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# Fas-Signaling and Effects on Receptor Tyrosine Kinase Signal Transduction in Human Breast Epithelial Cells

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**Fas-mediated cell death was examined in MCF-10AT preneoplastic human breast epithelial cells. Treatment with anti-Fas for 48 h induced apoptosis with cells exhibiting typical apoptotic features including loss of cell contact, condensation of chromatin, and increased staining of the nuclear membrane. DNA fragmentation occurred in response to anti-Fas treatment. Anti-Fas treatment resulted in decreased p53 protein levels, while bcl-2 and bax protein levels remained unaffected. Cells treated with anti-Fas also exhibited increased tyrosine phosphorylation of the c-met growth factor receptor tyrosine kinase. Immunoprecipitation experiments demonstrated that Fas associated with c-erbB2 and c-met in untreated cells. Treatment with anti-Fas, however, significantly decreased Fas-c-erbB2 and Fas-c-met association. Anti-Fas treatment of these cells caused a significant decrease in p120-GAP levels, ERK-1 levels, and phosphorylation, as well as Grb2-Sos1 and MEK-1-ERK-1 association. These results show that Fas-signaling exerted a suppressive effect on p53 levels and on downstream effectors of receptor tyrosine kinase signal transduction, thereby ensuring cell death.** 

Cancer is a disease of dysregulation of cell growth. Growth of a cell population is determined by a balance between an increase in cell number resulting from cell proliferation and a decrease in cell number which occurs as a result of cell death. Apoptosis, which is essential for the development and homeostasis of multicellular organisms, is characterized by morphologic alterations such as cell shrinkage, loss of cell-cell contact and membrane blebbing, and is usually accompanied by chromatin condensation and DNA fragmentation produced by endonucleases. Fas-mediated apoptosis plays an important regulatory role in the elimination of cells *in vivo.* Fas-signaling may be induced by ligation of the Fas receptors by Fas-ligand or by anti-Fas antibodies. Many tumor cells are capable of escaping Fas-mediated cell death by altering Fas receptor or Fas ligand expression (1). To date, a majority of research on Fas-mediated apoptosis has been focused towards hematopoietic cells (2-5); substantially less effort has been directed towards research on adherent cells and solid tumor cells, although Fas is expressed in a variety of tissues including liver, skin and breast (6-8). The cellular signaling pathways which lead to apoptosis remain the topic of intense investigation. In this context, even less is known about the interaction/relationship between Fas-signaling and protein tyrosine kinase signaling pathways, though early PTK activation has been suggested as playing an important role in Fas-signaling (9).

We examined Fas-mediated apoptosis in human breast epithelial cell and the effects of Fas-signaling/Fas-induced apoptosis on signal transduction effectors of the receptor tyrosine kinases (RTK). We report that Fas-mediated apoptosis occurs in MCF-10AT human breast epithelial cells, and that Fas-signaling stimulates c-met RTK tyrosine phosphorylation. In contrast, anti-Fas treatment suppresses the downstream effectors of the RTK signaling pathway, such as Grb2-Sos1 association, p120-GAP levels, MEK-1-ERK-1 association, ERK-1 levels and ERK-1 phosphorylation.

## MATERIALS AND METHODS

*Cell lines.* The MCF-10AT cell line is derived from MCF-10A cell line, which is a spontaneously immortalized, untransformed human breast epithelial cell line obtained from a patient with mild fibrocystic disease. MCF-10AT cells are MCF-10A cells that have been stably transfected with $T_{24}$ mutated c-Ha-ras and neomycin resistance gene. When transplanted into nude/beige mice, MCF-10AT cells are able to form persistent preneoplastic lesions, of which ~12-15% progress to carcinoma (10).

*Cell culture and chemical treatment.* MCF-10AT cells were cultured in DMEM/F12 media containing 10 $\mu$g/ml of human insulin,

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Abbreviations: RTK, receptor tyrosine kinase; PTK, protein tyrosine kinase; Sos, son of sevenless; GAP, GTPase activator protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-receptor kinase; MEK, MAPK/ERK kinase; PAGE, polyacrylamide gel electrophoresis; SH, src homology.



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20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 $\mu$g/ml hydrocortisone, 5% horse serum and 100 units/100 $\mu$g penicillin/streptomycin (GIBCO), to 100% confluence. Cells were grown to confluency and then treated with 200 ng/ml non-specific antibody (goat-anti-rat polyclonal IgG) or anti-Fas polyclonal antibody (Santa Cruz) for 48 h and analyzed for morphologic alterations, DNA fragmentation, protein expression, or association between signaling components using immunoprecipitation.

*Acridine orange staining.* Cells were washed with PBS three times and fixed with 100% cold methanol for 20 minutes at 4°C. Cells were washed 3 times with PBS, and stained with 0.005% acridine orange with gentle rocking for 3 to 5 minutes. Cells were then washed 3 times with PBS, and examined by fluorescence microscopy using a Nikon epifluorescence diaphot microscope equipped with 488 nm excitation filter.

*DNA fragmentation analysis.* $10^6$ cells were lysed in lysis buffer (0.5% Triton X-100, 5 mM Tris, pH 7.4, 5 mM EDTA) at 4°C for 30 minutes and the cell lysates were centrifuged at 27,000 $\times$g for 15 minutes. The supernatant was phenol-chloroform extracted three times and precipitated with 10.5 M ammonium acetate and 100% ethanol. Nucleic acids were centrifuged at 13,000 $\times$g for 15 minutes, and the pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), followed by RNAse (0.5 mg/ml) digestion at 37°C for 2 h. DNA was separated on a 1% agarose gel and examined by ethidium bromide staining.

*Immunoprecipitation.* Equal amounts of whole cell lysate (200 $\mu$g to 1 mg) were incubated with 1 $\mu$g/ml of PY20 anti-phosphotyrosine antibody (Transduction Laboratories) or monoclonal mouse anti-GAP $IgG_{2a}$ antibody (ICN), or rabbit anti-Grb2 polyclonal antibody (Santa Cruz Biotechnology), or rabbit anti-Fas polyclonal antibodies, or rabbit anti-ERK-1 polyclonal antibodies (Santa Cruz Biotechnology) in lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM $MnCl_2$, 100 mM ATP, 10 $\mu$g/ml leupeptin, 2 mM phenylmethyl sulfonyl fluoride, 200 units of aprotinin) at 4°C for 2 h. Protein A-sepharose was added and the samples were allowed to incubate for 1 h. The samples were centrifuged at 10,000 $\times$g and washed with lysis buffer 3 times and centrifuged. The pellet was resuspended in Western blot loading buffer (125 mM Tris-HCl, 2% SDS, 5% $\beta$-mercaptoethanol, 20% glycerol, 0.0025% bromophenol blue) and heated at 95°C for 5 min and centrifuged. The supernatant was loaded onto a 4 to 20% or a 5 to 10% gradient SDS-PAGE gel for Western blot analysis. To examine for protein association, immunoprecipitation with an antibody against one protein was performed, followed by Western blot analysis using an antibody against the associated protein.

*Western blot analysis.* Whole cell lysate (20 to 100 $\mu$g) or immunoprecipitate was separated using 5 to 10% or 5 to 20% gradient SDS-PAGE gel and transferred to a nitrocellulose membrane. Both membrane and gel were stained to check for even loading and transfer of protein. The membrane was blocked in PBST (1$\times$ PBS, 0.05% Tween 20), 5% nonfat dry milk at room temperature for 1 h, washed twice with PBST, and hybridized with 0.5 to 1 $\mu$g/ml of primary antibody in PBST containing 0.5% nonfat dry milk at room temperature for 1 to 16 h. The blots were washed twice with PBST and incubated with a 1:3000 to 1:5000 dilution of secondary antibody in PBST, 5% non-fat dry milk for 1 h, washed three times with PBST, and exposed and developed using the enhanced chemiluminescence (ECL) technique (Amersham).

## RESULTS AND DISCUSSION

Treatment of the MCF-10AT cells with anti-Fas antibody for 48 h caused a significant proportion (>90%) of cells to exhibit apoptotic features. Untreated cells and cells treated with non-specific antibody (goat-anti-rat IgG) maintained cell-cell contact, and a hexagonal cobble-stone like epithelial cell morphology. The nucleus of these cells stained evenly with acridine orange (Figs. 1A and 1B). In contrast, over 90% of the cells treated with anti-Fas showed a complete loss of cell-cell boundaries, in addition to the loss of the hexagonal cobble stone-like epithelial cell morphology. These morphologic changes were accompanied by increased staining of the nuclear membrane and uneven acridine orange staining of the nucleus, suggesting chromatin fragmentation (Fig. 1C). Anti-Fas treatment caused DNA fragmentation in these cells (Fig. 1D, lane 4), confirming the occurrence of apoptosis in anti-Fas treated cells and suggesting that activation of endonucleases occurred in response to anti-Fas treatment of these cells. In cells treated with non-specific antibody (goat-anti-rat IgG), no evidence of DNA fragmentation was detected (Fig. 1D, lane 3).

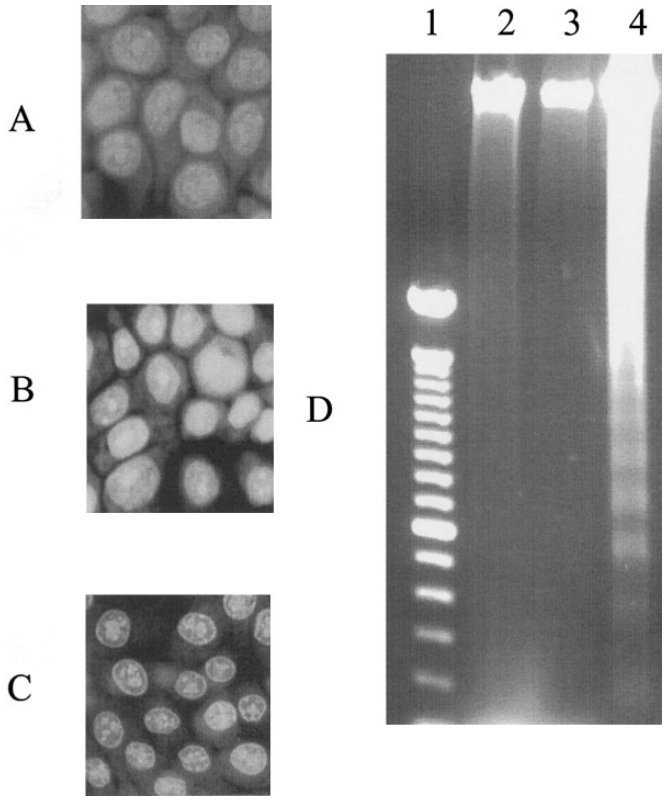


**FIG. 1.** Anti-Fas treatment induced apoptosis of MCF-10AT cells. MCF-10AT cells were grown to confluency, then left untreated or treated with 200 ng/ml of non-specific antibody (goat-anti-rat IgGs) or anti-Fas antibody for 48 h. Cells were stained with acridine orange to examine changes in nuclear morphology, or DNA was extracted to examine DNA fragmentation. (A) Untreated cells; (B) non-specific antibody treated cells; (C) anti-Fas treated cells; (D) DNA fragmentation analysis; lane 1, 100 bp DNA ladder; lane 2, untreated cells; lane 3, non-specific antibody (goat anti-rat IgG) treated cells; lane 4, anti-Fas treated cells.

To investigate further the mechanism of Fas-medi-

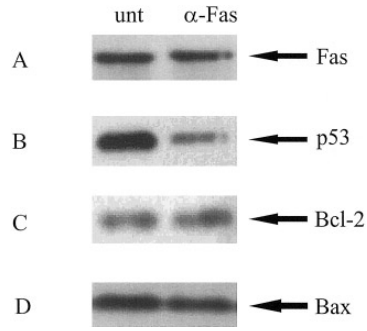


**FIG. 2.** Anti-Fas treatment effects on p53, bcl-2 and bax protein levels. MCF-10AT cells were grown to confluency, and treated as described under Materials and Methods. After harvesting, proteins were separated on a 7.5% SDS-PAGE gel, Western blotted with (A) anti-Fas, (B) anti-p53, (C) anti-bcl-2, or (D) anti-Bax antibody. Anti-Fas treatment decreased p53 protein levels, while Fas bcl-2 and bax levels remained unaffected.

ated apoptosis, the expression of Fas, p53, bcl-2, and Bax was examined in these cells. Fas expression was readily detected in the MCF-10AT cells, although the level of Fas protein was unaffected by anti-Fas treatment (Fig. 2A). Anti-Fas treated cells, however, exhibited significantly lower levels of p53 than untreated cells, suggesting that Fas-signaling had a suppressive role on p53 expression (Fig. 2B). Bcl-2 antagonizes apoptosis. Elevated levels of bcl-2 confer increased resistance to a number of cytotoxic drugs in lymphocytes (11). The activity of bcl-2 appears to be regulated by a 21 kDa protein, bax, which displays extensive homology to bcl-2, and both are capable of forming homodimers as well as heterodimers *in vivo.* Bax counters the death-repressing activity of bcl-2 (12, 13). In MCF-10AT cells, bcl-2 and bax levels were unaffected in response to anti-Fas treatment (Figs. 2C and 2D). The observation that signaling through Fas failed to down-regulate bcl-2 or upregulate bax suggests that Fas and bcl-2 regulate distinct pathways in MCF-10AT cells, which is consistent with previous findings in T-cells, where distinct regulation of apoptosis by Fas and bcl-2 was suggested (14).

Non-receptor protein tyrosine kinase activation has been shown to be an early event in Fas-signaling (9), however, the effect of Fas-signaling on receptor protein tyrosine kinase signaling remains unclear. Therefore the effects of anti-Fas treatment on RTK tyrosine phosphorylation, ras and MAPK activation were examined. Increased tyrosine phosphorylation of c-met was observed in cells undergoing apoptosis following anti-Fas treatment, whereas c-erbB2 tyrosine phosphorylation was only slightly elevated (Fig. 3A). This increase in c-met tyrosine phosphorylation was also observed at times prior to cells exhibiting apoptotic features (not shown). To examine whether these processes resulted from the physical interaction of Fas with c-erbB2 and c-met, immunoprecipitation experiments were performed to examine Fas association with c-erbB2 and c-met. The results showed that c-erbB2 and c-met were associated with Fas in untreated cells. However, Fas-c-erbB2 and Fas-c-met association was dramatically diminished by anti-Fas treatment (Fig. 3B).

To date, Fas has not been shown to contain previously known signaling motifs or catalytic activities, thus there is no obvious link between Fas signal transduction and PTK, JAKs-STATs or other known signal transduction pathways (15, 16). However, Fas is shown to physically interact with protein phosphatases, such as FAP-1 and the level of endogenous FAP-1 correlates with the Fas sensitivity of a cell (16). Fas has also been shown to physically interact with $p59^{fyn}$ and $p56^{lck}$ protein tyrosine kinases in T cells (15, 17, 18). In our studies, we showed that increased tyrosine phosphorylation of the c-met receptor tyrosine kinase and significant dissociation of Fas with c-erbB2 and c-met RTKs occurred in Fas-mediated signaling. These data suggest that Fas-signaling interact with RTK-mediated signaling, which has been shown to play a central role in cell proliferation and differentiation. Increased c-erbB2 and c-met tyrosine phosphorylation in response to anti-Fas treatment was also observed in MCF-10A cells (not shown). Since Fas lacks SH2 and SH3 domains, the association of Fas with RTKs may occur through interaction with the other effectors that are associated with Fas, such as the Fas-associated phosphatases FAP-1. The increase in RTK tyrosine phosphorylation, therefore, may be a result of disassociation of RTKs from Fas-phosphatase complex in response to anti-Fas stimulation.

To study further Fas-signaling effects on RTK signal transduction processes, Grb2-Sos association and p120-GAP levels were monitored. Tyrosine phosphorylation of the PTKs activates the signal transduction pathway, and ras is a critical effector in these processes. Ras is activated by son of sevenless (Sos), which upon activation of the PTKs, forms a complex with Grb-2 adaptor protein and Shc. This triplex is recruited

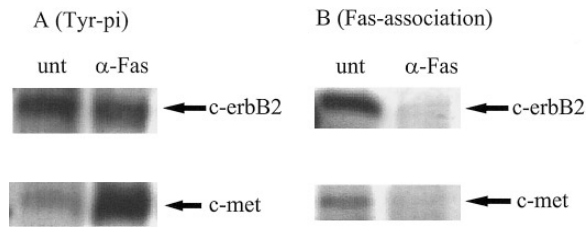


**FIG. 3.** Anti-Fas treatment effects on c-erbB2 and c-met tyrosine phosphorylation and Fas association with c-erbB2 or c-met. MCF-10AT cells were grown to confluency, and treated as described under Materials and Methods. Cell lysate was immunoprecipitated with anti-phosphotyrosine or anti-Fas antibody, followed by Western blot analysis with anti-c-erbB2 or anti-c-met antibody. (A) Anti-Fas treatment increased c-met tyrosine phosphorylation; (B) anti-Fas treatment decreased Fas association with c-erbB2 and c-met.

to the cytoplasmic domain of the activated PTKs, and becomes phosphorylated and activated. Sos exchanges ras-GDP for ras-GTP, thereby activating ras (19, 20). The association of phosphorylated Sos1 with Grb-2 was significantly decreased by anti-Fas treatment as compared to the untreated control (Fig. 4A), which suggests that decreased ras activation and ras signaling may occur with Fas-mediated signaling.

p120-GAP, the GTPase activator protein, functions to convert ras-GTP to ras-GDP when associated with ras (20, 21). p120-GAP also functions as a positive downstream effector of ras by a yet unknown mechanism, because oncogenic ras mutants require interaction with GAP to exert their transforming activity despite their lack of responsiveness to stimulation by GAPs (21). Therefore, the presence of p120-GAP in cells may be essential for cell proliferation induced by ras activation. A majority of the p120-GAP protein was present in complex with p21-ras in MCF-10AT cells. In cells treated with anti-Fas, however, p120-GAP levels were decreased dramatically, relative to p120-GAP levels in the untreated cells (Fig. 4B). This suggests that p120-GAP function may be essential for cell survival, and that Fas-mediated signaling possibly functions through abolishing GAP protein expression. These data support the analysis of p120-GAP as a positive effector in ras signaling, and that p120-GAP interaction with ras is required for ras signaling and cell proliferation.

MAPK activation is the critical link between the ras-raf signaling and downstream signaling to the nucleus. Ras activation transduces signals to raf and MAPKK/MEK, and activated MEK associates with MAPK, resulting in the phosphorylation and activation of ERKs/MAPK. Activated MAPK translocates into the nucleus and activates transcription factors, which regulate genes controlling cell proliferation and differentiation (22, 23). Therefore, MEK-1-MAPK association and MAPK levels were examined to study the role of Fas on MEK and MAPK signaling. Cells treated with anti-Fas exhibited a significantly decreased association of MEK-1 with ERK-1/MAPK (Fig. 5A), suggesting decreased activation of ERK-1 in anti-Fas treated cells.

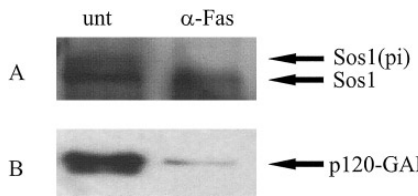


**FIG. 4.** Anti-Fas treatment effects on Grb2-Sos1 association and p120-GAP levels. MCF-10AT cells were grown to confluency, and treated as described under Materials and Methods. Cell lysate was immunoprecipitated with anti-Grb2 or anti-GAP antibody, followed by Western blot analysis with anti-Sos1 (A), or anti-GAP (B) antibody, respectively. (A) Anti-Fas treatment decreased Grb2-Sos1 association; (B) anti-Fas treatment decreased p120-GAP levels.

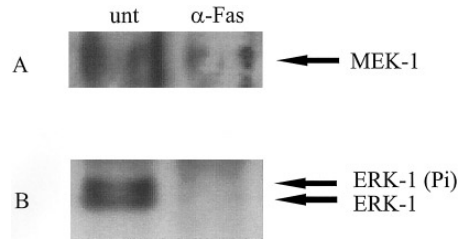


**FIG. 5.** Anti-Fas treatment effects on MEK-1-ERK-1 association, and on ERK-1 levels and ERK-1 phosphorylation. MCF-10AT cells were grown to confluency and treated as described under Materials and Methods. Cell lysate was immunoprecipitated with anti-ERK-1 antibody, followed by Western blot analysis with anti-MEK-1 (A) or anti-ERK-1 (B) antibody. (A) Anti-Fas treatment decreased MEK-1-ERK-1 association; (B) anti-Fas treatment decreased ERK-1 levels and ERK-1 phosphorylation.

Cells treated with anti-Fas exhibited a dramatic decline in ERK-1/MAPK levels and phosphorylation, relative to untreated cells (Fig. 5B). These data show that MAPK function was abolished with Fas-mediated apoptosis, suggesting that Fas-signaling may function through inhibiting ras-raf-MEK-ERK signaling, even though Fas-signaling initially activated PTK tyrosine phosphorylation. Thus, Fas-signaling and growth factor signaling produce opposing effects on the downstream effectors of PTKs, although both share the same initial event, PTK tyrosine phosphorylation.

The studies presented herein illustrate a link between Fas-signaling and signal transduction processes of the RTKs. Fas-signaling exerted a dramatic suppressive effect on ras-raf-MEK-MAPK signaling, although it stimulated the initial step of signal transduction, the tyrosine phosphorylation of the RTKs. The negative effects of Fas on ras-MAPK signal transduction processes would appear to inhibit further any possibility of the cell mounting a proliferative response, thereby ensuring a terminal outcome regardless of the potential for stimulation of PTK signaling.

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