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Nuclear targeting of Bax during apoptosis in human colorectal cancer cells

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Homeostasis in colonic epithelial cells is regulated by the balance between proliferative activity and cell loss by apoptosis. Because epithelial cells at the apex of colonic crypts undergo apoptosis and proliferative activity is usually restricted to the base of the crypts, it has been proposed that the limited availability of growth factor-signals at the upper portions of the crypts may trigger apoptosis. In the present studies, we investigate the mechanism of apoptosis mediated by growth factor deprivation in colorectal carcinoma cells by delineating the possible involvement of Bax and its subcellular localization. We report that inhibition of epidermal growth factor receptor (EGFR) tyrosine kinase activity and downregulation of EGFR by anti-EGFR mAb 225 induces apoptosis in human colorectal carcinoma DiFi and FET cells. Induction of apoptosis was preceded by enhanced expression of newly synthesized Bax protein, and required protein synthesis. In the mAb 225-treated cells, Bax was redistributed from the cytosol to the nucleus and subsequently, to the nuclear membranes. The observed induction of Bax expression by mAb 225 was not associated with p53 induction. However, mAb 225 treatment also triggered relocalization of p53 from the cytosol to a nuclear membrane-bound form. Induction of Bax and its redistribution to the nucleus of DiFi cells during apoptosis was also demonstrated in response to butyrate, a physiological relevant molecule in colonic epithelial cells as it is the principal short-chain fatty acid produced by bacterial fermentation of dietary fiber in colonic epithelium. Using immunofluorescence and confocal microscopy, we observed that Bax is predominantly localized in the cytosol, but during apoptosis it is localized both inside and along the nuclear membrane. Taken together, these findings suggest that apoptosis induced by growth factor-deprivation or butyrate may involve the subcellular redistribution of Bax in human colorectal carcinoma cells.

Keywords: Apoptosis; Bax; colorectal cancer; growth factor receptor

Introduction

Homeostasis in colonic epithelial cells is regulated by a balance between proliferative activity at the base of the crypt and cell loss at the apex of the crypt and in the lumen (Gavrieli *et al*., 1992). Apoptosis is a physiological mechanism of cell loss that is dependent on both pre-existing proteins and *de novo* protein synthesis (Reed 1997; Korsmeyer 1995; Boise *et al*., 1995). Apoptosis appears to have an integral role in the normal colon *in vivo* (Hockenbery *et al*., 1991; Krajewski *et al*., 1994), and deregulation of apoptosis may be involved in the development of colorectal cancer (Bedi *et al*., 1995).

Recent studies have established the essential role of growth factors that activate epidermal growth factor receptors (EGFR) in stimulating proliferation of colonic epithelium (Brattain *et al*., 1994). A number of epithelial tumor cells with increased expression of EGFR also produce its ligands, and therefore, leading to enhanced autocrine growth stimulation (Kumar and Mendelsohn 1991; Van de Vijver *et al*., 1991; Atlas *et al*., 1992). EGFR and TGF-alpha are expressed in human colorectal carcinomas (Malden *et al*., 1989) and in a number of colorectal carcinoma-derived human cell lines (Coffey *et al*., 1986; Markowitz *et al*., 1990; Untawalwe *et al*., 1993). In recent years, approaches involving interference with and/or blocking of EGFR-mediated autocrine growth stimulation by anti-EGFR mAbs have been the subject of active investigation in an effort to control cell proliferation (Mendelsohn 1990). Furthermore, growth factors may also contribute towards cell survival since growth factor withdrawal can lead to apoptosis (Oltvai *et al*., 1993; White 1996). There are numerous examples demonstrating that EGF can prevent apoptosis in response to a wide variety of stimuli (Coles *et al*., 1993; Luciano *et al*., 1994; Nakajima *et al*., 1994; Fabregat *et al*., 1996; Garcia-Lloret *et al*., 1996), and inhibition of EGFR expression can induce apoptosis (Englert *et al*., 1995). Since activation of EGFRs may be involved in stimulating colonic epithelium proliferation and in antagonizing apoptosis, one potential mechanism to suppress colorectal cancer may include modulation of apoptotic pathway(s) by inhibiting EGFR using anti-EGFR mAbs.

The occurrence of apoptosis is a highly regulated process. One important regulator of apoptosis is Bcl-2, a 26 kDa protein that protects cells against apoptosis in a variety of experimental systems (Korsmeyer 1995; Mandal and Kumar, 1996; Mandal *et al*., 1996). Bcl-2 protein is primarily localized to the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membranes (Krajewski *et al*., 1993; Lithgow *et al*., 1994). A number of Bcl-2 homologues have been identified, including Bcl-X_L which suppresses apoptosis (Boise *et al*., 1993) and Bax which promotes apoptosis (Oltvai *et al*., 1993). Since Bcl-2 can influence Ca^{++} across membranes (Baffy *et al*., 1993; Lam *et al*., 1994), it has been also proposed that Bcl-2 or its family members may be involved in trafficking of ions (Reed 1994). In an analysis of the three-dimensional structure of Bcl-X_L, Muchmore *et al*. (1996) demonstrated significant

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similarity with the pore-forming domains of bacterial toxins and proposed that Bcl-X_L or its family members may be involved in regulating ion-channels across membranes. In support of this proposal, Schendel *et al*. (1997) demonstrated the ability of recombinant Bcl-2 to form ion channels using a synthetic lipid bilayer-based assay.

Recent studies suggest that cells derived from a variety of human cancers, including colorectal (Bedi *et al*., 1995), have a decreased capacity to undergo apoptosis in response to various physiological stimuli (Carson and Ribeiro, 1993; Thompson 1995). Thus, a defect in apoptosis may be involved in the aberrant survival and/or development of colorectal cancer cells. Colonic epithelial cells at the apex of crypts, where apoptosis is active, display strong Bax immunoreactivity (Krajewski *et al*., 1994). Bax forms homodimers and also heterodimers with anti-apoptotic family members such as Bcl-2, Bcl-X_L, and Mcl-1 (Boise *et al*., 1995). Current models suggest that the ratios of anti- to pro-apoptotic proteins play a regulatory role in apoptosis (Oltvai *et al*., 1993; Sedlak *et al*., 1995). Both Bax and Bcl-2 appear to be regulated independently, as Bcl-2 overexpression does not affect Bax induction (Miyashita *et al*., 1994). The overexpression of Bax has been shown to accelerate apoptosis in response to a variety of stimuli, including growth factor withdrawal (Oltvai *et al*., 1993; Brady *et al*., 1996a).

Earlier we showed that inhibition of EGFR tyrosine phosphorylation by anti-EGFR mAb 225 induces apoptosis in DiFi human colorectal carcinoma cells (Wu *et al*., 1995). In the present study, we investigate the mechanism of EGFR-mediated apoptosis in colorectal carcinoma cells by delineating the involvement of Bax. We report experiments utilizing mAb 225 against the EGFR which blocks binding of ligand and prevents ligand-induced activation of receptor tyrosine kinase (Kawamoto *et al*., 1983). We demonstrate that mAb 225-induced apoptosis in colorectal cancer cells was preceded by enhanced expression of newly synthesized Bax protein, a redistribution of Bax from the cytosol to the nucleus and subsequently to the nuclear membranes. In addition, both induction and redistribution of Bax in colorectal carcinoma cells was also demonstrated by butyrate, a physiological relevant molecule in colonic epithelium.

Results

MAb 225 induces apoptosis in colorectal carcinoma DiFi and FET cells

Results in Figure 1A demonstrate that mAb 225 treatment of DiFi cells inhibited the constitutive activation of EGFR (upper panel) and also reduced EGFR expression (middle panel). As an internal control, the lower portion of the EGFR blot was reprobed with an unrelated anti-Ku80 mAb (lower panel), and there was no effect of mAb 225 on the expression of Ku80. The observed inhibition of EGFR-signaling by mAb 225 was accompanied by induction of apoptosis as determined by a quantitative ELISA-based assay (Figure 1B). To quantitate apoptosis, DiFi cells treated with or without mAb 225 were stained with propidium iodide and analysed by FACS. MAb treatment (24 h) of DiFi cells enhanced the percentage of cells in the pre-Go apoptotic polulation by 36.9% compared to control cells (Figure 1C). Treatment with mAb 225 also caused a reduction in the viability of DiFi cells by 45% compared to control cells in a time-dependent manner (Figure 1D). These results are

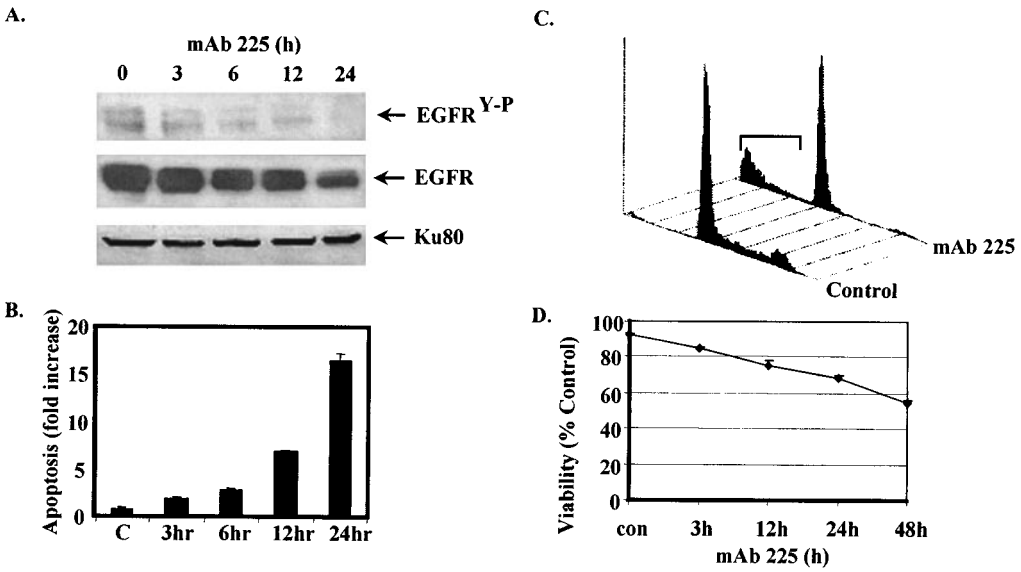

Figure 1 Inhibition of EGFR by mAb 225 induces apoptosis in human colorectal carcinoma cells. (**A**) DiFi cells were cultured with 20 nM mAb 225 for different times, and cell lysates (30 μg protein) were analysed by SDS–PAGE and immunoblotted with an anti-phosphotyrosine mAb PY-20 (upper panel) and then reprobed with an anti-EGFR mAb (middle panel). As an internal control, the lower part of the EGFR blot was immunoblotted with an unrelated anti-Ku80 mAb (lower panel). (**B**) Cell lysates from DiFi cells treated with 20 nM mAb 225 were quantitatively assayed for the induction of apoptosis using the ELISA assay (**C**) FACS profile of DiFi cells treated with 20 nM mAb 225 for 24 h. Bar in mAb treated cells shows apoptotic cells. (**D**) DiFi cells were treated with 20 nM mAb 225 for different times and cellular viability was determined by trypan blue staining ($n=3$). Results shown are representative of three or more independent experiments

consistent with our earlier report that mAb 225-inhibits the growth of DiFi cells (Wu *et al*., 1995).

The observed induction of apoptosis by anti-EGFR mAb 225 was not a restricted phenomenon in DiFi cells with high EGFR ($1.5-2\times10^6$/cell) (Olive *et al*., 1993), as downregulation of EGFR by mAb 225 also induces apoptosis in another colorectal carcinoma FET cells (Figure 2A, upper two blots) which have low EGF binding sites [6.0×10^3, high affinity; 4.26×10^4, low affinity (Huang *et al*., 1992)]. Immunoblotting of the lower portion of the EGFR blot was with an anti-poly(ADP-ribose)polymerase (PARP) mAb revealed its proteolytic cleavage into 85 kD fragment (Figure 2A). The observed PARP cleavage was a specific apoptotic effect of mAb 225, as reprobing the PARP blot with an unrelated anti-Ku80 mAb indicated that there was no effect of mAb 225 on Ku80 expression (Figure 2A, lower blot). Induction of apoptosis in FET cells by mAb 225 was also confirmed by DNA fragmentation (Figure 2B), and 26% increased appearance of pre-Go apoptotic polulation in cells treated for 24 h with mAb 225, compared to untreated cells (Figure 2C). Consistent with these results, mAb 225 also reduced the viability of FET cells by 41% compared to control cells in a time-dependent manner (Figure 2D).

Mab 225-induced apoptosis is accompanied by increased expression of Bax

Since apoptosis is regulated by the ratios between pro- and anti-apoptotic gene product, we hypothesized that mAb 225-induced apoptosis may involve Bax and thus modulate the ratio of Bax to Bcl-2 in favor of cell death. Therefore, we examined the effect of mAb 225 on the expression of Bax in DiFi cells. As shown in Figure 3A, treatment of DiFi cells with mAb 225 induced increased levels of Bax in a time-dependent manner starting at 3 h post-treatment. As an internal control, the upper portion of the Bax blot was blotted with an unrelated mAb against heat-shock protein-70 (Figure 3A). Quantitation of Bax induction in relation to HSP-70 expression indicated that there was seven fold increase in the expression of Bax in cells treated with mAb 225 for 12 h, compared to untreated cells (Figure 3B). To further characterize the induction of Bax in mAb 225-treated cells, we examined the synthesis of Bax protein. DiFi cells were labeled with ^{35}S-methionine for the last 3 h before preparing cell extracts. MAb 225 treatment resulted in the increased expression of newly synthesized Bax within 3 h of treatment (Figure 3C).

Since apoptosis in other systems has been shown to be protein synthesis-dependent (Brady *et al*. 1996b), we

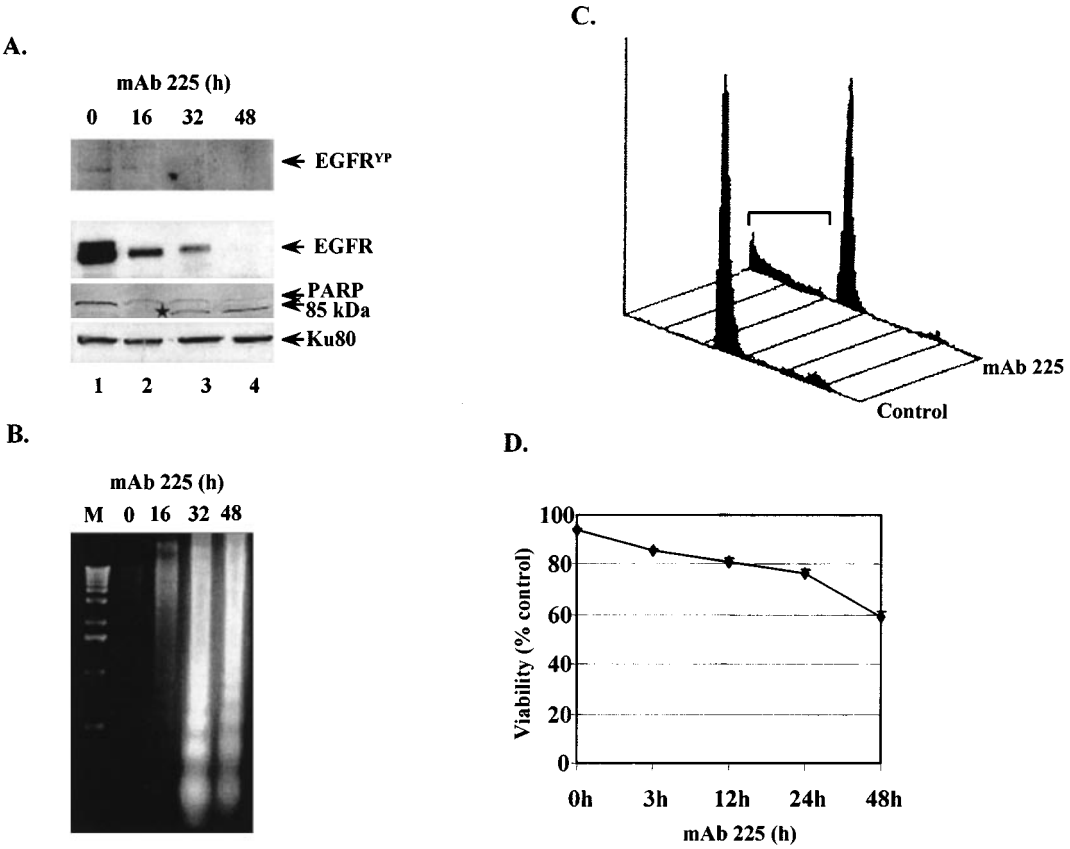

Figure 2 (**A**) FET cells were cultured with 20 nM mAb 225 for different times, and cell lysates (30 μg protein) were analysed by SDS–PAGE and immunoblotted with an anti-phosphotyrosine mAb PY-20 (upper panel) and then reprobed with an anti-EGFR mAb (second panel). As an internal control, the lower part of the EGFR blot was immunoblotted with an anti-PARP mAb (third panel) and then reprobed with an unrelated anti-Ku80 mAb (fourth panel). (**B**) Induction of DNA-fragmentation in FET cells by 20 nM mAb 225. (**C**) FACS profile of DiFi cells treated with 20 nM mAb 225 for 24 h. Bar in mAb treated cells shows apoptotic cells. (**D**) DiFi cells were treated with 20 nM mAb 225 for different times and cellular viability was determined by trypan blue staining ($n=3$). Results shown are representative of three or more independent experiments

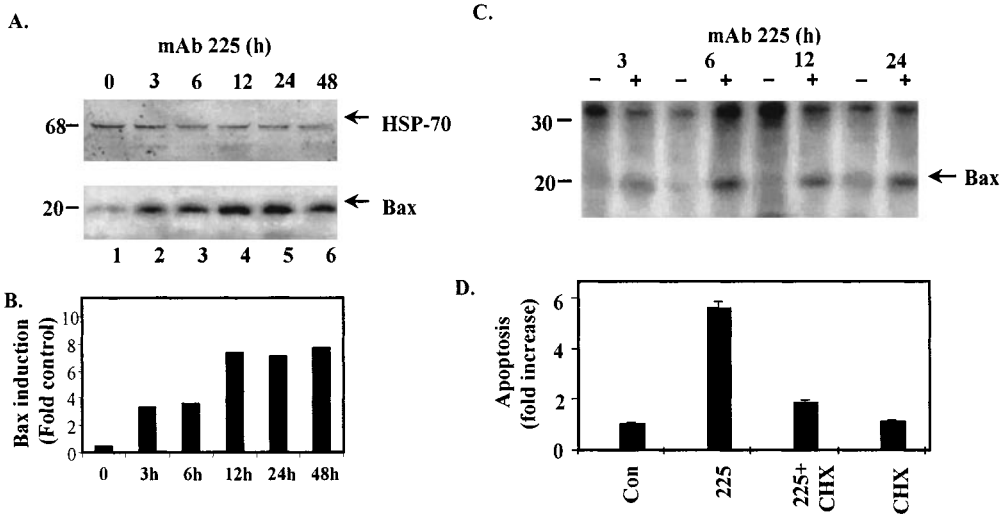

Figure 3 MAb 225 induces expression of Bax. (**A**) DiFi cells in culture were treated with 20 nM mAb 225 for the indicated times. Cell extracts (30 μg protein) were analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab (lower panel). As an internal control, the upper portion of the Bax blot was blotted with an unrelated anti-HSP70 mAb (upper panel). (**B**) Ratios of HSP-70 to Bax bands are presented in the lower panel. (**C**) DiFi cells were treated with (+) or without (−) 20 nM mAb 225 for the indicated times and metabolically labeled with ^{35}S-methionine for the final 3 h. Lysates containing equal amount of TCA perceptible c.p.m. were immunoprecipitated with an anti-Bax Ab and analysed by SDS–PAGE. (**D**) DiFi cells were treated with mAb 225 with or without 30 μg cycloheximide (CHX) per ml for 16 h. Cell lysates were assayed for the induction of apoptosis. The induction of Bax was observed in five separate experiments

investigated whether mAb 225-mediated apoptosis require on-going protein synthesis. DiFi cells were treated with mAb 225 in the presence or absence of cycloheximide, and cell extracts were assayed for the induction of apoptosis. As shown in Figure 3D, exposure of DiFi cells to cycloheximide prevented mAb 225-induced apoptosis. As expected, cycloheximide treatment of DiFi cells also prevented the mAb 225-induced increase in Bax expression (data not shown).

MAb 225 and butyrate trigger nuclear translocation of Bax

Bcl-2 family members have been proposed to be involved in membrane functions (Reed 1994; Schendel *et al*., 1997). Since very little is known about the subcellular localization of Bax under growth-factor receptor-mediated apoptosis in colorectal carcinoma cells, we next explored the distribution of Bax in DiFi cells triggered to undergo apoptosis by the inhibition of EGFR. DiFi cells were treated with mAb 225 for varying lengths of time, soluble and membrane-bound fractions were prepared from the cytosolic and nuclear departments, and immunoblotted with an anti-Bax Ab or with an unrelated anti-Ku80 mAb (Figure 4B). Results in Figure 4A demonstrated that mAb 225-induced Bax exists predominantly in the soluble form in both the cytosolic and, to a lesser extent, the nuclear compartments. The nuclear accumulation of Bax increased over a period of 24 h. The loss of soluble nuclear Bax at 48 h after mAb225 treatment was accompanied by an increase in the membrane-bound form (Figure 4C, lane 10). Quantitation of Bax expression indicated that about 29–37% of total

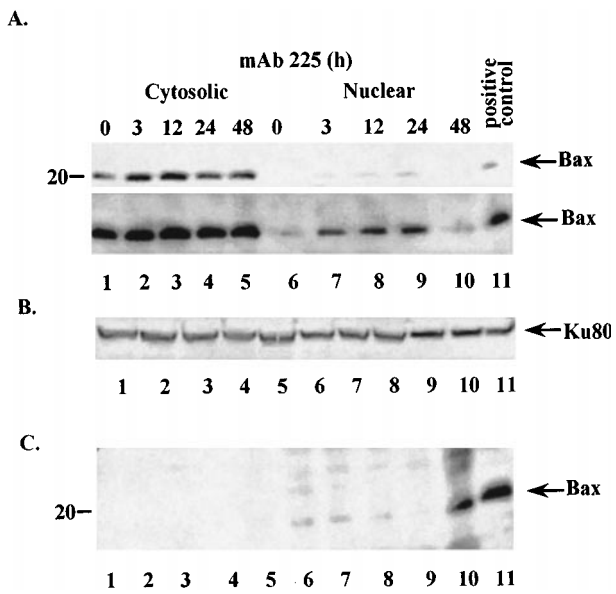

Figure 4 MAb 225 triggers nuclear translocation of Bax in colorectal carcinoma DiFi cells. (**A**) DiFi cells were treated with 20 nM mAb 225 for the indicated times, and subjected to the subcellular fractionation as described in the Materials and methods. Soluble cytosolic and nuclear fractions were analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. Lane 11, Bax positive control. The lower panel is an overexposed autorad of the upper panel. (**B**) Cell lysates from the above experiment was immunoblotted with an unrelated anti-Ku80 Ab. (**C**) Membranes from the cytosol and nuclear fraction were solubilized and analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. The purity of the nuclear and cytosolic extracts was confirmed by immunoblotting the control extracts with nuclear enzyme PARP or cytosolic Caspase-3 (data not shown). Similar results were obtained in three independent experiments

induced Bax was translocated to the nuclear compartment in mAb 225-treated cells. Results in Figure 5A indicate that the observed induction of proapoptotic Bax protein was not a restricted effect of mAb 225 in DiFi cells, as mAb 225 could effectively induce and translocate Bax in FET cells which were also sensitive to apoptosis by mAb 225. The purity of the cytoplasmic and nuclear fraction was routinely determined by immunoblotting the control extracts with the nuclear proteins such as DNA-PK or PARP, and with cytoplasmic protein such as CPP32 (Figure 5C). To further explore the generality of our finding, we next examined the effect of butyrate, a physiological relevant molecule in colonic epithelial cells (Cumming, 1981), and known to induce apoptosis in DiFi cells (Mandal *et al*., 1997), on the expression of Bax. As illustrated in Figure 6, butyrate induced the expression of Bax as early as 6 h post-treatment, and this was accompanied by subsequent increase in the Bax accumulation in the nuclear compartment and subsequently (Panel A), to the nuclear membranes (Panels B).

To further characterize the subcellular localization of Bax in mAb 225-treated cells, we next examined the

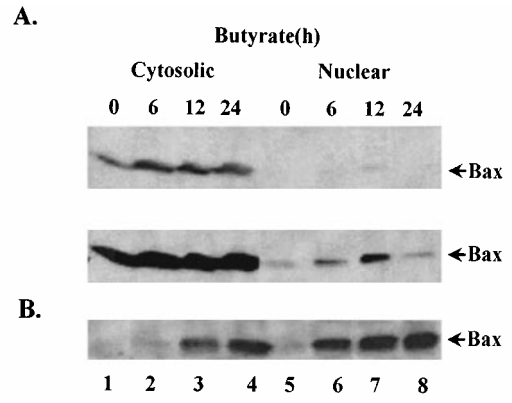

Figure 6 Butyrate induces and triggers nuclear translocation of Bax in DiFi cells. (**A**) Cells were treated with 3 mM butyrate for the indicated times, and subjected to the subcellular fractionation as described in the Materials and methods. Soluble cytosolic and nuclear fractions were analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. The lower panel is an overexposed autorad of the upper panel. (**B**) Membranes from the cytosol and nuclear fraction were solubilized and analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. These experiments were repeated two times with similar results

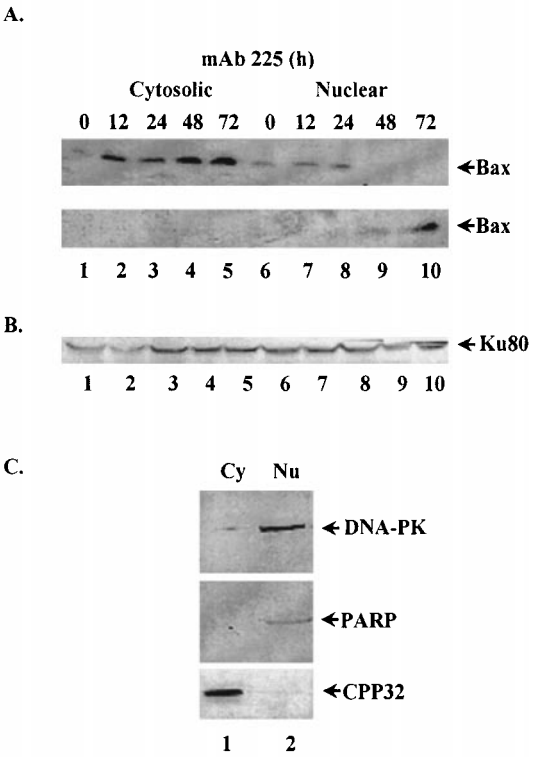

Figure 5 MAb 225 induces the expression of Bax and its translocation to the nucleus in colorectal carcinoma FET cells. (**A**) cells were treated with 20 nM mAb 225 for the indicated times, and subjected to the subcellular fractionation as described in the Materials and methods. Soluble cytosolic and nuclear fractions were analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. Lower panel, Membranes from the cytosol and nuclear fraction were solubilized and analysed by SDS–PAGE and immunoblotted with an anti-Bax Ab. (**B**) Cell lysates from the above experiment was immunoblotted with an unrelated anti-Ku80 Ab. (**C**) The purity of the nuclear and cytosolic extracts was confirmed by immunoblotting the control extracts with antibodies against nuclear enzymes such as PARP or DNA-PK, and cytosolic CPP32. Similar results were obtained in two times

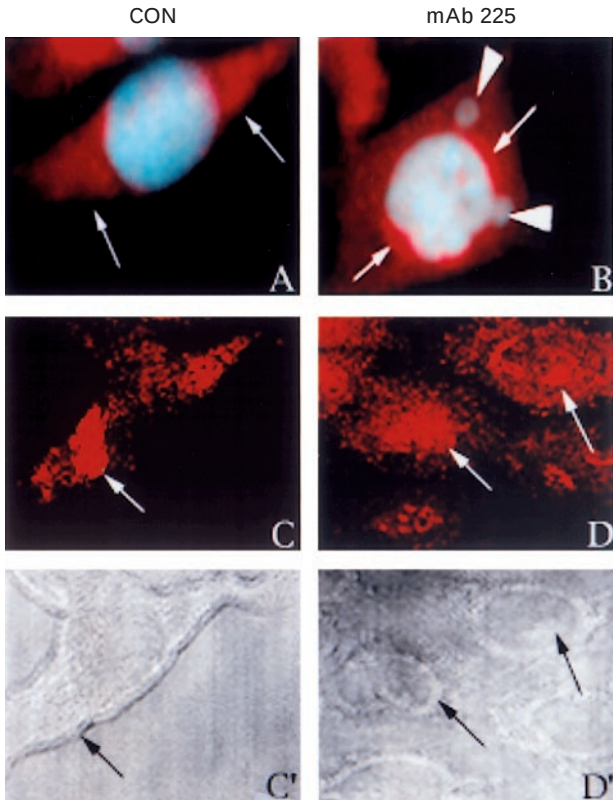

Figure 7 Subcellular localization of Bax by immunofluorescence and confocal imaging. (**A** and **B**) Immunofluorescence. (**A**) Bax-staining (red) in control, untreated DiFi cells. (**B**) Bax-staining in DiFi cells treated with 20 nM mAb 225 for 6 h. Arrows, Bax; Arrowheads, nuclear membrane blebbing. (**C** and **D**) confocal imaging. DiFi cells were treated with 30 nM mAb 225 (Panels D and D′) or without mAb (Panels C and C′) for 12 h. White arrows, Bax; and black arrows, Bax location in the same field under transmission mode. Note that treatment of DiFi cells with mAb 225 resulted in the nuclear immunostaining of Bax (compare panel D with panel C). These experiments were repeated two times

redistribution of the Bax protein by indirect immunofluorescence. For identification of nuclei, we used the DNA intercalating agent DAPi (blue staining). The Bax protein was detected using a TRITC-coupled secondary antibody against the polyclonal anti-Bax antibody (red staining). The Bax protein was primarily localized in the cytoplasm in DiFi cells (Figure 7A). The observed Bax staining was specific, as prior incubation of anti-Bax Ab with the peptide against which it was raised, completely blocked Bax-associated immunofluorescence and incubation with the secondary antibody alone showed a very low background (data not shown). Following the apoptotic signal, Bax translocated around the nucleus as shown by its intense perinuclear accumulation except at the points of nuclear membrane blebbing (Figure 7B). To confirm the results of immunofluorescence studies, we next examined the effect of mAb 225 on the distribution of Bax in DiFi cells by confocal microscopy. The results in Figure 7C demonstrate that Bax was primarily localized in the cytosol in DiFi (Panel C is the corresponding transmitted mode photograph). In mAb 225-treated DiFi cells, the Bax-immunostaining was intensely localized both around and inside (shown by white arrows in Panel D) the nucleus. Taken together, our results demonstrated that mAb 225-induced apoptosis was accompanied by alteration in the subcellular distribution of Bax.

Effect of mAb 225 on the expression and subcellular localization of p53

Since Bax has been shown to be induced by p53, we next examined whether the observed induction of Bax in mAb 225-treated DiFi cells was dependent on p53. As shown in Figure 8A, there was no induction of p53 by mAb 225, and the levels of p53 were actually reduced in 225-treated cells. Data in Figure 8B illustratate that DiFi cells have both wild-type and mutated p53 as mAbs specific for total p53 (D0-1 mAb, lanes 1 and 2) and mutated p53 (240 mAb, lanes 3 and 4) effectively immunoprecipitated p53 from ^{35}S-labeled cell extracts. Similar results were obtained when unlabeled cell extracts were immunoblotted with anti-p53 mAbs after immunoprecipitation with DO-1 or 240 mAbs (data not shown). The data in Figure 8B also confirmed that mAb 225-treatment reduced the levels of p53 (Figure 8A). To understand the basis of the reduced levels of p53 in mAb 225-treated cells, we hypothesized that mAb 225-induced apoptosis may involve redistribution of subcellular localization of p53. Next, we examined the distribution of p53 in soluble and membrane fractions prepared from the cytosolic and nuclear compartments. Soluble p53 was found localized primarily in the nucleus with some cytosolic localization, and mAb 225 treatment resulted in a modest time-dependent decrease in the levels of soluble

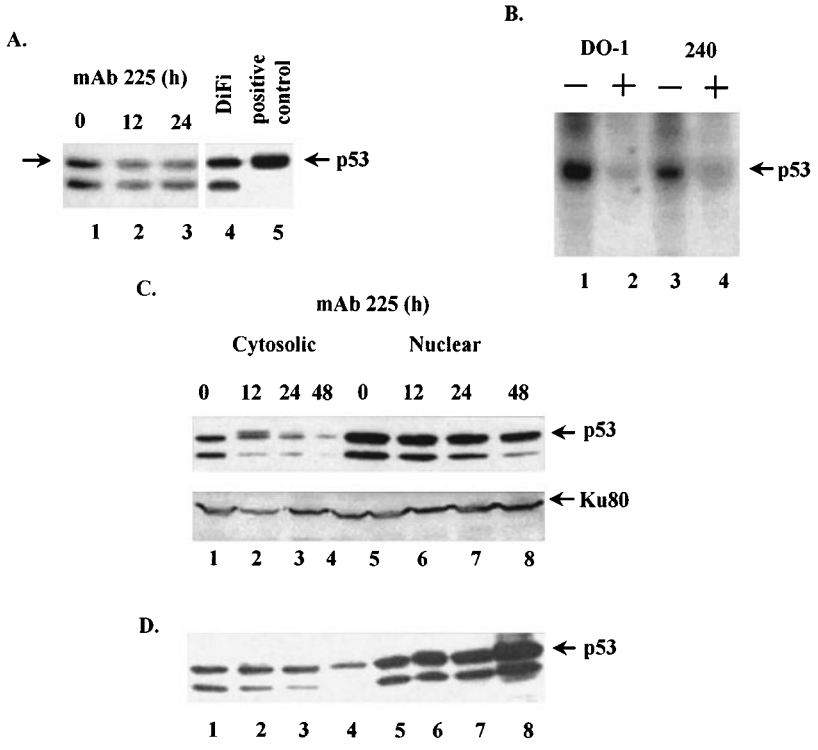

Figure 8 Effect of mAb 225 on the expression and subcellular localization of p53. (**A**) DiFi cells were treated with 20 nM mAb 225 for 24 h (lane 3), 12 h (lane 2), or 0 h (lane 1), and cell extracts were analysed by SDS–PAGE and immunoblotted with an anti-p53 mAb (clone DO-1) that recognizes the total (wild-type and mutated) p53. The DO-1 mAb used here immunoreacted with an additional lower band in DiFi cell extract (lane 4) compared to positive control (lane 5). (**B**) Lysates from DiFi cells metabolically labeled with ^{35}S-methionine and treated with (+) or without (−) 20 nM mAb 225 were immunoprecipitated with either anti-p53 D0-1 mAb (lanes 1 and 2) or anti-p53 240 mAb (lanes 3 and 4) and analysed by SDS–PAGE. An autoradiograph of a dried gel is shown here. (**C**) DiFi cells were treated with 20 nM mAb 225 for the indicated times, and subjected to the subcellular fractionation as described in the Materials and methods. Soluble cytosolic and nuclear fractions were analysed by SDS–PAGE and immunoblotted with an anti-p53 mAb DO-1. Lower panel, cell lysates from the above experiment were immunoblotted with an unrelated anti-Ku80 Ab. (**D**) Crude membranes from the cytosol and nuclear fraction were solubilized by SDS–PAGE and immunoblotted with an anti-p53 mAb D0-1. Results shown were repeated three times

p53 in both cytosolic and nuclear fractions (Figure 8C). In contrast, there was a significant time-dependent increase in the levels of membrane-bound p53 in the nuclei of mAb-treated cells (Figure 8D). Studies of p53 localization have shown it to be present in both the cytosol and the nucleus, and translocation both inside and outside of the nucleus can occur following different stimuli (Ostermeyer *et al*., 1996; Middeler *et al*., 1997). Comparison of the kinetics of p53 translocation with that of newly synthesized Bax in mAb 225-treated cells (Figure 3B) indicated that induction of Bax was an early effect and p53 translocation was a late response of mAb 225. In brief, although mAb 225-treatment did not induce increased p53 expression, it is possible that the observed redistribution of p53 from the soluble cytosolic form to the nucleus could constitute an apoptotic signal.

Discussion

To understand the mechanism of EGFR-induced apoptosis in human colorectal carcinoma cells, we investigated the effect of inhibition of EGFR-signaling by mAb 225 in DiFi and FET cells by examining the role of Bax pathway. It was observed that mAb 225-induced apoptosis was accompanied by the induction of expression of newly synthesized Bax. The mechanism of observed induction of increased Bax protein remains to be delineated. This may occur at the transcriptional level and/or post-transcriptional level and may involve enhanced stability of Bax protein. Our finding that mAb 225-mediated apoptosis in DiFi cells was accompanied by alteration in the subcellular distribution of Bax, is important, as it suggests that Bax redistribution may play a role in apoptosis. We have demonstrated that Bax is predominantly localized in the cytoplasm, but following the inhibition of EGFR-signaling by mAb 225, it translocates to the nucleus and subsequently to the nuclear membrane. This finding is in agreement with a recent report by Hsu *et al*. (1997) (who reported their results while this study was in progress), demonstrating the cytosolic-to-membrane redistribution of Bax during dexamethasone or gamma-irradiation-induced apoptosis in murine thymocytes. Furthermore, in addition to biochemical methods of cell fractionation, we have also used double-immunofluorescence and confocal microscopic methods to document the *in situ* localization of Bax. We have now demonstrated that following an apoptotic signal, a significant amount of Bax accumulates both inside and around the nucleus. The observed induction expression of Bax and its translocation to the nucleus during apoptosis was not an event limited to DiFi cells, as induction of apoptosis in FET cells by mAb 225 or induction of apoptosis in DiFi cells by butyrate (a physiological relevant molecule in colonic epithelial cells) was also accompanied by increased expression and redistribution of Bax to the nucleus. At the moment, we do not know the physiological significance of our finding. In this context, it is important to note that on the basis of structural similarity between the alpha-helical models of Bcl-X_L and diphtheria toxin, it has been proposed that Bcl-X_L and its family members may be involved in regulating the ion-channels across membranes (Muchmore *et al*., 1996; Schendel *et al*., 1997). In the same vein, it is possible that the observed intense localization of Bax around the nucleus may influence the ion-channels across the nuclear membrane in a manner that promotes apoptosis.

Another notable finding in this study was the redistribution of p53 from the soluble to the membrane bound form in the nucleus during apoptosis. Our results showed that the increased expression of Bax in mAb 225-treated DiFi cells was not related to enhanced expression of p53. In contrast, there was a time-dependent-decrease in the level of soluble p53. Inspite of the fact that p53 is a nuclear protein, there is considerable evidence that it also is present in the cytosol, and that p53 can be translocated both inside and outside of the nucleus following different stimuli (Ostermeyer *et al*., 1996; Middeler *et al*., 1997). It also has been shown that mutant p53 accumulates in the nucleus and wild-type p53 translocates to the nucleus. Since DiFi cells have both wild-type and mutant p53 (Figure 5B), it is possible that the observed translocation of p53 from the cytosol to the nucleus may be due to wild-type p53. The possible role of the observed redistribution of p53 from the soluble to the membrane bound and its potential role in mAb 225-mediated apoptosis remains to be established.

Data from the literature indicated that Bax lacks the nuclear localization sequence but has a putative carboxyl-terminal transmembrane region (Oltvai *et al*., 1993), implying that it has the potential to insert into membranes as demonstrated here in DiFi cells undergoing apoptosis following blockade of EGF receptor tyrosine kinase. Taken together our observations raise new questions: (i) why Bax exists in the soluble form, inspite of the presence of a c-terminal hydrophobic region; (ii) how Bax translocates to the nucleus; and (iii) whether Bax redistribution depends on its phosphorylation status or on the phosphorylation of other proteins. Regardless of the mechanism of Bax redistribution, our results presented here have clearly demonstrated that induction of apoptosis in human colorectal carcinoma cells by either inhibition of EGFR function or butyrate may involve the subcellular redistribution of Bax in human colorectal carcinoma cells.

Materials and methods

Cell cultures, cell extracts and immunoblotting

Human colorectal carcinoma DiFi cells (Olive *et al*., 1993; Wu *et al*., 1995; Mandal *et al*., 1997; Mandel and Kumar, 1997) and FET cells (Huang *et al*., 1992) were maintained in DMEM-F12 (1 : 1) supplemented with 10% fetal calf serum. All experiments were performed with cells in logarithmic phase by controling the plating density. Cell lysates (30 μg protein) were resolved on a 7% or 10% SDS–PAGE followed by probing with the desired antibodies, and immune complexes were detected by using an ECL method. The Bax antibody and its peptide were obtained from the Santa Cruz. Anti-p53 mAbs (DO-1 and 240) were obtained from the Neomarkers.

Metabolic labeling with ^{35}S-methionine

An equal number of cells were treated with or without mAb 225 for varying periods of time, followed by

metabolic labeling with 100 μCi/ml ^{35}S-methionine in methionine-free medium containing 2% FBS during the last 3 h of culture. Cell extracts containing an equal amount of total trichloroacetic acid perceptible counts were immunoprecipitated with the desired mAb, resolved on a SDS-polyacrylamide gel, and analysed by autoradiography (Kumar *et al*., 1991; Fan *et al*., 1993).

Apoptosis assays

Apoptotic cell death was quantitated by an ELISA assay (Boehringer) that measures cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptotic DNA fragmentation, and not free histone or DNA that could be released during nonapoptotic cell death (Mandal and Kumar, 1996; Mandal *et al*., 1997; Leist *et al*., 1997; Szabo *et al*., 1997; Dimmeler *et al*., 1997). After the desired treatment, cytoplasmic extracts were made from both floating and attached cells according to the manufacturer's protocol. Briefly, wells were first coated with anti-histone antibody, loaded with cytoplasmic extracts, and followed by incubation with anti-DNA second antibody conjugated with peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) as a substrate.

DNA fragmentation assay

For DNA fragmentation assay, low molecular weight DNA was isolated. Briefly, cells (3×10^6/plate) were seeded in 100 mm plates, and treated as desired. Both floating and attached cells were scraped and collected in media, washed $3\times$ with PBS, and resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; and 0.5% Triton X-100). After 30 min incubation on ice, the lysates were centrifuged at 12 000 r.p.m. (Eppendorf) for 10 min. Low molecular weight DNA in the supernatant was extracted with an equal volume of phenol/chloroform for 1 h at 4°C. Ammonium acetate (2 M) was added to the aqueous phase and DNA was precipitated with 2 vol of ethanol at −20°C overnight. DNA was treated with RNase A (1 mg/ml) at 37°C for 1 h and total DNA was analysed using 1.5% agarose gel, and visualized by ethidium bromide staining of gel (Mandal *et al*., 1996).

Cell cycle analysis

The percentage of cells in different phases of the cell cycle will be determined by staining with propidium iodide (Sigma) and passed through the beam of an argon ion laser turned to 514 (FAC/Scan; Becton Dickinson) as described (Mandal and Kumar, 1997).

Subcellular fractionation

Cytosolic and nuclear extracts were prepared as described (Kumar and Korutla, 1995; Mandal *et al*., 1996). Briefly, cells were washed with phosphate-buffered saline, and pelleted by centrifugation. Cells were incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride) and homogenized in a loose fitting glass homogenizer with 15 strokes. Nuclei were separated from the cytosolic fraction by centrifugation at 5000 r.p.m. for 5 min at 4°C. The cytosolic fraction was centrifuged at 40 000 r.p.m. for 30 min to pellet the crude membranes. Nuclei were washed and resuspended in buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGDA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride), lysed with vigorously shaking for 20 min, and centrifuged at 15 000 r.p.m. for 30 min to collect the soluble nuclear fraction. Crude cytosolic and nuclear membranes were resuspended in a volume of lysis buffer equal to that used to resuspend the cells and nuclei. The purity of the nuclear and cytoplasmic extracts was assessed by immunoblotting the control extracts with mAbs against the nuclear enzymes such as PARP and DNA-PK, or against CPP-32 which is predominantly localized in the cytoplasm (Krajewska *et al*., 1997; Posmantur *et al*., 1997; Krajewski *et al*., 1997).

Immunofluorescence and confocal studies

Cellular localization of Bax and p53 was determined using indirect immunofluorescence (Martin *et al*., 1987) with some modifications (Bandyopadhyay *et al*., 1998). Briefly, cells grown on glass coverslips were fixed in methanol at −20°C for 10 min. Several dilutions of each antibody were used to obtain the optimal results. DNA was stained with DAPi (blue). For staining of Bax, DiFi cells were treated with anti-Bax antibody followed by TRITC-labeled secondary antibody (Molecular Probes). For controls, cells were treated only with secondary antibody, omitting the primary antibody, and no signals were detected. In addition, no signals were observed after addition of the anti-Bax antibody in the presence of Bax peptide. Confocal microscopy was performed using a Zeiss laser scanning confocal microscope. Each image represents Z-sections at the same cellular level and magnification. In some of the cases shown here, the transmission mode was also used on the same microscopic field to visualize the entire cell.

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