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Letter to the Editor

Restoration of sensitivity to anoikis in Ras-transformed rat intestinal epithelial cells by a Ras inhibitor

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Dear Editor,

Ras proteins control key signaling pathways that regulate cell growth, migration, and survival.[1,2] Activating mutations in *ras[SRS1]* genes or alterations in upstream signaling components that activate Ras proteins are common in most human tumors.[2] The activated Ras contributes to cell transformation and maintenance of the transformed tumor phenotype.[1] This is manifested by uncontrolled growth of the tumor cells and by their ability to survive under stressful conditions such as loss of matrix attachment.[1,2] Activated Ras provides survival signals and interferes with apoptosis in tumor cells.[2–4] Ras regulates $p19^{ARF}$ and Mdm2, which control p53, a key factor in apoptosis. Activated Ras also inhibits c-myc, which induces apoptosis in the absence of survival factors. In addition, Ras regulates NF-κB, which transcriptionally activates the expression of antiapototic members of the Bcl-2 and IAP families. Ras activates phosphoinositide 3-kinase (PI3-K) and downstream effectors, including the PKB/Akt. PI3-K positively regulates the antiapototic Bcl-2 protein while Akt inhibits the activity of Bad, a proapototic member of the Bcl-2 family, and regulates gene expression by inactivating a number of forkhead family transcription factors that can induce proapoptotic genes.[2–4] In view of the above findings, therapies that target Ras can be expected to induce apoptosis of tumor cells. This possibility was not examined with inhibitors that affect directly the Ras protein. We showed previously that farnesylthiosalicylic acid (FTS) is a direct Ras inhibitor.[5] FTS removes all active Ras isoforms from the cell membrane facilitating Ras degradation.[5] This leads to the inhibition of anchorage-dependent growth of Ras-transformed cells without cytotoxicity.[5] Thus, the inhibition of active Ras by FTS, and presumably also the neglect of Ras survival signals, are not sufficient by themselves to induce death of transformed cells. However, because cancer cells can possess means of survival in addition to Ras activation, an additional stress might be needed to promote FTS-induced apoptosis. Here, we examined whether inhibition of Ras by FTS can induce death of Ras-transformed rat intestinal epithelial-1 (RIE-1/Ras) cells that were sensitized to apoptosis by detaching them from the matrix, a process termed anoikis.

Untransformed RIE-1 cells can undergo anoikis and activated Ras inhibits this type of apoptotic death in RIE-1 cells.[6] To investigate the effects of FTS on resistance to anoikis, RIE-1 or RIE-1/Ras cells were grown in suspension on plates coated with PolyHeme.[6] Cell viability was then estimated by an assay based on MTS.[6] Results of a typical experiment (Figure 1a(i)) demonstrate that RIE-1/Neo (vector control) cells died within 15–24 h, whereas RIE-1/H-Ras(61L) and RIE-1/K-Ras(12V) cells were fully protected from detachment-induced cell death. RIE-1/Neo cell death was inhibited by the general caspase inhibitor *t*-butyloxycarbony-asp-fluoromethylketone (Boc-asp-fmk), suggesting that they underwent caspase-dependent apoptotic death (Figure 1a(i)). We next examined the effects of FTS on RIE-1/Ras cells. The cells were treated with 12.5 μM FTS immediately after being plated on PolyHeme-coated plates. Unlike in the untreated RIE-1/Ras cells, massive death was observed in the FTS-treated cells (Figure 1a(i)). The FTS-induced cell death was time (Figure 1a(i)) and dose dependent (Figure 1a(ii)); approximately, 50% of the cells died within 36 h at 12.5 μM FTS. Boc-asp-fmk provided significant protection (Figure 1a(i)). In contrast, 3-methyl adenine (10 mM), an inhibitor of autophagic cell death did not protect the RIE-1/Ras cells from FTS-induced death (not shown). To further substantiate the findings that RIE-1/Neo cells underwent anoikis and that FTS eliminated the ability of Ras to protect RIE-1/Ras cells from anoikis, we subjected the cells to Hoechst 33258 dye exclusion staining. In the absence of FTS, the numbers of Hoechst-stained nuclei (representing dead cells) were markedly increased after 24 h in the suspended RIE-1/Neo cells. Treatment with Boc-asp-fmk decreased the number of cells with Hoechst-stained nuclei; 72 and 11% of the RIE-1/Neo cells died in the absence and in the presence of Boc-asp-fmk, respectively. In similar experiments carried out with RIE-1/H-Ras(61L) cells, we found that FTS induced a marked increase in Hoechst-stained nuclei and that this effect was inhibited by Boc-asp-fmk; 5% of the control RIE-1/H-Ras(61L) cells, 52% of the FTS-treated cells, and 11% of the cells treated with FTS and Boc-asp-fmk died under these conditions. Similar effects were observed with RIE-1/K-Ras(12V) cells. Importantly, FTS had no cytotoxic effects in RIE-1/Ras cells grown under anchorage-dependent conditions. Unlike FTS, inhibitors of enzymes known to be involved in survival/apoptosis pathways had little or no effect on RIE-1/Ras cell survival. The protein kinase C inhibitor GF109203X (5 μM), the p38 inhibitor SB203580 (10 μM), and the PI3-K inhibitor LY294002 (10 μM) did not affect the survival of these cells. The MEK inhibitor U012650 (50 μM), however, induced a small (25−28%) but significant cell death ($P<0.05$), consistent with a partial involvement of the Raf/MEK/ERK cascade in Ras protection from anoikis. Indeed, of three prominent Ras pathways, namely, Raf/MEK/ERK, PI3-K/PKB, and RalGEF/Ral, we found that in RIE-1/Ras cells only the Raf pathway was

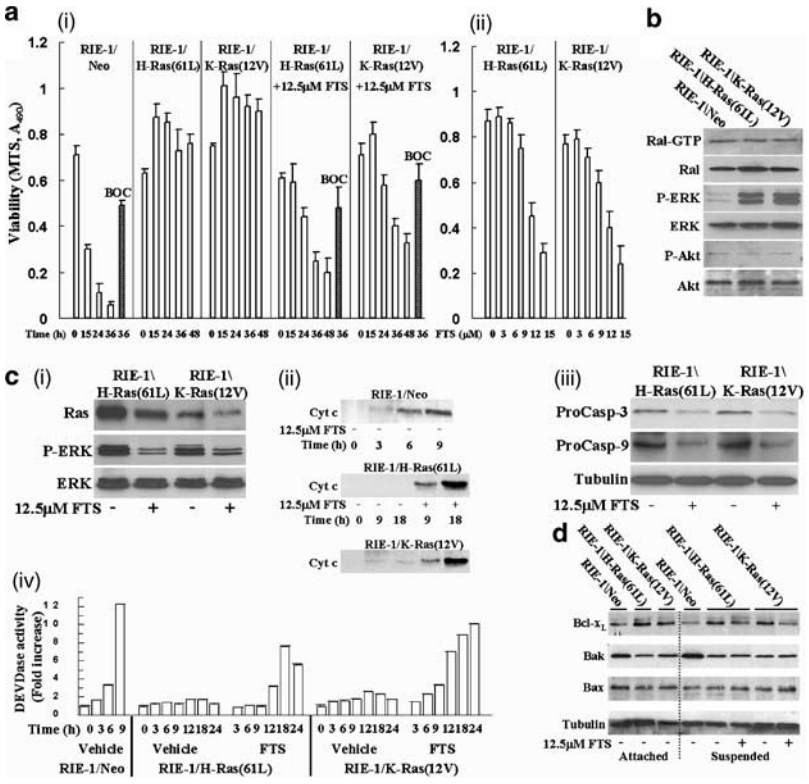

Figure 1 (**a**) *Ras* anoikis resistance is inhibited by FTS. RIE/Neo and RIE-1/Ras cells were plated on PolyHeme-coated plates [6] (3×10^4 cells per well in 96-well plates) and incubated in suspension for the indicated time periods in the absence or in the presence of 12.5 μM FTS with or without 100 μM Boc-asp-fmk (BOC, striped bar) (i), or for 36 h with the indicated concentrations of FTS (ii). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.[6] The absorbance values (A_{490}) recorded in four separate experiments (mean $\pm$ S.D.) are shown. (**b**) Ras upregulates phospho-ERK, but not Ral-GTP or phospho-Akt in RIE-1 cells. Cells (6×10^6 cells per 10 cm plate) were grown under anchorage-dependent conditions,[6] then homogenized. Aliquots of the homogenates were immunoblotted with anti-ERK, phospho-ERK, Akt, phospho-Akt, and Ral A antibodies (Abs), or subjected to the GST-RalBD pull-down assay[13] followed by immunoblotting with anti-Ral A Ab, enhanced chemiluminescence and densitometry as detailed earlier.[14] Typical immunoblots are shown. All experiments here and below were performed in triplicate and data provided represent means $\pm$ S.D. Only phospho-ERK was higher in RIE-1/H-Ras(61L) and RIE-1/K-Ras(12V) cells than in RIE-1/Neo cells (6 ± 1- and 8 ± 2.5-fold, respectively). (**c**) FTS downregulates Ras and phosph-ERK and induces cytochrome *c* release and caspase activation. RIE-1/Neo or RIE-1/Ras cells cells (6×10^6 cells per 10 cm plate) were grown in suspension as in (**a**) for 9 h (i and iii) or for the indicated periods of time (ii and iv) in the absence or in the presence of FTS, then homogenized. (i) Aliquots of the homogenates were immunoblotted with anti-Ras, ERK, and phospho-ERK Abs. FTS induced a decrease of 60 ± 10 and 51 ± 8%, respectively, in the amounts of H-Ras(61L) and K-Ras 4B(12V), and a decrease of 69 ± 12 and 60 ± 8% of phospho-ERK in the RIE-1/H-Ras(61L) and RIE-1/K-Ras 4B(12V) cells, respectively. (ii) Cytochrome *c* in the cytosolic fractions of the cells was determined by immunoblotting with anticytochrome *c* Ab as described earlier.[15] Similar results were obtained in two additional experiments. (iii) Aliquots of the homogenates were immunoblotted with anticaspase-3 and -9 antibodies as detailed.[16] FTS induced a decrease (means $\pm$ S.D.) of 29 ± 6 and 56 ± 4% in the amounts of procaspase-3 and -9, respectively, in RIE-1/H-Ras(61L) cells and 32 ± 5 and 51 ± 7%, respectively, in RIE-1/K-Ras 4B(12V) cells. (iv) Caspase-3-like activity was determined in the cytosolic fractions of the cells with the substrate Ac-DEVD-7-AMC as described earlier.[16] Data represent the fold increase in DEVDase activity relative to zero time. (**d**) Ras upregulates and FTS downregulates Bcl-x_L in RIE-1/Ras cells. RIE-1/Neo cells or RIE-1/Ras were grown under anchorage-dependent conditions as in (**b**) or in suspension with or without FTS as detailed in (**c**). Aliquots of cell homogenates were subjected to immunoblotting with anti-Bax, anti-Bak and anti-Bcl-x_L Abs as described.[16] Ras induced an increase of 2.3 ± 0.24- and 2.8 ± 0.15-fold in Bcl-x_L in RIE-1/H-Ras(61L) and RIE-1/K-Ras 4B(12V) cells, respectively, and a corresponding decrease of 56 ± 16 and 54 ± 12% in Bak. FTS induced a decrease of 44 ± 15 and $33\% \pm 13$ in Bcl-x_L in RIE-1/H-Ras(61L) and RIE-1/K-Ras 4B(12V) cells, respectively

upregulated relative to RIE-1/Neo cells (Figure 1b). This was shown by the amounts of phospho-ERK, phospho-PKB, and Ral-GTP, determined as readouts of the corresponding pathways (Figure 1b). Under anoikis-promoting conditions, FTS induced a decrease in the upregulated phospho-ERK of the RIE-1/Ras cells (Figure 1c(i)). Together, these results suggest that mediation of Ras protection from anoikis in RIE-1/Ras cells might occur through more than one Ras pathway.[6] Nevertheless, downregulation of the activated Ras by FTS (Figure 1c(i)), which occurred rapidly (within 9 h), clearly coincided with the restoration of anoikis sensitivity in the RIE-1/Ras cells.

We next examined the effect of FTS on release of cytochrome *c* from the mitochondria to the cytosol, a well-known mechanism of mitochondria-dependent caspase activation.[7,8] RIE-1/H-Ras(61L) and RIE-1/K-Ras(12V) cells were grown in suspension in the absence and in the presence of 12.5 μM FTS, and the apparent amount of cytochrome *c* in their cytosolic fraction was then determined with anticytochrome *c* antibodies. No cytochrome *c* was detected in the cytosol of control RIE-1/Ras cells, whereas appreciable amounts were detected in the cytosol of the FTS-treated cells 9 and 18 h after the treatment (Figure 1c(ii)). RIE-1/Neo cells, in the absence of FTS, also exhibited cytochrome *c* release, which was detected 3–9 h after detachment (Figure 1c(ii)). Thus, under anoikis-promoting conditions, RIE-1/Ras cells with FTS and RIE-1 cells without FTS appear to share in common cytochrome *c* release. This would be expected to induce activation of caspases. The typical executioner

caspase-3 enzyme was indeed activated in RIE-1/Ras cells undergoing anoikis in the presence of FTS. This was evident from the decrease in procaspase-3 (the caspase-3 precursor) (Figure 1c(iii)) and from the increase in caspase-3-like (DEVDase) activity (Figure 1c(iv)) observed in the FTS-treated RIE-1/Ras cells in suspension. Caspase-3 activation was also evident in RIE-1/Neo cells undergoing anoikis (Figure 1c(iv)). FTS treatment of suspended RIE-1/Ras cells also induced a decrease in procaspase-9 (Figure 1c(iii)), a caspase that is activated in a multimeric complex with Apaf-1 and cytochrome *c*, and activates caspase-3.[7–9] Taken together, these results suggested that FTS releases the RIE-1/Ras cells from the Ras-induced blockade of an apoptotic pathway. It was then of interest to determine whether Ras and FTS have opposing effects on factors that regulate cytochrome *c* release. The typical antiapoptotic Bcl-2 family members Bcl-2 or Bcl-x_L prevent cytochrome *c* release, whereas proapoptotic members such as Bax and Bak promote cytochrome *c* release.[10] We found that the activated H-Ras (61L) or the activated K-Ras (12V) did not alter the expression levels of Bax, but significantly decreased those of Bak (Figure 1d). We could not detect Bcl-2 in RIE-1/Neo or in RIE-1/Ras cells, but detected significant expression of Bcl-x_L (Figure 1d). Bcl-x_L expression was upregulated in the RIE-1/Ras cells (Figure 1d). We next examined whether FTS affects expression of the above-mentioned Bcl-2 family members in RIE-1/Ras cells under anoikis-promoting conditions. We found that the inhibitor had no effect on the expression levels of Bax or Bak, but downregulated the expression levels of Bcl-x_L (Figure 1d). Taken together, the results suggest that in RIE-1/Ras cells FTS and Ras have opposing effects on Bcl-x_L, an antiapoptotic factor known to prevent cytochrome *c* release.[9,10]

The present study showed that the Ras inhibitor FTS cancels resistance to anoikis in Ras-transformed RIE-1/H-Ras(61L) and RIE-1/K-Ras(12V) cells. Without loss of attachment to the matrix, the FTS-treated RIE-1/Ras cells did not die, even though activated Ras was downregulated. Thus, inhibition of Ras in RIE-1/Ras cells is not by itself sufficient to induce cell death. We showed that oncogenic Ras provided survival signals that blocked anoikis in RIE-1/Ras cells, and that FTS, which removed the activated Ras, restored sensitivity to anoikis because these survival signals had disappeared. Indeed, a Ras signal that upregulates the antiapoptotic factor Bcl-x_L in RIE-1/Ras cells was strongly decreased in the FTS-treated RIE-1/Ras cells (Figure 1d). Consistent with the above notion, anoikis of naive RIE-1 cells proceeded, as expected, at a faster pace than that of FTS-treated RIE-1/Ras cells (Figure 1a(i)), which apparently required that Ras and its survival signals first be removed. This is clearly reflected in the much faster activation of the apoptotic apparatus in RIE-1/Neo cells than in FTS-treated RIE-1/Ras cells; in the former, both cytochrome *c* release (Figure 1c(ii)) and the increase in DEVDase activity (Figure 1c(iv)) after detachment from the matrix appeared earlier than in the latter. Evidently, FTS indeed restored sensitivity of the RIE-1/Ras cells to anoikis via a distinctive time-dependent sequence of events: removal of activated Ras and downregulation of the antiapoptotic factor Bcl-x_L within 9 h was followed by release of cytochrome *c* within 9–18 h and a subsequent increase in DEVDase activity within 12–24 h. In RIE-1/Neo cells, which contain high levels of the proapototic factor Bak and low levels of Bcl-x_L (Figure 1d), the release of cytochrome *c* and the increase in DEVDase activity occurred within 3–9 h after detachment from the matrix, even in the absence of the Ras inhibitor. Interestingly, in rat intestinal epithelial IEC-18 cells oncogenic Ras blocks anoikis via signals that prevent downregulation of Bcl-x_L and by signals that downregulate Bak.[11] Thus in both, RIE-1 cells and IEC-18 cells, oncogenic Ras seems to activate pathways that lead to the increase in the amounts of Bcl-x_L, known to prevent cytochrome *c* release and to inhibit anoikis. However, treatment of RIE-1/Ras cells with FTS did not alter Bak expression levels in our study (Figure 1d). This cannot be interpreted to mean that Bak activity is not altered by Ras inhibition, because Bak regulation may occur through conformational changes[9] that are not detectable by the anti-Bak antibody used here. In line with earlier studies,[6] our experiments showed that inhibitors of prominent signaling pathways downstream of Ras had little or no effect on Ras-induced protection from anoikis in RIE-1/Ras cells. The large amounts of phospho-ERK in RIE-1/Ras cells (Figure 1b) might suggest that the Raf/MEK/ERK pathway is needed for protection from anoikis, consistent with the partial anoikis-promoting effect of the MEK inhibitor. Studies have shown, however, that H-Ras(12V) effector domain mutants activating only a single Ras effector fail to block RIE-1 anoikis.[6] Moreover, neither the expression of activated Raf, PI3K, Ral-GDS, Rac or Rho nor coexpression of activated PI3K and Raf could protect RIE-1 cells from anoikis.[6] Taken together, these data point to the possibility that Ras-induced blocking of anoikis in RIE-1/Ras cells might occur through more than one Ras pathway or that unknown effectors mediate the effect. Such a Ras effector pathway would lead to stabilization of Bcl-x_L. This possibility is supported by the demonstration that stabilization of Bcl-x_L was not mediated by PI3-K or by Raf in ICE-18 cells,[11] as well as by the present finding that the Ras-induced increase in Bcl-x_L was inhibited by FTS in RIE-1/Ras cells in which FTS, but not LY294002 or U0126, blocked survival. Finally, it is worth noting that the activated Ras that disables apoptosis of tumor cells also inhibits apoptosis induced by drugs.[4] These activities of Ras are probably related to one other, in line with the knowledge that resistance to apoptosis during tumor development and after drug treatment are very similar in nature.[4] Thus, Ras inhibitors can be expected to promote both apoptosis of malignant cells and an increase in sensitivity to cytotoxic drugs. Recent experiments indeed showed that FTS-treated pancreatic tumor Panc-1 cells and colon carcinoma SW480 cells acquired sensitivity to cytotoxic drugs.[12] These data and the present finding that FTS canceled Ras-induced resistance to anoikis point to the potential use of Ras inhibitors such as FTS to alleviate cancer.

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