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Oncogenic Ras, but not ^{V600E}B-RAF, protects from cholesterol depletion-induced apoptosis through the PI3K/AKT pathway in colorectal cancer cells

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Cholesterol is necessary for proliferation and survival of transformed cells. Here we analyse the effect of cholesterol depletion on apoptosis and the mechanisms underlying this effect in colorectal cancer cells carrying oncogenic Ras or ^{V600E}B-RAF mutations. We show that chronic cholesterol depletion achieved with lipoprotein-deficient serum (LPDS) and 25-hydroxycholesterol (25-HC) treatment results in a significant increase in apoptosis in HT-29 and Colo-205 cells containing the ^{V600E}B-RAF mutation, but not in HCT-116 and LoVo cells harbouring the ^{G13D}Ras mutation, or BE cells, which possess two mutations, ^{G13D}Ras and ^{G463V}B-RAF. We also demonstrate that oncogenic Ras protects from apoptosis induced by cholesterol depletion through constitutive activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway. The specific activation of the PI3K/AKT pathway by overexpression of the ^{V12}RasC40 mutant or a constitutively active AKT decreases the LPDS plus 25-HC-induced apoptosis in HT-29 cells, whereas PI3K inhibition or abrogation of AKT expression renders HCT-116 sensitive to cholesterol depletion-induced apoptosis. Moreover, our data show that LPDS plus 25-HC increases the activity of c-Jun N-terminal kinase proteins only in HT-29 cells and that the inhibition of this kinase blocks the apoptosis induced by LPDS plus 25-HC. Finally, we demonstrate that AKT hyperactivation by oncogenic Ras protects from apoptosis, preventing the activation of c-Jun N-terminal kinase by cholesterol depletion. Thus, our data demonstrate that low levels of cholesterol induce apoptosis in colorectal cancer cells without oncogenic Ras mutations. These results reveal a novel molecular characteristic of colon tumours containing Ras or B-RAF mutations and should help in defining new targets for cancer therapy.

Introduction

Cholesterol is involved in a broad spectrum of cellular functions and an adequate cholesterol supply is needed from endogenous synthesis or exogenous sources to support cell proliferation and survival.

There is increasing evidence that cholesterol content may be a critical determinant of tumour progression. We know that its homeostasis is abnormal in malignant cells. Thus, both their content and biosynthesis are increased in different proliferating tumoural cells compared with normal cells, including colorectal cancer cells (1,2). Colon cancer cells show high HMG-CoA reductase levels and the activity and expression of this enzyme is increased several fold in colonic tumours compared with normal tissues (3,4). Moreover, it has also been shown that inhibition of HMG-CoA reductase by statins suppresses DNA synthesis in colorectal cancer cell lines and other transformed cells and reduces the growth of various types of tumour (5–7). Consistent with this possibility, some studies have linked long-term cholesterol-lowering therapy to lower cancer risk. Moreover, some clinical studies showed that the use of statins reduces the risk of breast and colorectal cancer (5,7,8), although some other studies of patients receiving statins and two recent meta-analyses (9,10) found no such evidence.

The relationship between lowering cholesterol levels and tumoural cell growth alterations are mainly due to induction of apoptosis. Thus, statin administration has been shown to inhibit cell proliferation, to increase apoptosis and sensitize cancer cells to radiochemotherapy (11,12). However, the effects of inhibition of cholesterol biosynthesis by statins are dependent on the model used. Thus, acute lymphoid leukaemias seem to be resistant to statins (12), and some preclinical data in rodents indicated that statins might increase the incidence of different tumours (11). Moreover, inhibition of cholesterol production, but not of non-sterol isoprenoid products, was also reported to induce cell death (13), and cholesterol enrichment of the neuronal plasma membrane protects cortical neurons from apoptosis (14). In this sense, acute methyl-β-cyclodextrin (MβCD)-induced cholesterol depletion from plasma membrane induces apoptosis in many cell lines (15,16). Furthermore, it has also been reported that many cholesterol effects, including apoptosis, are due to oxygenated derivatives of cholesterol (17,18). These effects are consistent with previous results from our laboratory showing that cholesterol depletion, but not mevalonate-generated non-sterol products, induces apoptosis in NIH3T3 cells (19).

Different pathways have been shown to either mediate or protect cells from apoptosis. Thus, the members of the mitogen-activated protein kinase (MAPK) family c-Jun N-terminal kinase (JNK) and p38 MAPK are important mediators of apoptotic signalling (20). JNK activation is required for induction of apoptosis by a number of different stress stimuli and chemotherapeutic agents (21,22). On the other hand, the serine/threonine kinase AKT, the downstream effector of the phosphatidylinositol-3 kinase (PI3K), has been found to be involved in promoting cell survival in the presence of different apoptotic stimuli. Thus, several studies have suggested that increased constitutive phosphorylation of AKT is associated with decreased apoptosis, whereas its inhibition increased it (23).

Activating mutations in the K-Ras gene are detected in about 30–40% of colorectal tumours (24), whereas activating mutations in B-RAF were recently described as an alternative oncogenic event in about 5–20% colorectal tumours without K-Ras mutations (25). Both promote cell proliferation by stimulating the RAF/MEK/ERK kinase cascade. Ras-induced transformation, however, is dependent on its capacity to activate further downstream signalling pathways, such as PI3K/AKT, that protect transformed cells from apoptosis (26) and B-RAF has also been shown to protect apoptosis through an ERK-dependent mechanism (27,28).

In this study, we analyse the effects of cholesterol starvation on apoptosis of colorectal cancer cells harbouring oncogenic Ras and/or B-RAF mutations and the mechanisms underlying this effect. We demonstrate that chronic cholesterol depletion triggers apoptosis through activation of the JNK pathway in cells without Ras mutants, whereas oncogenic B-RAF does not protects from this effect. We also show that oncogenic Ras suppresses JNK activation induced by low cholesterol levels through the constitutive activation of the PI3K/AKT pathway. Thus, our results show that depending on the genetic background, changes in cholesterol levels induce an apoptotic response and highlight the potential of cholesterol pathway inhibition as a possible tool in colorectal cancer therapy.

Abbreviations: FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; 25-HC, 25-hydroxycholesterol; JNK, c-Jun N-terminal kinase; LPDS, lipoprotein-deficient serum; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; PARP, Poly (ADP-ribose) polymerase; PI3K, phosphatidylinositol-3 kinase.

Materials and methods

Materials and expression vectors

Antibodies included anti-dual-phosphorylated JNK (Thr 183/Tyr 185; Promega, Promega Biotech Ibérica, Madrid, Spain); anti-phospho-AKT (Ser473),

anti-phospho-p38 MAPK (Thr 180/Tyr 182), anti-AKT1 and anti-total AKT were purchased from Cell Signalling Technology (Izasa S.A., Barcelona, Spain); anti-JNK1 and anti-B-RAF (Santa Cruz Biotechnology, Quimigen S.L., Madrid, Spain), anti-Poly (ADP-ribose) polymerase (PARP) and anti-C-RAF (BD Biosciences, San Agustín de Guadalix, Madrid, Spain), anti-tubulin (Sigma, Sigma-Aldrich Ibérica S.A., Tres Cantos, Madrid, Spain), anti-HA tag 12CA5 (Abcam, Cambridge, UK) and peroxidase-conjugated secondary antibodies (Dako, Dako diagnostic S.A., San Just Desvern, Barcelona, Spain). The p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the PI3K inhibitors LY294002 and wortmannin were from Calbiochem (Merk Chemicals España, Mollet del Valles, Barcelona, Spain). Cholesterol and 25-hydroxycholesterol (25-HC) were from Sigma. [γ-^{32}P]ATP was from Amersham Biosciences (GE healthcare, Cerdanyola, Barcelona, Spain). The *myc*-epitope-tagged ^{V12G}Ras expression vector (pEFm/V12Ras) and the 9E10 antibody have been described previously (29). The point mutations ^{V12}Ras T35S (^{V12}RasS35), ^{V12}Ras E37G (^{V12}RasG37) and ^{V12}Ras Y40C (^{V12}RasC40) were introduced into this vector using polymerase chain reaction with sequence verification by automated dideoxy sequencing procedures. The expression construct containing the constitutively active pcDNA3-HA-AKT1 (AKTca, with Thr-to-Asp and Ser-to-Asp mutations at codons 308 and 473) and the expression vector pEFm^{N28S}RalB (^{N28}Ral) were kindly provided by Dr R. Marais.

Isolation of lipoprotein-deficient serum

Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum (FBS) by ultracentrifugal flotation in potassium bromide at 1.210 g/ml density (19). After extensive dialysis against 50 mM Tris–HCl, 150 mM NaCl, and 0.01% ethylenediaminetetraacetic acid, pH 7.4, LPDS was sterilized by filtration (Millex-GV 0.22 mm; Millipore, Millipore Ibérica, Madrid, Spain) and stored at –20°C.

Cell culture and alteration of cell cholesterol content

Human colorectal cancer cell lines HCT-116, LoVo, HT-29, Colo-205 and BE were maintained in Dulbecco's Modified Eagle Medium supplemented with 5% FBS. For the different treatments, cells were washed with saline and incubated with fresh medium containing 5% FBS or 5% LPDS with 25-HC, alone or with cholesterol (see figure legends). Cholesterol and 25-HC were dissolved in ethanol; control cells were incubated with the same volume of ethanol used in the different treatments.

Determination of free cholesterol

Free cholesterol concentration was determined by a fluorimetric assay, as described (19). Lipids were extracted from cells with chloroform–methanol 2:1 (vol/vol); chloroform was evaporated under N_2 flow and an enzymatic mixture added. After incubation (30 min, 37°C), fluorescence was measured in a fluorimeter (excitation 325 nm, emission 415 nm). Proteins were determined by Bradford method and values are expressed as microgram free cholesterol/milligram cell protein.

Cell transfection

For expression vectors, cells were seeded in six-well plates (2.5×10^5 cells per 35-mm well) and transfected after 18–20 h with the appropriate constructs using LipofectAMINE (Invitrogen, Prat de Llobregat, Barcelona, Spain) according to manufacturer's protocols. After 24 h, cells were treated as stated in figure legends and harvested. For siRNA, cells were seeded at 2.5×10^5 cells

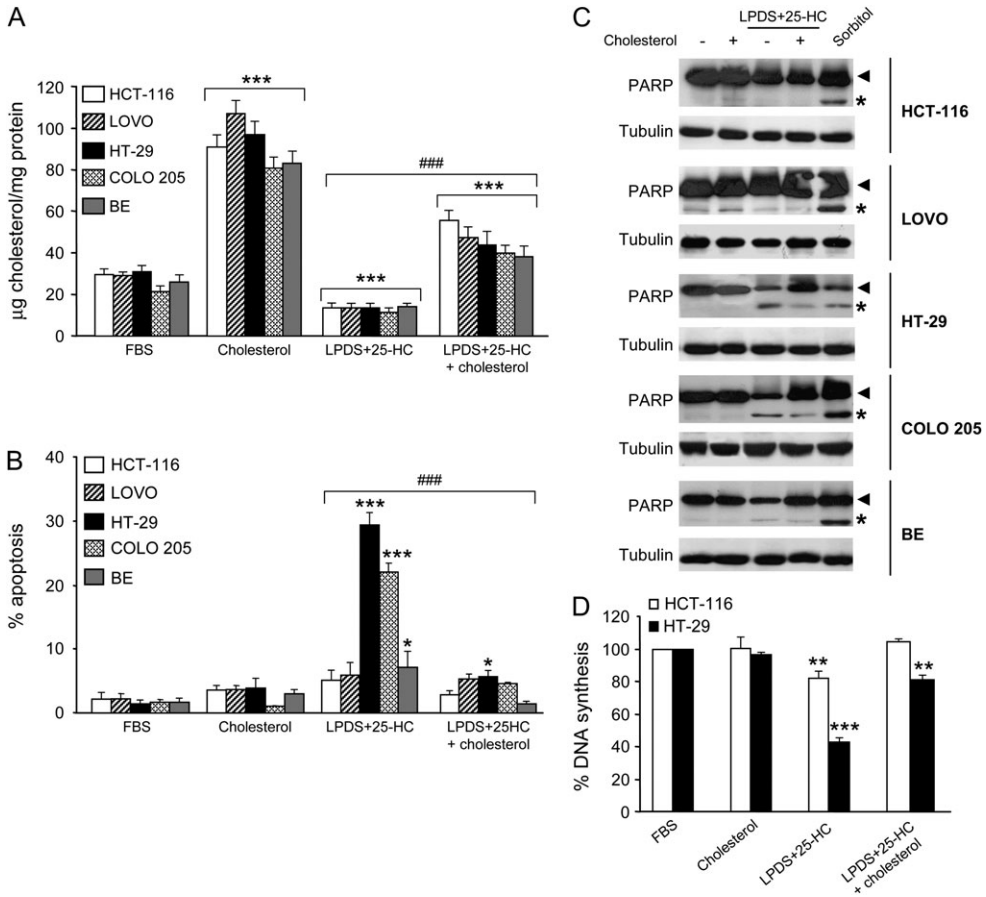

Fig. 1. Cholesterol depletion induces apoptosis only in colorectal cancer cells without oncogenic Ras. **(A)** Cholesterol cell content in colorectal cancer cells incubated with 5% FBS or 5% LPDS plus 2.5 μM 25-HC, in the absence or presence of 25 μg/ml of cholesterol for 48 h. Data are shown as the mean ± SEM of three independent experiments performed in duplicate. **(B)** The sub-G1 fractions of propidium iodide-stained colorectal cancer cells treated as in (A) were analysed by fluorescence activated cell sorting (FACS). Results shown are the mean ± SEM of three independent experiments performed in duplicate. **(C)** Western blot showing PARP cleavage in colorectal cancer cells treated as in (A). The arrows indicate full-length PARP and the asterisk the cleavage product. Blots were reprobed with anti-tubulin antibody as loading control; sorbitol was used as a positive control. The results show a representative experiment repeated three times with similar results. **(D)** [^{3}H]thymidine incorporation into HCT-116 and HT-29 cells treated as in (A) for 24 h. Data are the mean ± SEM of three independent experiments performed in duplicate. $^{*}0.01 < P < 0.05$, $^{***}P < 0.001$, significant differences compared with the corresponding FBS control. $^{\#\#\#}P < 0.001$, significant differences compared with the corresponding LPDS plus 25-HC control.

per 35-mm well the day before transfection. Cells were transfected using LipofectAMINE in 1 ml of OPTIMEM with 100 nM AKT1-specific (5′-AAGCCCUCAGAACAAUCCGAU-3′), B-RAF-specific (5′-AACAGUCUACAAGGGAAAGUG-3′), C-RAF-specific (5′-AAUAGUUCAGCAGUUUGGCUA-3′) or Silencer™ negative control#1 siRNA (Ambion). After 6 h incubation with the RNA complex, 1 ml of medium containing FBS was added. Cells were harvested at the indicated times after the transfection (see figure legends).

Cell lysis and western blot analysis

After incubation, cells were harvested into 200 µl lysis buffer (20 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 100 mM KCl, 1% Triton X-100, 0.3% 2-mercaptoethanol, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$) supplemented with protease inhibitors. Cell extracts were normalized for protein content. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and western blot analysis was performed following standard protocols using the indicated antibodies.

JNK activity assay

Briefly, cell lysate protein (∼200 µg) was incubated with 5 µg GST-c-Jun (1-135) coupled to glutathione agarose (4°C, 2 h, with constant rotation). Agarose beads were washed three times with buffer A (20 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.1 mM ethylene glycol tetraacetic acid, 0.1% 2-mercaptoethanol, 0.05% Triton X-100), and then incubated (20 min, 30°C) in 30 µl modified buffer A (standard preparation plus 10 mM $MgCl_2$, 50 µM ATP, 1 µCi of [γ-^{32}P] ATP); the reaction was terminated by addition of 15 µl of 2× SDS-loading buffer. Phosphorylated GST-c-Jun was resolved in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, dried and visualized by autoradiography. Relative JNK protein concentrations were determined by immunoblotting the top of the gels with anti-JNK antibody.

Flow cytometric analysis of apoptosis

Apoptosis was identified and quantified by flow cytometry with propidium iodide staining. Adherent and floating cells were collected after treatment, washed with ice-cold phosphate-buffered saline and fixed with 70% ice-cold ethanol (30 min, 4°C). Fixed cells were washed twice with phosphate-buffered saline and treated with RNase (1 mg/ml; 30 min, 37°C). Cellular DNA was stained with 5 ng/ml propidium iodide in phosphate-buffered saline and cells were analysed on a FACScan flow cytometer (Becton Dickinson, BD Biosciences, San Agustín de Guadalix, Madrid, Spain). Percentages of cells in different cell-cycle phases were calculated from DNA histograms. Cells with sub-G_1 DNA content were considered apoptotic.

Statistical analysis

All data are expressed as means ± SEM. In statistical analysis, the Student's *t*-test was performed using the SSCStat software (V2.18, University of Reading, UK).

Results

Depletion of cholesterol cell content specifically induces apoptosis in colorectal cancer cells without oncogenic Ras

LPDS is an acceptor of cholesterol that diminishes cell cholesterol content resulting in a parallel activation of HMG-CoA reductase to increase mevalonate synthesis (30,31). Addition of cholesterol or 25-HC in the presence of LPDS inhibits HMG-CoA reductase activity by a feedback mechanism, but whereas exogenous cholesterol increases cholesterol cell content, 25-HC does not (30,31). Firstly, to confirm that our treatments modified cell cholesterol levels as in other cell types (19,31), we assessed the level of cellular cholesterol in the colorectal cancer cell lines HCT-116 and LoVo harbouring the ^{G13D}Ras mutation, HT-29 and Colo-205 containing the ^{V600E}B-RAF mutation and BE, which possess two mutations ^{G13D}Ras and ^{G463V}B-RAF, that were treated for 48 h with medium supplemented with 5% FBS or 5% LPDS in combination with 25-HC, in the absence or presence of exogenous cholesterol. As expected, the incubation of the cells with LPDS plus 25-HC (LPDS-HC) decreased cellular cholesterol content by ∼50% when compared with control cells incubated in FBS (Figure 1A). We also found a significant increase by about 3-fold in cellular cholesterol levels when the cells were incubated with exogenous cholesterol (Figure 1A). Moreover, the addition of exogenous cholesterol reversed the cholesterol decrease induced by LPDS-HC (Figure 1A). These data indicate that these treatments modulate colorectal cancer cell cholesterol content.

Secondly, we analysed how cholesterol cell content affected apoptosis in these colorectal cancer cells. We observed that low levels of cholesterol, achieved by treatment with LPDS-HC, resulted in a marginal increase of apoptosis in HCT-116, LoVo and BE cells, carrying oncogenic forms of Ras, whereas this treatment significantly increases apoptosis levels in HT-29 and Colo-205 cells, which only contain a B-RAF mutation (Figure 1B). Furthermore, the addition of exogenous cholesterol to HT-29 and Colo-205 cells reduced LPDS-HC-induced apoptosis by ∼90%, near control cell values (Figure 1B), indicating that the effect of LPDS and 25-HC is mediated by the changes in the cholesterol cell content and not to non-sterol-derived product from its biosynthesis. To confirm the role of cholesterol levels in apoptosis, we examined the effect of LPDS-HC on PARP cleavage, an endogenous substrate of caspase-3. As expected, the low cholesterol levels achieved by this treatment increased the proteolysis of PARP in HT-29 and Colo-205 cells but not in HCT-116, LoVo and BE cells, and exogenous cholesterol reversed this effect (Figure 1C). These data were consistent with the decrease in DNA synthesis observed in HT-29 cells after cholesterol depletion and the reversion by addition of cholesterol, whereas no effect was observed in HCT-116 cells (Figure 1D).

These findings suggest that apoptotic death of these cells is triggered by a decrease in cell cholesterol levels and that oncogenic Ras but not B-RAF protects from the apoptosis induced by cholesterol depletion.

Oncogenic Ras protects from cholesterol depletion-induced apoptosis through PI3K activation

To analyse the mechanism by which oncogenic Ras protects from low cholesterol induced apoptosis, we examined the effects of LPDS-HC treatment on apoptosis of HT-29 cells that overexpressed different oncogenic Ras mutants that selectively activate the RAF, PI3K or RalGDS signalling pathways (32). HT-29 cells were transfected with different expression plasmids encoding for ^{V12G}Ras (^{V12}Ras), a mutant that selectively activates RAF (^{V12}RasG37), a mutant that selectively activates RalGDS (^{V12}RasS35) or a mutant that activates PI3K but not RAF or RalGDS (^{V12}RasC40), and then incubated with LPDS-HC

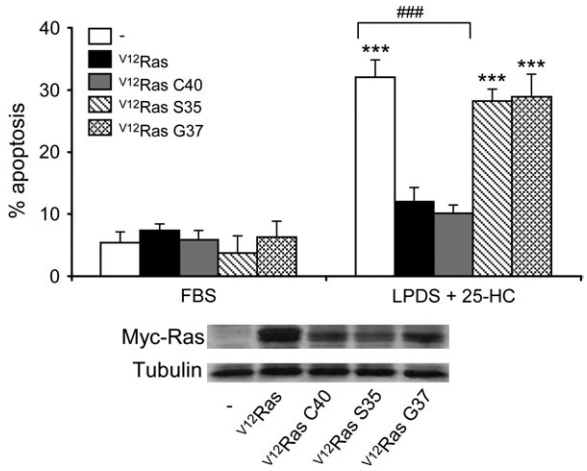

Fig. 2. Oncogenic Ras protects from cholesterol depletion-induced apoptosis through PI3K activation. HT-29 cells were transfected with ^{V12}Ras, ^{V12}RasC40, ^{V12}RasS35 or ^{V12}RasG37 expression vector, as indicated. At 24 h after transfection, cells were left untreated or incubated with LPDS plus 25-HC for 48 h. Apoptosis was identified and quantified by FACS with propidium iodide staining. Results shown are the mean ± SEM of three independent experiments performed in duplicate. Representative western blot for myc-tagged Ras mutants, using the 9E10 antibody, is shown together with the reprobed membrane with tubulin antibody to check their expression. $^{***}P < 0.001$, significant differences compared with the corresponding FBS control. $^{\#\#\#}P < 0.001$, significant differences compared with LPDS plus 25-HC transfected with the empty vector.

(Figure 2). As predicted, ^{V12}Ras overexpression significantly decreased the cholesterol depletion-induced apoptosis close to the basal levels. Moreover, we also found that the RAF or RalGDS effectors mutants did not show any effect, whereas the overexpression of the effector mutant that specifically activates PI3K almost completely reversed the apoptosis induced by low cellular cholesterol levels (Figure 2). These data indicate that oncogenic Ras protects from the apoptosis induced by cholesterol starvation presumably activating the PI3K/AKT pathway but not the RalGDS or RAF/MEK/ERK pathways. In this sense, we observed that neither inhibition of MEK with specific inhibitors or overexpression of a dominant-negative RalB sensitized HCT-116 to the lack of cholesterol (data not shown).

To test the role of the PI3K/AKT pathway, we analysed the effect of PI3K inhibition with the specific inhibitors LY294002 and wortmannin on the LPDS-HC-induced apoptosis in HCT-116 and HT-29 cells. As shown in Figure 3A, both LY294002 and wortmannin sensitized HCT-116 cells to LPDS-HC treatment and induced apoptosis to a similar level as that found in HT-29 cells (Figure 3A). In HT-29 cells, however, neither one of these inhibitors exerted any effect in basal conditions nor did they modify the cholesterol depletion-induced apoptosis (Figure 3A). We next examined the phosphorylation of AKT in both cell lines. We observed that HCT-116 cells show a constitutive phosphorylation of AKT, whereas HT-29 cells do not show basal AKT phosphorylation (Figure 3B). In both cases, changes in cholesterol cell content did not modify the AKT phosphorylation status, although in HCT-116 cells the treatment with LY294002 completely abolished the phosphorylation of this protein (Figure 3B). These data suggest that the constitutive activation of the PI3K/AKT pathway by oncogenic Ras in HCT-116 could be responsible for the protection of apoptosis induced by low cholesterol levels. To further demonstrate that PI3K/AKT pathway activation leads to lack of cytotoxicity in response to cell cholesterol depletion, we studied the direct role of AKT on apoptosis induced by LPDS-HC. We examined whether overexpression of a constitutive active form of AKT1 (AKTca) could prevent the apoptosis induced by cholesterol depletion in HT-29 cells (Figure 3C). As expected, AKTca expression significantly decreased LPDS-HC-induced cell death by ~60% in these cells (Figure 3C). On the other hand, HCT-116 cells were treated with siRNA oligonucleotides specific for AKT1 that deplete the expression of this protein and an oligo control that did not affect AKT1 expression (Figure 3D). AKT1 depletion did not have any effect on basal condition but sensitized HCT-116 cells to LPDS-HC treatment and significantly increased apoptosis to similar levels of those observed in HT-29 cells, which lack AKT1 activity. Taken together, these data suggest that the activation of the constitutive PI3K/AKT pathway by oncogenic Ras protects from apoptosis induced by cholesterol depletion in colorectal cancer cells.

Cholesterol depletion induces apoptosis through a JNK-dependent mechanism

We have recently demonstrated that cholesterol-depleted NIH3T3 cells showed increased JNK and p38 MAPK activity (19), and it is well known that both MAPKs transduce cell death signalling in many cell types (20). We next investigated if the increase in apoptosis induced by low cholesterol cell content was mediated by these pathways in

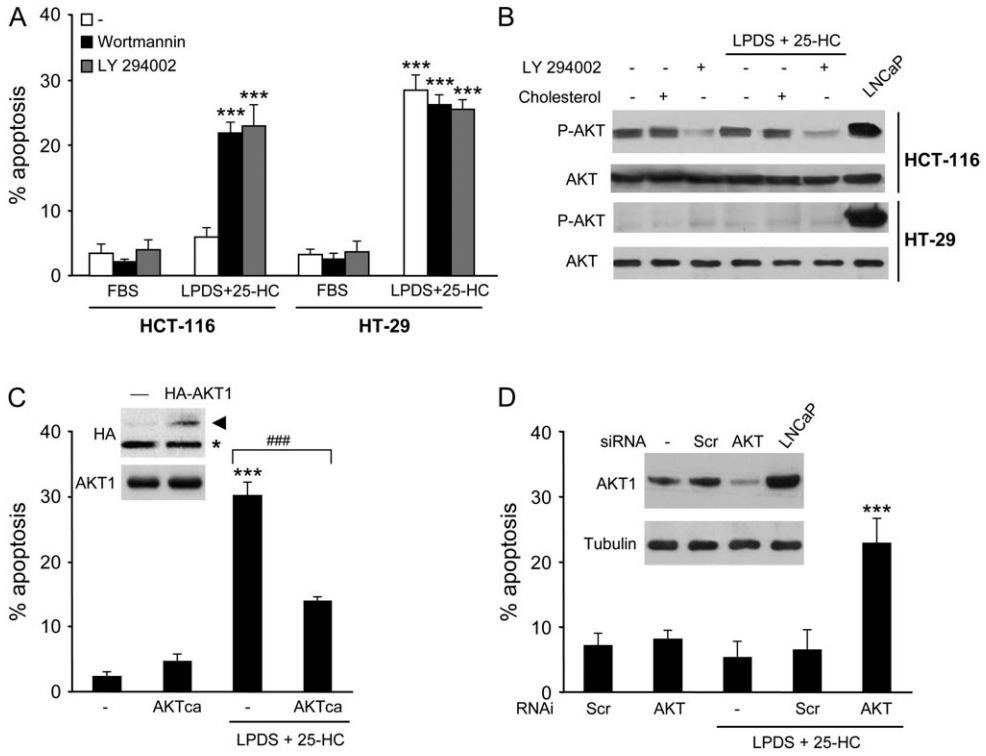

Fig. 3. Active PI3K/AKT pathway protects colorectal cancer cells from cholesterol depletion-induced apoptosis. **(A)** HCT-116 and HT-29 cells were treated with FBS or LPDS plus 25-HC, in the presence or absence of 20 μM LY294002 or 100 nM wortmannin, for 48 h. Proportion of sub-G1 cells quantified by FACS. Data showing the mean ± SEM are complied from three independent experiments performed in duplicate. **(B)** Representative western blot for phospho-AKT (P-AKT) and total AKT (AKT), as control, in untreated or LPDS plus 25-HC-treated cells, in the absence or presence of cholesterol and with or without LY294002; prostate LNCaP cells were used as positive control. **(C)** The sub-G1 fractions from HT-29 cells transfected with empty vector or a constitutively active AKT1 construct (AKTca) and then incubated with FBS or LPDS plus 25-HC for 48 h. Data are the mean ± SEM of three independent experiments performed in duplicate. Inset, representative western blot showing ectopically expressed HA-AKT1 and total AKT1 expressions. The arrows indicate HA-AKT1 and the asterisk a non-specific band. **(D)** Sub-G1 fraction measurements in HCT-116 cells untreated or treated with LPDS plus 25-HC, in which AKT1 was depleted (AKT) compared with cells transfected with a scrambled control (Scr). Data are the mean ± SEM of three independent experiments performed in duplicate. Inset, representative western blot for AKT and tubulin expressions. $^{***}P < 0.001$, significant differences compared with the corresponding control. $^{\#\#\#}P < 0.001$, significant differences compared with LPDS plus 25-HC transfected with empty vector.

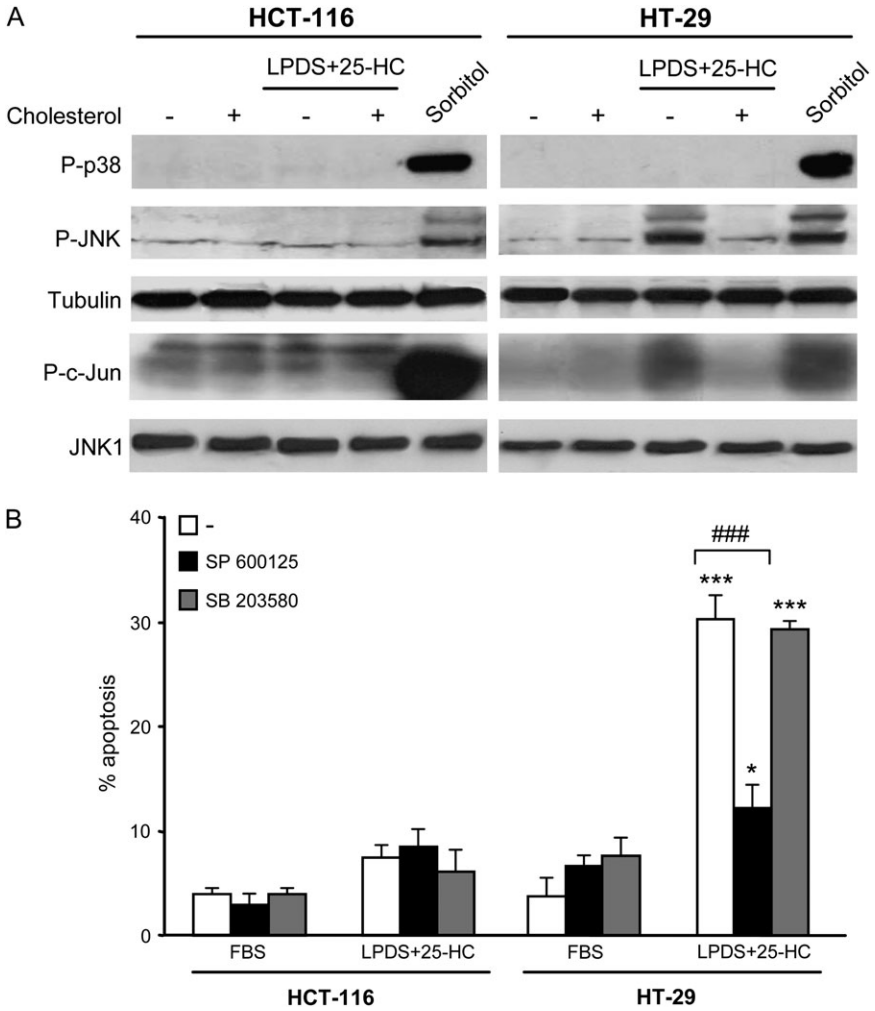

Fig. 4. JNK mediates cholesterol depletion-induced apoptosis. **(A)** HCT-116 and HT-29 cells were incubated with or LPDS plus 25-HC for 48 h, alone or with cholesterol. Phosphorylation of p38 (P-p38) and JNK (P-JNK) were detected by western blot in the same extracts using appropriate phospho-specific antibodies. For each pair of rows, an image of the phospho-specific blot is shown with the reprobed membrane with anti-tubulin antibody as control. For JNK activation, endogenous JNK was pulled down with GST-c-Jun coupled to glutathione agarose, and kinase activity was measured (P-c-Jun). To ensure equivalent protein loading, the tops of the gels were transferred and blotted with anti-JNK1 antibody as control (JNK1). Sorbitol was used as a positive control. Similar results were obtained in three independent experiments. **(B)** Sub-G1 fractions were identified and quantified by flow cytometry with propidium iodide staining of HCT-116 and HT-29 cells incubated for 48 h in FBS or LPDS plus 25-HC with or without 1 μM SB203580 or 10 μM SP600125. Results shown are the mean ± SEM of three independent experiments performed in duplicate. $^{*}0.01 < P < 0.05$, $^{***}P < 0.001$, significant differences compared with the corresponding FBS control. $^{\#\#\#}P < 0.001$, significant decrease compared with control (untreated LPDS plus 25-HC).

colorectal cancer cells. We first examined the effects of LPDS-HC treatment on the activity of these kinases (Figure 4). The results clearly indicate that LPDS-HC treatment did not increase the phosphorylation of p38 MAPK neither in HCT-116 nor HT-29 cells (Figure 4A). However, low cholesterol levels had a different effect on JNK activation in these cell lines. LPDS-HC treatment did not modify either phosphorylation or JNK activity in HCT-116 cells, whereas both were activated in HT-29 cells. Moreover, these effects were inhibited by simultaneous addition of cholesterol to the medium (Figure 4A).

Since this increase in JNK activity by low cholesterol levels in HT-29 cells is parallel to that previously observed in apoptosis in the same cells, we next examined the role of JNK and p38 MAPK activation on LPDS-HC-induced apoptosis. We determined apoptosis levels in HCT-116 and HT-29 cells, untreated or pretreated with specific inhibitors for these MAPKs, followed by LPDS-HC treatment (Figure 4B). As expected, cell exposure to the p38 MAPK inhibitor SB203580 did not alter LPDS-HC-induced apoptosis levels (Figure 4B). Nonetheless, although the JNK inhibitor SP600125 did not exert any effect in HCT-116 cells with low cholesterol cell content (Figure 4B), it abrogated the effect of LPDS-HC on apoptosis almost completely in HT-29 cells (Figure 4B). These results indicate that cholesterol deficiency-induced apoptosis in cells without oncogenic Ras is mediated though the activation of the JNK signalling pathway.

Constitutive AKT activity impairs JNK activation by low cell cholesterol levels

Finally, since we have observed a protective role of constitutive active PI3K/AKT pathway on apoptosis induced by low cellular cholesterol levels and it seems that this cell death is mediated by the activation of JNK proteins, we studied whether there is any relationship between both pathways. For this purpose, we examined the levels of JNK phosphorylation in HCT-116 cells after inhibition of AKT activity using the PI3K inhibitor LY294402 or specific siRNA oligonucleotides for AKT (Figure 5). As we have shown before, cholesterol depletion did not have any effect on JNK phosphorylation in these cells. However, the treatment of the HCT-116 cells with LY294002 inhibitor, which increases cell death in these cells (Figure 3B), allows LPDS-HC to increase JNK phosphorylation (Figure 5A). Similar effect was also observed in cells treated with specific AKT1 siRNA. LPDS-HC treatment increased JNK phosphorylation in cells with AKT ablation (Figure 5B), whereas the lack of RAF proteins expression with specific siRNA or RalB activity by the overexpression of the

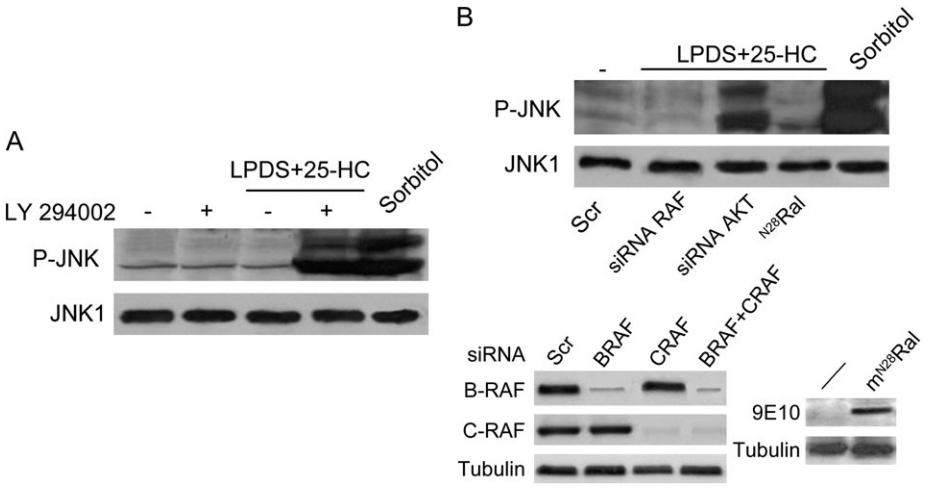

Fig. 5. Constitutive AKT activity abrogates JNK activation by cholesterol depletion. **(A)** Western blot for phospho-JNK in HCT-116 cells treated with FBS or LPDS plus 25-HC for 48 h, in the absence or presence of LY294002. **(B)** Upper panel, western blot for phospho-JNK in LPDS plus 25-HC-treated HCT-116 cells, in which the B-RAF and C-RAF (RNAi RAF) or AKT1 (RNAi AKT) were depleted with specific siRNA oligonucleotide probes, or a dominant-negative RalB construct (^{N28}Ral) was overexpressed. In all cases, sorbitol was used as positive control and an image of the phospho-specific blot is shown with the reprobed membrane with anti-JNK1 antibody as control. Bottom left panel, representative western blot showing B-RAF, C-RAF and tubulin expressions 72 h after transfection with siRNA oligonucleotides specific for B-RAF or C-RAF and a scrambled oligo control (Src). Bottom right panel, representative western blot for ectopically expressed myc-tagged ^{N28}RalB mutant, using the antibody 9E10, is shown together with the membrane reprobed with tubulin. Blots are representative of experiments performed three times with similar results.

dominant-negative ^{N28}RalB construct did not show any effect (Figure 5B), indicating the specificity of the PI3K/AKT pathway in preventing the induction of JNK activity by cholesterol depletion.

Discussion

This study shows that cellular cholesterol-depleting treatment with LPDS-HC increases the level of apoptosis in HT-29 and Colo-205 cells harbouring oncogenic B-RAF mutation, but not in HCT-116 and LoVo harbouring oncogenic Ras mutation or BE cells carrying both, through a mechanism involving JNK activation. We also demonstrate that oncogenic Ras, but not oncogenic B-RAF, protects from apoptosis induced by cholesterol depletion by constitutive activation of the PI3K/AKT pathway, which in turn impairs JNK activation. All these effects are the result of alterations in cholesterol levels and are not mediated by mevalonate-generated non-sterol products, since all were reversed by restoring cell cholesterol.

In this study, our data indicating that alterations in cholesterol levels modulate apoptosis agrees with the previous finding that cholesterol enrichment of the neuronal plasma membrane protects cortical neurons from apoptosis and that cholesterol depletion renders cells more vulnerable to cytotoxic effects (13,14). In this sense, Michikawa and Yanagisawa showed that addition of free cholesterol or cholesterol via β-VLDL completely rescued neurons from compactin-induced cell death, indicating that cholesterol deficiency, but not of non-sterol isoprenoid products, is crucial for the toxicity induced by inhibition of HMG-CoA reductase (13). Many studies have similarly shown that specific cholesterol depletion from plasma membrane using MβCD induces apoptosis in various cell types including different tumoural cells (15,16). Furthermore, we have shown previously that low levels of cholesterol induced by LPDS-HC treatment increases apoptosis in NIH3T3 and that this effect is not related to the mevalonate synthesis rate but rather to changes in cell cholesterol content, since it was not reversed by concomitant treatment with farnesyl or geranylgeranyl pyrophosphate (19).

Here, we demonstrate that cholesterol depletion activates JNK in colorectal cancer cells lacking oncogenic Ras, and does not exert any effect on p38 MAPK activity independently of Ras status. We and others have shown previously that p38 MAPK activity increases following depletion of membrane cholesterol (19,33); however, it has also been shown that low cell cholesterol levels do not induce p38 MAPK phosphorylation in other cell types (34), indicating that this effect may be cell type specific. In this sense, our data are similar with those showed by Ramacle-Bonnet *et al.* indicating that cholesterol depletion from plasma membrane, using MβCD treatment, does not affect IGF-I-induced p38 MAPK activity in HT-29 cells (35). To date, only few reports have addressed the role of cholesterol in the JNK pathway. Thus, it has been shown that MβCD activates JNK activity and that this effect is significantly inhibited by cholesterol repletion (34,36). To our knowledge, this is the first report showing that low cholesterol levels promote JNK activation in the absence of stimuli in cancer cells. We still do not know the mechanism by which cholesterol depletion activates JNK. Although, there is no complete connection, many JNK activators can be modulated by changes in cell cholesterol content, such as signalling mediated by cell-surface receptors like TNFαR (36), increase in reactive oxygen species or modification of the activity of the GTPases Rac and Cdc42 (37). Thus, we propose that cholesterol depletion may trigger a cell stress signal that modifies any one of these pathways and activates JNK proteins, possibly as a result of alterations in the distribution and/or activity of molecules otherwise concentrated within lipid domains at the plasma membrane.

Our data also demonstrate that oncogenic Ras, but not oncogenic B-RAF, protects from apoptosis induced by cholesterol depletion through JNK activation in colorectal cancer cells and that the protective role of oncogenic Ras is exerted through constitutive activation of the PI3K/AKT pathway. Expression of oncogenic ^{V12}Ras, ^{V12}RasC40 or AKTca significantly decreases the cholesterol depletion-induced apoptosis in HT-29 cells, whereas abrogation of AKT expression in HCT-116 cells renders these cells sensitive to apoptosis by cholesterol depletion. Moreover, the treatment with the specific JNK inhibitor SP600125 does not modify apoptosis in basal conditions in both cell lines, which is consistent with the findings of a previous study in HT-29 cells (38), but significantly decreases it in HT-29 cholesterol-depleted cells. Mutation in the B-RAF gene may contribute to colorectal carcinogenesis by upregulating the antiapoptotic role of the RAS/RAF/MEK/ERK pathway, but the relationship between B-RAF mutation and apoptosis is not so clear. Although B-RAF provides proliferation and survival signals in colorectal carcinoma cells displaying ^{V600E}B-RAF but not K-Ras mutations (28), it

has been recently shown that only the simultaneous suppression of B-RAF and Rac1b dramatically decreased colorectal cancer cell viability through impaired cell-cycle progression and increased apoptosis (27). However, the protective role of Ras and AKT in apoptosis has been widely described (23,26). Inhibition of this pathway results in the suppression of cell proliferation and/or the induction of apoptosis in several types of cancer cells. Thus, PI3K inhibitors LY294002 and wortmannin show antitumourigenic activity in human colon cancer cells *in vivo* and can cause induction of apoptosis and cell growth arrest in different colorectal cancer cells lines (39,40).

Substantial evidence has related AKT activity with cholesterol depletion and apoptosis. Thus, it has been shown that oxysterols can induce apoptosis through inactivation of AKT, with the concomitant activation of the proapoptotic Bad and downregulation of the antiapoptotic Bcl-X_L, both regulated by this protein (18). Moreover, membrane cholesterol depletion inhibits AKT phosphorylation and increases apoptosis in different stimulated cancer cells (15,16,35). We have shown here, however, that in the absence of stimuli, changes in cholesterol content do not modify AKT activity. Interestingly, it has been recently found that in prostate cancer cells, two different AKT subpopulations exist, a cholesterol depletion sensitive and a cholesterol insensitive population, depending on the tendency to reside in cholesterol-rich membrane microdomains (41).

We show that inhibition of PI3K activity and abrogation of AKT expression render HCT-116 cells susceptible to cholesterol depletion allowing to LPDS-HC to activate JNK. Many studies have demonstrated JNK inhibition by AKT in different cell types (42,43). We still do not know the mechanism by which AKT exerts this function in these cells; possible candidates would be the inhibition of the JNK-activating proteins ASK1, MKK4 or MLK3 or induction of the inhibitor JNK-interacting protein-1 (42,43). Moreover, AKT activates the transcription factor nuclear factor kappa-B, which can inhibit JNK-mediated apoptosis increasing the expression of inhibitors of the JNK pathway, such as the inhibitor of caspases XIAP (44) or the MKK7 inhibitor Gadd45b/Myd118 (45). Finally, it has also been shown that PI3K/AKT pathway is involved in fatty acid and cholesterol accumulation in some tumours. Porsttman *et al.* (46) demonstrated that AKT increases the synthesis of the transcription factors sterol responsive element-binding protein, responsible of the expression of enzymes involved in lipid and cholesterol homeostasis after changes in cellular cholesterol content . Thus, the constitutive AKT activation may counteract the lack of cholesterol on sterol responsive element-binding proteins, and ultimately impair JNK activation in these cells.

To our knowledge, this is the first report showing that low cholesterol levels promote apoptosis in colorectal cancer cells lacking oncogenic Ras but not in those carrying mutations in this protein. Our data establish that constitutive activation of the PI3K/AKT pathway by this oncogene protects colorectal cancer cells from JNK-induced apoptosis activated by cholesterol depletion.

To reduce incidence of colon cancer, combinations of chemopreventive agents are increasingly being studied in an attempt to amplify preventive effects or/and to reduce side effects of known effective agents. It is therefore important to develop alternative therapeutic strategies with improved efficacy and tolerability to overcome unsuccessful current available therapies because they frequently lead to resistance or unacceptable levels of toxicity. Thus, strategies that induce apoptosis selectively in cancer cells, such as cholesterol depletion, together with PI3K inhibitors should greatly increase the chemopreventive effects or allow their use at lower doses, and are therefore highly desirable.

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References

1. Rao,K.N. (1995) The significance of the cholesterol biosynthetic pathway in cell growth and carcinogenesis (review). *Anticancer Res.*, **15**, 309–314.
2. Tosi,M.R. *et al*. (2005) Cholesteryl esters in malignancy. *Clin. Chim. Acta*, **359**, 27–45.
3. Notarnicola,M. *et al*. (2004) Up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in left-sided human colon cancer. *Anticancer Res.*, **24**, 3837–3842.
4. Caruso,M.G. *et al*. (2005) Biochemical changes of mevalonate pathway in human colorectal cancer. *Anticancer Res.*, **25**, 3393–3397.
5. Agarwal,B. *et al*. (1999) Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. *Clin. Cancer Res.*, **5**, 2223–2229.
6. Alonso,D.F. *et al*. (1998) Reduction of mouse mammary tumor formation and metastasis by lovastatin, an inhibitor of the mevalonate pathway of cholesterol synthesis. *Breast Cancer Res. Treat.*, **50**, 83–93.
7. Kim,K.P. *et al*. (2004) Combinatorial chemoprevention: efficacy of lovostatin and exisulind on the formation and progression of aberrant crypt foci. *Anticancer Res.*, **24**, 1805–1811.
8. Poynter,J.N. *et al*. (2005) Statins and the risk of colorectal cancer. *N. Engl. J. Med.*, **352**, 2184–2192.
9. Bonovas,S. *et al*. (2005) Use of statins and breast cancer: a meta-analysis of seven randomized clinical trials and nine observational studies. *J. Clin. Oncol.*, **23**, 8606–8612.
10. Dale,K.M. *et al*. (2006) Statins and cancer risk: a meta-analysis. *Jama*, **295**, 74–80.
11. Demierre,M.F. *et al*. (2005) Statins and cancer prevention. *Nat. Rev. Cancer*, **5**, 930–942.
12. Dimitroulakos,J. *et al*. (1999) Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: a potential therapeutic approach. *Blood*, **93**, 1308–1318.
13. Michikawa,M. *et al*. (1999) Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. *J. Neurochem.*, **72**, 2278–2285.
14. Sponne,I. *et al*. (2004) Membrane cholesterol interferes with neuronal apoptosis induced by soluble oligomers but not fibrils of amyloid-beta peptide. *Faseb J.*, **18**, 836–838.
15. Li,Y.C. *et al*. (2006) Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. *Am. J. Pathol.*, **168**, 1107–1118; quiz 1404–1405.
16. Oh,H.Y. *et al*. (2007) Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERK signal transduction. *Prostate*, **67**, 1061–1069.
17. Yang,L. *et al*. (2000) 25-Hydroxycholesterol activates a cytochrome c release-mediated caspase cascade. *Biochem. Biophys. Res. Commun.*, **278**, 557–563.
18. Rusinol,A.E. *et al*. (2004) AKT/protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. *J. Biol. Chem.*, **279**, 1392–1399.
19. Calleros,L. *et al*. (2006) RhoA and p38 MAPK mediate apoptosis induced by cellular cholesterol depletion. *Apoptosis*, **11**, 1161–1173.
20. Wada,T. *et al*. (2004) Mitogen-activated protein kinases in apoptosis regulation. *Oncogene*, **23**, 2838–2849.
21. Osborn,M.T. *et al*. (1996) Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. *J. Biol. Chem.*, **271**, 30950–30955.
22. Stadheim,T.A. *et al*. (2002) c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for mitoxantrone- and anisomycin-induced apoptosis in HL-60 cells. *Leuk. Res.*, **26**, 55–65.
23. Song,G. *et al*. (2005) The activation of Akt/PKB signaling pathway and cell survival. *J. Cell Mol. Med.*, **9**, 59–71.
24. Andreyev,H.J. *et al*. (1998) Kirsten Ras mutation in patients with colorectal cancer: the multicenter ''RASCAL'' study. *J. Natl. Cancer Inst.*, **90**, 675–684.
25. Davies,H. *et al*. (2002) Mutations of the BRAF gene in human cancer. *Nature*, **417**, 949–954.
26. Downward,J. (2003) Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer*, **3**, 11–22.

27. Matos,P. *et al*. (2008) B-Raf(V600E) cooperates with alternative spliced Rac1b to sustain colorectal cancer cell survival. *Gastroenterology*, **135**, 899–906.
28. Preto,A. *et al*. (2008) BRAF provides proliferation and survival signals in MSI colorectal carcinoma cells displaying BRAF(V600E) but not KRAS mutations. *J. Pathol.*, **214**, 320–327.
29. Chiloeches,A. *et al*. (2001) S338 phosphorylation of Raf-1 is independent of phosphatidylinositol 3-kinase and Pak3. *Mol. Cell Biol.*, **21**, 2423–2434.
30. Goldstein,J.L. *et al*. (1990) Regulation of the mevalonate pathway. *Nature*, **343**, 425–430.
31. Chiloeches,A. *et al*. (1997) Effect of mevalonate availability on the association of G-protein alpha-subunits with the plasma membrane in GH4C1 cells. *FEBS Lett.*, **401**, 68–72.
32. White,M.A. *et al*. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell*, **80**, 533–541.
33. Jans,R. *et al*. (2004) Cholesterol depletion upregulates involucrin expression in epidermal keratinocytes through activation of p38. *J. Invest. Dermatol.*, **123**, 564–573.
34. Kim,S. *et al*. (2007) Cholesterol inhibits MMP-9 expression in human epidermal keratinocytes and HaCaT cells. *FEBS Lett.*, **581**, 3869–3874.
35. Remacle-Bonnet,M. *et al*. (2005) Membrane rafts segregate pro- from anti-apoptotic insulin-like growth factor-I receptor signaling in colon carcinoma cells stimulated by members of the tumor necrosis factor superfamily. *Am. J. Pathol.*, **167**, 761–773.
36. Habelhah,H. *et al*. (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. *EMBO J.*, **23**, 322–332.
37. Nofer,J.R. *et al*. (2003) Involvement of Cdc42 signaling in apoA-I-induced cholesterol efflux. *J. Biol. Chem.*, **278**, 53055–53062.
38. Xia,H.H. *et al*. (2006) Induction of apoptosis and cell cycle arrest by a specific c-Jun NH2-terminal kinase (JNK) inhibitor, SP-600125, in gastrointestinal cancers. *Cancer Lett.*, **241**, 268–274.
39. Semba,S. *et al*. (2002) The in vitro and in vivo effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3′-kinase, in human colon cancer cells. *Clin. Cancer Res.*, **8**, 1957–1963.
40. Yamaguchi,K. *et al*. (2004) Identification of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel downstream target of phosphatidylinositol 3-kinase/AKT/GSK-3beta pathway. *J. Biol. Chem.*, **279**, 49617–49623.
41. Adam,R.M. *et al*. (2007) Cholesterol sensitivity of endogenous and myristoylated Akt. *Cancer Res.*, **67**, 6238–6246.
42. Yuan,Z.Q. *et al*. (2003) AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. *J. Biol. Chem.*, **278**, 23432–23440.
43. Barthwal,M.K. *et al*. (2003) Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. *J. Biol. Chem.*, **278**, 3897–3902.
44. Tang,G. *et al*. (2001) Inhibition of JNK activation through NF-kappaB target genes. *Nature*, **414**, 313–317.
45. Deng,Y. *et al*. (2003) A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell*, **115**, 61–70.
46. Porstmann,T. *et al*. (2005) PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. *Oncogene*, **24**, 6465–6481.

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