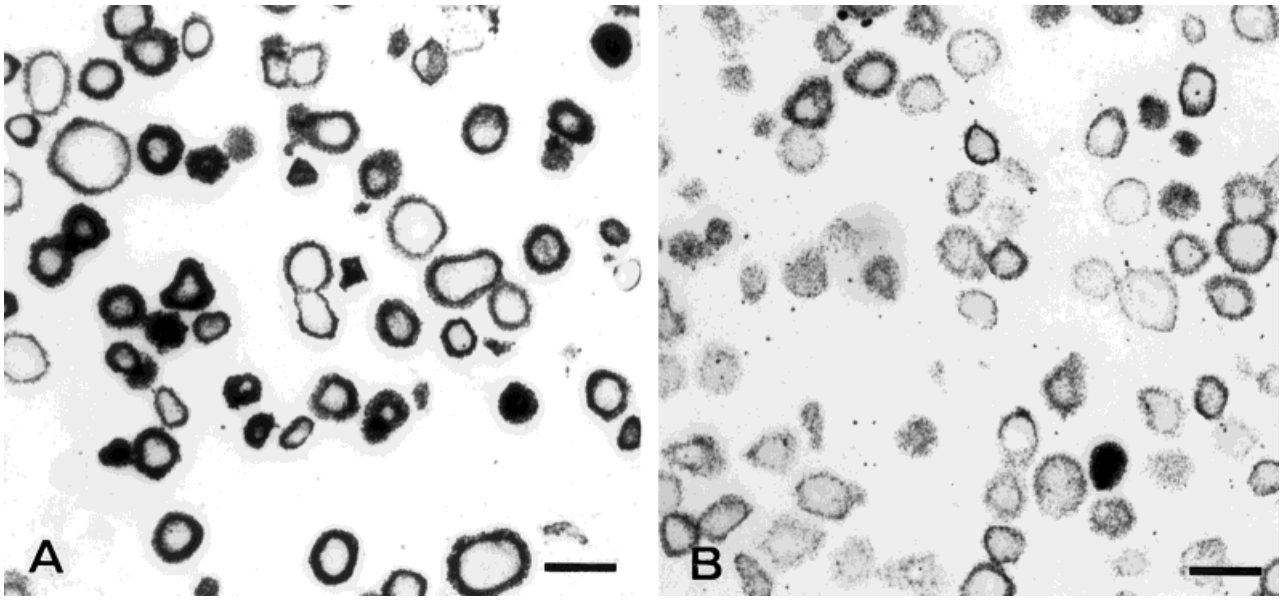

FIGURE 1 – WiDr cells treated for 48 hr with vehicle alone (*a*) or 10 µM quercetin (*b*). Localization of p21-ras proteins was done in prefixed and permeabilizated cells with anti-pan-ras Ab1 MAb (10 µg/ml) followed by goat-anti-mouse gold conjugated antibody (1:100). Semi-thin sections from LR-White embedded cells were treated by silver enhancement and observed without counterstaining. Scale bars = 13 µm.

to establish background staining. After washing, cells were resuspended in PBS-BSA and used for flow cytometric analysis. This was carried out using an EPICS XL flow cytometer (Coulter, Miami, FL). Forward and side scatter signals served to exclude unwanted events (*i.e.*, debris and cell clumps) from analysis. FITC-generated fluorescence signal was collected in log mode. Data are displayed as fluorescence histogram or as fluorescence intensity, *i.e.*, the mean channel of the fluorescence histogram, computed by the cytometer's software. At least 5,000 viable cells were analyzed for each experimental condition.

RESULTS

As shown in Figure 1*a*, the cytoplasm of WiDr colon cancer cells was stained by anti-pan-ras MAb and both the pattern and intensity of staining appeared heterogeneous. However, many cells were clearly outlined, consistent with a localization of p21-ras at the cell membrane. Treatment with 10 µM quercetin for 48 hr produced a clear reduction of cell immunostaining whose intensity varied among cells (Fig. 1*b*).

Time-dependent reduction of steady-state levels of p21-ras was analyzed by Western blot analysis. As shown in Figure 2, 10 µM quercetin lowered in a time-dependent fashion the amount of p21-ras in Colo-320HSR cells. The inhibitory effect of quercetin became evident after 10 hr of treatment reaching about 50% and 20% of control after 24 hr and 48 hr of treatment, respectively. Colon cancer cell lines analyzed by Western blot assay after 48 hr of treatment with 10 µM quercetin showed a clear reduction of p21-ras content, being the order of their relative sensitivity to quercetin: Colo-320HSR = Colo-205 > WiDr > LS-174 (Fig. 3).

When analyzed by flow-cytometry, the fluorescence intensity of cancer cells stained with anti-pan-ras MAb was strikingly higher than in samples stained with normal mouse immunoglobulin (Fig. 4). Furthermore, cells treated with 10 µM quercetin for 48 hr showed a significant reduction of the specific p21-ras immunofluorescent staining. The magnitude of this reduction varied among the cell line tested (Figs. 4, 5). In Colo-320HSR and Colo-205 cells treated for 48 hr with quercetin concentrations ranging between 10^{-9} M and 10^{-5} M, the specific p21-ras immunofluorescence was inhibited in a concentration-dependent manner, being the flavonoid concentration giving 50% reduction about 0.5 µM (Fig. 6). In order to ascertain whether the expression of all 3 p21-ras proteins is inhibited by quercetin, Colo-320HSR cells treated with 10 µM quercetin for 48 hr, were immunostained with specific anti K-, anti N-, and anti H-ras MAbs and analyzed by flow cytometry. As

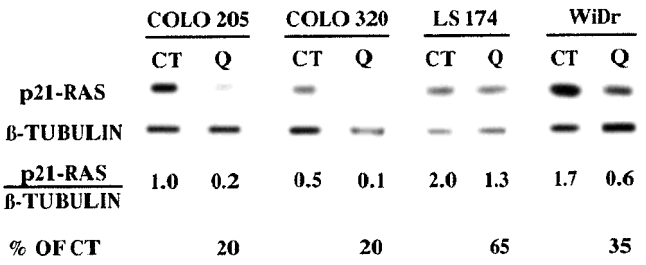

FIGURE 3 – Western blot analysis of lysates from colon cancer cell lines treated for 48 hr with vehicle alone (CT) or 10 µM quercetin (Q). The p21-ras band intensities from both CT and Q were normalized for protein loading by reprobing immunoblots with anti-ß-tubulin MAb. Shown is 1 out of 2 similar experiments.

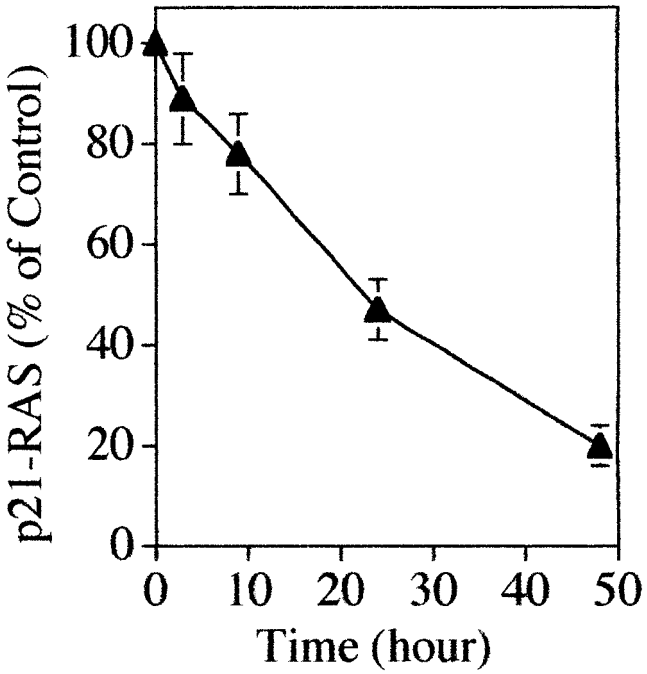

FIGURE 2 – Time-course of quercetin inhibitory effect on the levels of p21-ras proteins in Colo-320HSR cells. Cells treated with wehicle alone or 10 µM quercetin were harvested at the indicated times and processed by Western blot analysis as indicated in Material and Methods. The p21-ras band intensities from both control and treated samples were normalized for protein loading by reprobing immunoblots with anti-ß-tubulin MAb. Results are expressed as mean ± S.E. of triplicate determinations. Shown is 1 out of 3 similar experiments.

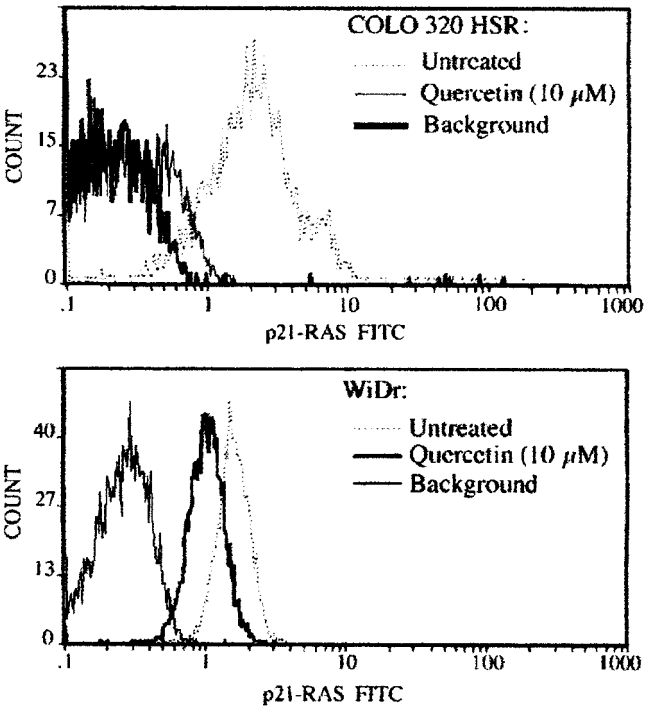

FIGURE 4 – Colo-320HSR and WiDr cells were cultured in the presence of vehicle alone (untreated) or 10 µM quercetin for 48 hr and then examined for their content of p21-ras. The fluorescence histogram of quercetin-treated cells is clearly shifted to the left as compared to untreated sample indicating a reduction of p21-ras expression. This was more evident with Colo-320HSR cells in which the fluorescence signal of quercetin-treated sample was almost superimposable to that of background. The unimodal shape of the fluorescence histograms indicates that p21-ras is homogeneously expressed in the whole cell line and homogeneously modulated by quercetin. Data refer to 1 experiment representative of 3 performed in duplicate.

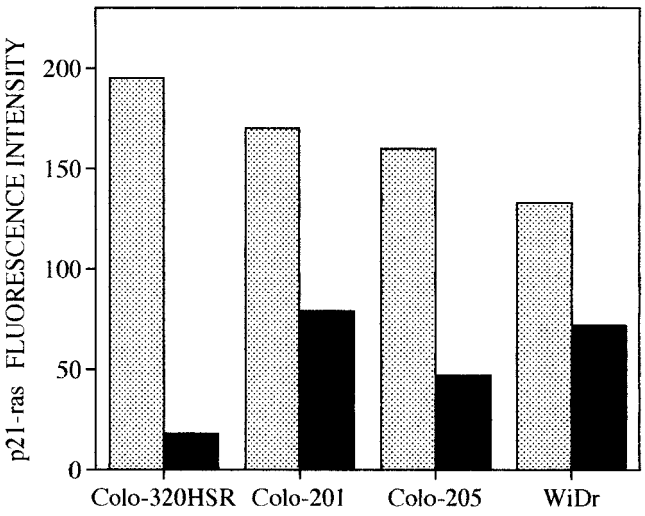

FIGURE 5 – Quercetin inhibition of p21-ras expression in different colon cancer cell lines. Cells were cultured for 48 hr in the presence of vehicle alone (dotted bars) or 10 μM quercetin (closed bars). Data are expressed as mean channel fluorescence subtracted background in untreated and treated samples and are representative of 1 out of 3 experiments performed in duplicate.

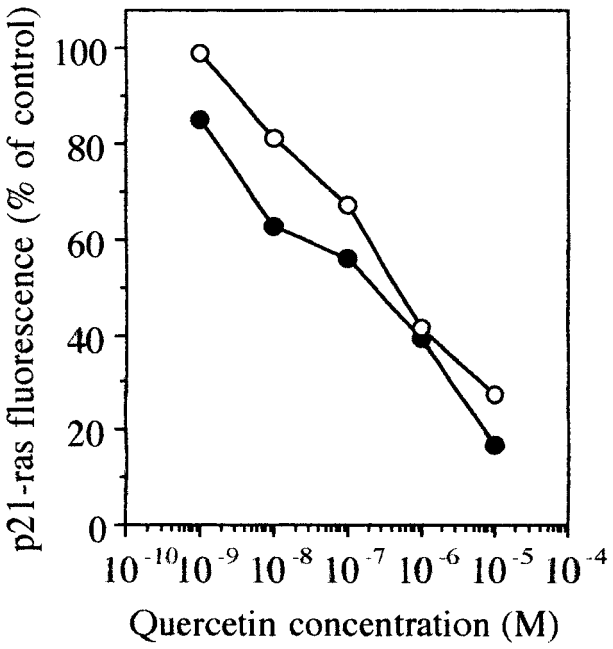

FIGURE 6 – Concentration dependence of quercetin inhibitory effect on p21-ras expression in Colo-320HSR (closed circle) and Colo-205 (open circle) colon cancer cell lines. Cells were cultured in the presence of vehicle alone (control) or various quercetin concentrations for 48 hr. Data are expressed as percentage of the value for control cells and are representative of 2 experiments performed in duplicate.

shown in Figure 7, quercetin inhibited to a similar extent the specific immunofluorescent staining of the 3 ras proteins.

Since quercetin inhibits the progression of colon cancer cells from G_1 to S phase of the cell cycle (Hosokawa *et al.*, 1990; Ranelletti *et al.*, 1992) and the intensity of p21-ras expression could be cell-cycle-dependent (Czerniak *et al.*, 1987), we studied by biparametric (DNA *vs.* p21-ras) flow cytometry the effects of 10 μM quercetin on both p21-ras content and distribution of WiDr

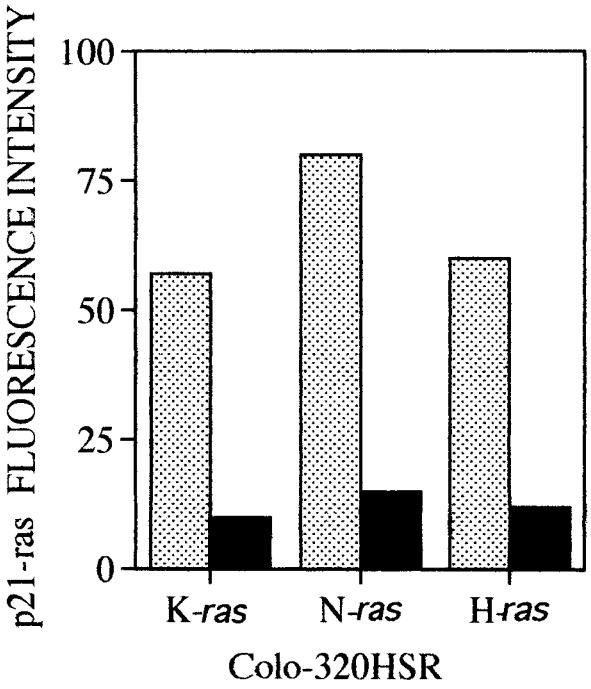

FIGURE 7 – Perturbation of K-, N- and H-ras protein expression induced in Colo-320HSR cells by quercetin. Cells were cultured for 48 hr in the presence of vehicle alone (dotted bars) or 10 μM quercetin (closed bars). Data are expressed as mean channel fluorescence subtracted background in untreated and treated samples and are representative of 1 out of 3 experiments performed in duplicate. All p21-ras species are negatively modulated by quercetin in a similar manner.

cells in the diverse phases of the cell cycle. The results obtained from the analysis of scattergrams (not shown) revealed that p21-ras levels of untreated WiDr cells increased during G_1 phase and reached a maximum during G_2-M phase. Compared with control cells, quercetin-treated cells contained lower amounts of p21-ras regardless of the cell-cycle position. Quercetin increased the percentage of cells in the non-proliferative compartments (G_0-G_1) of the cell cycle (Table I).

Northern blot analysis of K-ras mRNA in Colo-320HSR, Colo-205 and WiDr cells treated with 10 μM quercetin is shown in Figure 8. Quantitative analysis of the blot revealed that quercetin produced an early (30 min) reduction of the steady state levels of K-ras mRNA in all cancer cell lines tested and that the inhibitory effect was also present after 360 min of treatment (Table II). Furthermore, the steady state levels of H- and N-ras mRNAs were similarly inhibited by quercetin (Table II).

In order to verify the effectiveness of quercetin in inhibiting *in vitro* p21-ras expression in human primary colorectal tumors, we analyzed by immunohistochemistry 4 primary tumors cultured for 24 hr with vehicle alone or 10 μM quercetin. The findings relative to one of these tumors (case 4) are shown in Figure 9. In untreated specimens, cancer cells were stained by anti-pan-ras MAb and the pattern of staining was relatively diffuse. However, intense staining was particularly evident at the apical border of cells lining the lumen of the glandular-like structures (Fig. 9*a*). Tumor specimens cultured in the presence of quercetin showed a reduction in the staining intensity of cancer cells (Fig. 9*b*). To control the specificity for p21-ras of the flavonoid inhibitory action, we assayed, in a parallel experiment, the effect of quercetin on the expression of cytokeratins on the mucosa neighbouring the tumor tissue. As compared to vehicle-treated sample (Fig. 9*c*), quercetin-treated specimen (Fig. 9*d*) did not show any appreciable reduction in the intensity of staining for cytokeratins. The immunohistochemical

TABLE I – EFFECT OF 10 μM QUERCETIN ON CELL-CYCLE DISTRIBUTION OF WiDr CELLS AND ON p21-ras CONTENT IN THE DIVERSE PHASES OF THE CELL CYCLE AFTER 48 HR TREATMENT

Phase	Untreated		Quercetin-treated	
G_0/G_1	45%[1]	14[2]	60%	9
S	53%	17	39%	12
G_2/M	2%	20	1%	13

[1]Percent cell in cycle phase.–[2]p21-ras expressed as mean channel of the fluorescence histogram.

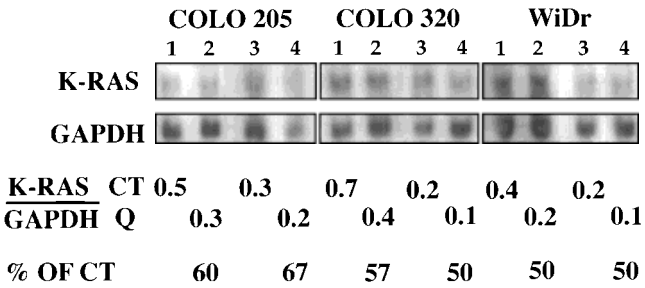

FIGURE 8 – Quercetin (10 μM) inhibition of K-ras mRNA levels in Colo-205, Colo-320HSR and WiDr cells. Northern blot analysis of total RNA (20 μg per lane) from cells treated for 30 min (lanes 1 and 2) or 360 min (lanes 3 and 4) with vehicle alone (CT: lanes 1 and 3) or 10 μM quercetin (Q: lanes 2 and 4). RNA was hybridized sequentially with ^{32}P-labeled K-ras c-DNA and 40 mer human GAPDH probes. K-ras band intensities of both CT and Q were normalized by dividing by signal intensities of the respective GAPDH bands. Results are expressed as percent of Q *vs.* CT values. Shown is 1 out of 2 experiments (see Table II).

analysis of the other 3 tumors gave similar results and the inhibitory effect of quercetin did not appear to depend on the differentiative state of the tumor (data not shown).

To further confirm the inhibitory effect of quercetin on p21-ras expression in primary colorectal tumors, both untreated and quercetin-treated fragments of one of the biopsy specimens utilized for immunohistochemical experiments (case 4) were assayed by Western blot analysis. In untreated sample, as assessed by m.w. markers runned in the same gel, the anti pan ras MAb (Ab1) revealed a strong band of 21 kDa and a very weak one of about 23 kDa. In quercetin-treated fragments there was a clear reduction of the intensity of the 21 kDa band to about 50% of the control value (Fig. 10). The cytokeratin band intensities confirmed that an equal amount of protein was loaded on each lane.

DISCUSSION

We show here, using immunocytochemistry, flow cytometry, Western and Northern blot analyses, that quercetin inhibits the expression of p21-ras in human colon cancer cells.

Immunocytochemical studies revealed that quercetin reduced the steady state levels of p21-ras proteins in both cancer cell lines and primary colon cancer cells. These findings were confirmed by Western blot and flow cytometric analyses showing that the inhibition of p21-ras expression by quercetin was time- and concentration-dependent. Moreover, quercetin appeared similarly effective in inhibiting the expression of K-, H- and N-ras proteins. Consistently with the reported half-life for p21-ras proteins (Ulsh and Shih, 1984), time-course experiments revealed that 24 hr treatment with 10 μM quercetin reduced the steady state level of p21-ras to about 50% of control. The effect of quercetin on *ras* oncogene expression was not dependent on the cell-cycle position of colon cancer cells. This finding is very similar to that observed by flow cytometry in an MTV-EJ*ras* cell line treated with coumarin (Kahn *et al.*, 1994).

The inhibitory action of quercetin was specific and not merely a consequence of an overall inhibition of protein synthesis since the difference in p21-ras band intensities appeared still more evident when immunoblot data from untreated and quercetin-treated samples were normalized for loading on the basis of ß-tubulin or cytokeratin concentrations. Furthermore, as revealed by immunocytochemistry, while quercetin reduced p21-ras immunostaining, it did not inhibit cytokeratin expression in colon epithelial cells.

It has been observed that flavonoid-structured molecules reverted the transformed phenotype and inhibited the oncogene *ras*-driven cell growth in NIH 3T3 cells transformed by transfection with v-H-*ras* (Kuo and Yang, 1995) and in NIH 3T3 cells transformed by transfection with activated human H-, K- or N-*ras* oncogenes (Avila *et al.*, 1996), respectively. However, in both cases, the flavonoid effects were not accompanied by changes in the level of p21-ras proteins. The present data show that quercetin treatment greatly reduced the amount of p21-ras proteins both in colon cancer cell lines and in primary colorectal tumors. Furthermore, in LS-174 and WiDr colon cancer cells, the quercetin efficacy to reduce p21-ras expression was positively correlated with its growth-inhibitory potential (Ranelletti *et al.*, 1992, and this work). The reasons for the difference in the quercetin effect on p21-ras expression between *ras*-transfected NIH 3T3 cells and human colon cancer cells remain unclear. However, in oncogenic *ras*-driven cell lines, it has been observed that the flavonoid-structured 7-hydroxy-coumarin decreased the amount of p21-ras in rat mammary RBA cells but not in T-24 human bladder carcinoma cells (Lü *et al.*, 1996). This finding raises the possibility that in different cell types, p21-ras expression can be regulated at multiple sites by mechanisms which are differently sensitive to flavonoids.

Quercetin produced in colon cancer cells an early (30 min) reduction of the steady state levels of K-, H- and N-ras mRNAs; this inhibitory effect was also present after 6 hr of flavonoid treatment. Csokay *et al.* (1997) have reported that quercetin produced an early (1 hr) downregulation of c-myc and K-ras mRNAs in K562 cells. However, our results indicate that colon cancer cells are more sensitive than K562 cells to the inhibitory effect of quercetin on K-ras mRNA levels. This finding is consistent with the greatest growth inhibitory effect of quercetin on colon cancer cells (Ranelletti *et al.*, 1992) as compared with that in K562 cells (Csokay *et al.*, 1997).

Inhibition of *ras* expression by quercetin could result from a lowered rate of oncogene transcription, rapid degradation of the transcripts, or both transcriptional and post-transcriptional processes. Actually, RNA hybridization analyses, revealing that quercetin significantly reduced the steady state levels of p21-ras mRNA, suggest a pre-translational control, although quercetin alterations of p21-ras protein stability leading to enhanced degradation could not be ruled out. It has been observed that quercetin suppresses the transcriptional activation of the MDR1 gene caused by arsenite in the human hepatocarcinoma cell line HepG2 (Kioka *et al.*, 1992) and that transcriptional activation of heat shock protein genes is suppressed by quercetin through inhibition of heat shock factor activation (Lee *et al.*, 1994). The flavonoid-structured 7-hydroxycoumarin modulated *ras* oncogene expression by interfering with the ras mRNA-splicing process (Zänker, 1993). On the other hand, both TGF-β induction (Scambia *et al.*, 1994) and p53 inhibition (Avila *et al.*, 1994) by quercetin do not affect steady state levels of mRNAs. At present, our data do not allow discriminating between a transcriptional and a post-transcriptional control of p21-ras mRNA levels by quercetin. Quercetin can reduce the cell antioxidant potential in Caco-2 human intestinal cells by inhibiting the expression of metallothionein (Kuo *et al.*, 1998). Relative to the mechanism of action of quercetin on p21-ras expression, this finding may be very interesting, since Miller *et al.* (1993)

TABLE II – NORTHERN BLOT ANALYSIS OF K-, H- AND N-ras mRNAs OF COLON CANCER CELLS TREATED WITH 10 μM QUERCETIN

	Experiment	K-ras		H-ras		N-ras	
		30[1]	360	30	360	30	360
Colo-320HSR	1	57%[2]	50%	37%	60%	70%	50%
	2	50%	65%	45%	54%	63%	45%
WiDr	1	50%	50%	47%	67%	N.D.[3]	
	2	63%	52%	55%	58%	N.D.	
Colo-205	1	60%	67%	N.D.		N.D.	
	2	55%	71%	N.D.		N.D.	

[1]Minutes of treatment.–[2]Results are expressed as percent of control band intensity (cells treated with vehicle alone). The signal intensity of band in each lane was normalized for loading by dividing by signal intensity of the respective glyceraldehyde-3-phosphate dehydrogenase band.–[3]Not done.

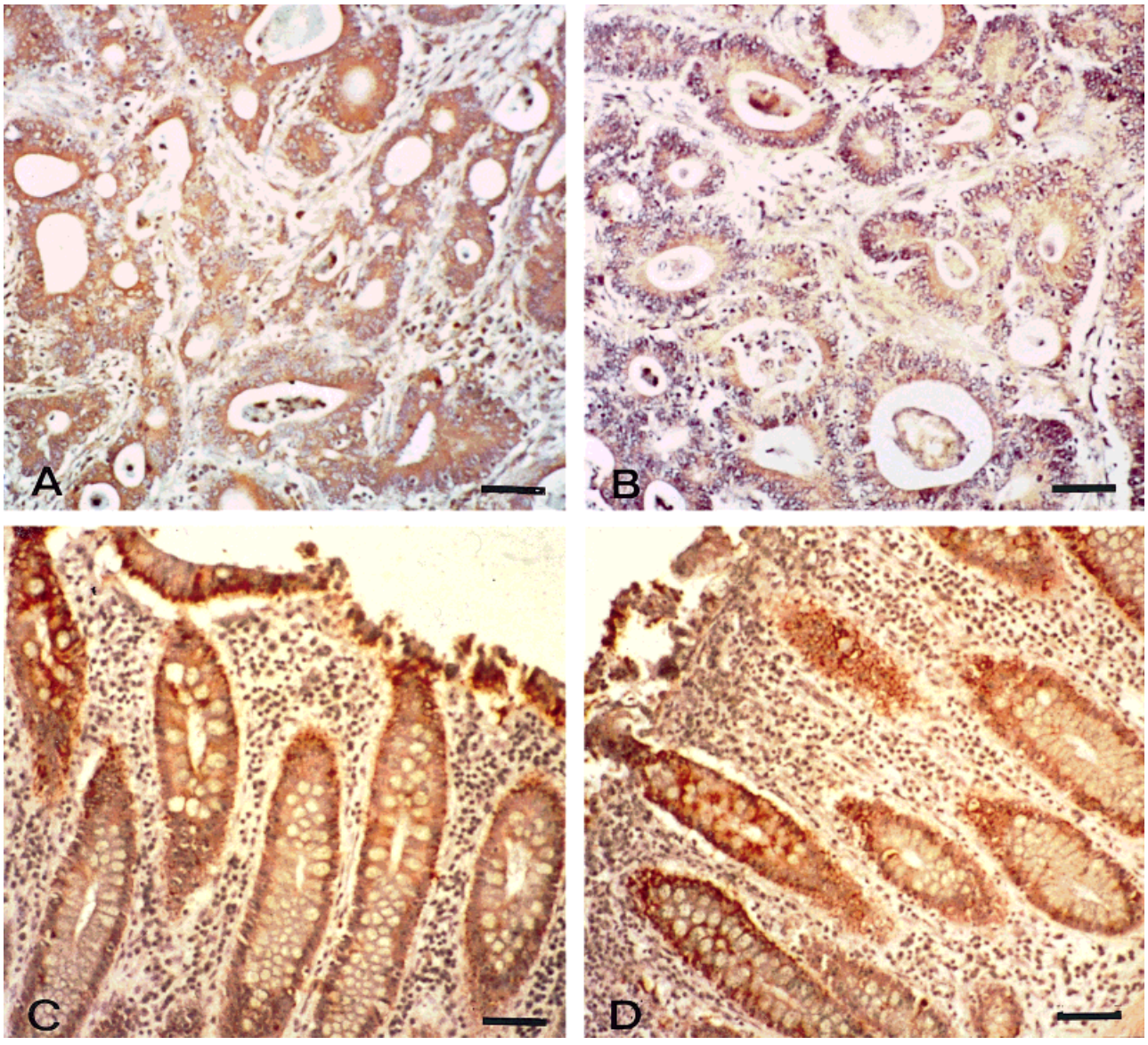

FIGURE 9 – Localization of p21-ras in a human rectal adenocarcinoma (*a* and *b*) and of cytokeratins in the mucosa neighbouring the tumor tissue (*c* and *d*) (case 4, Material and Methods). Immediately after surgical resection, samples from both tissues were incubated with vehicle alone (*a* and *c*) or 10 μM quercetin (*b* and *d*) for 24 hr. Immunoperoxidase staining was carried out with anti-pan-ras MAb (*a* and *b*) or with anti-cytokeratin MAb (*c* and *d*) on paraffin sections as outlined in Material and Methods. Hematoxylin counterstaining. Scale bars *a* and *b* = 80 μm; *c* and *d* = 40 μm.

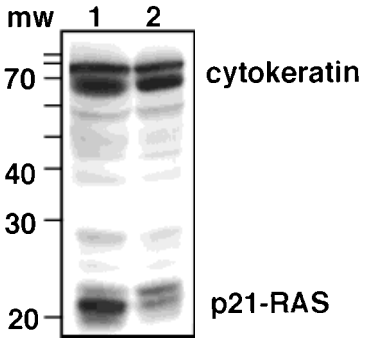

FIGURE 10 – Western blot analysis of lysates from some of the tumor fragments utilized for immunohistochemical experiment shown in Figure 9. Tumor specimens were cultured for 24 hr in the presence of vehicle alone (lane 1) or 10 μM quercetin (lane 2). To detect p21-ras and cytokeratin expression, anti-pan-ras Ab1 MAb and AE1/AE3 anti-cytokeratins MAb were utilized, respectively. Quercetin downregulates the expression of p21-*ras*. The cytokeratin signal intensities confirmed an equal amount of protein was loaded on each lane.

demonstrated that downregulation of *ras* expression can be achieved through modulation of the cellular reductive environment.

Many different mechanisms for the protective effect of flavonoids as anticarcinogens have been suggested. Quercetin and certain related flavonoids are inhibitors of carcinogenesis, as showed in azoxymethane-induced colonic neoplasia in mice and rats (Deschner *et al.*, 1993; Matsukawa *et al.*, 1997). The expression of biochemically active p21-ras protein significantly increases during the development of azoxymethane-induced colon carcinogenesis and chemopreventive agents reduce both colon tumor formation and the expression of biochemically active p21-ras proteins (Singh *et al.*, 1993). Taken together, these observations suggest that the inhibitory effect of quercetin on p21-ras expression can be used as a reliable intermediate biomarker of the efficacy of the chemopreventive action of this flavonoid on azoxymethane-induced colon carcinogenesis.

It is widely accepted that human colorectal carcinogenesis involves a pathway, the adenoma-carcinoma sequence, which proceeds through a series of genetic alterations, including the activation of *ras*. The role of *ras* gene mutations in the conversion from adenoma to carcinoma in the colorectum remains unclear. However, *ras* gene mutations may play a critical role in colorectal tumorigenesis by itself (Shaw *et al.*, 1991) and/or by cooperating with *p53* gene alterations (Shaw *et al.*, 1991; Bell *et al.*, 1993). Moreover, dominantly acting *ras* oncogenes may contribute to the growth of colorectal tumors also indirectly by facilitating tumor angiogenesis through an upregulation of the vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) expression (Rak *et al.*, 1995). Since our results have shown that quercetin downregulates the expression of p21-ras in colon cancer cells, it is possible to hypothesize that this flavonoid can act as a chemopreventive agent in colorectal carcinogenesis. Moreover, the observation that in MDA-MB468 cell line quercetin inhibits the expression of mutant p53 (Avila *et al.*, 1994) may be even more interesting and could further support the possibility that this flavonoid may block important points in the process of tumor promotion.

Finally, we speculate that the putative regulatory roles of quercetin might partially explain the correlation between high dietary intake of flavonoids in humans and a lower incidence of cancer of the stomach and colon in these populations (Haenszel *et al.*, 1980).

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