Fibroblast Growth Factor-2 Suppression of Tumor Necrosis Factor α-Mediated Apoptosis Requires Ras and the Activation of Mitogen-activated Protein Kinase

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Treatment of L929 cells with tumor necrosis factor α (TNFα) activates a programmed cell death pathway resulting in apoptosis. We investigated the intracellular signaling pathways activated in L929 cells by TNFα. TNFα robustly activates Jun kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family. In addition, p42MAPK is activated, but a 10-fold greater concentration of TNFα was required for substantial MAPK activation than was needed for maximal JNK stimulation. Simultaneous treatment of L929 cells with fibroblast growth factor (FGF-2) significantly reduced the apoptotic response to TNFα. FGF-2 substantially activated the Raf/MEK/MAPK (where MEK is mitogen-activated protein kinase kinase) pathway but did not affect TNFα activation of JNK. These results indicate that although JNK may play an important role in transmitting the TNFα signal from the cell surface to the nucleus, activation of the JNK pathway is not sufficient to induce apoptosis. Expression of dominant-negative Asn-17 Ras in L929 cells diminished the FGF-2 stimulation of p42MAPK and eliminated the protective effect of FGF-2. Asn-17 Ras expression did not affect JNK activity and had no effect on TNFα activation of JNK. Pharmacological inhibition of MEK-1 activity by incubation of cells with the compound PD 098059 blocked p42MAPK activation and FGF-2 protection against apoptosis. Interestingly, activated Val-12 Ras expression substantially enhanced TNFα-mediated apoptosis in L929 cells, but Val-12 Ras did not constitutively activate MAPK in L929 cells and FGF-2 partially protected Val-12 Ras-expressing cells from TNFα-mediated apoptosis. Our data indicate that activation of the MAPK pathway mediates an FGF-2 protective effect against apoptosis and highlights the important role that integration of multiple intracellular signaling pathways plays in the regulation of cell growth and death.

Tumor necrosis factor α (TNFα) is a multifunctional cyto-

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1 The abbreviations used are: TNFα, tumor necrosis factor α; p55TNFR, 55-kDa tumor necrosis factor receptor; p75TNFR, 75-kDa tumor necrosis factor receptor; MAPK, mitogen-activated protein kinase; JNK, Jun kinase; FGF-2, fibroblast growth factor-2; MEK, mitogen-activated protein kinase kinase; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; GST-Jun, glutathione S-transferase-c-Jun; EGFR, epidermal growth factor receptor; PAGES, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; IFTG, isopropyl-1-thio-β-D-galactopyranoside; PD, MEK-1 inhibitor PD 098059.
FGF-2 Suppression of TNF-α-Mediated Apoptosis

FGF-2 Suppression of TNF-α-Mediated Apoptosis—TNF-α activates a cell death program resulting in the apoptosis of L929 cells (33). Fig. 1A shows that treatment of L929 cells overnight with TNF-α resulted in substantial cell death using the neutral red assay as a measure of viable cells (see “Materials and Methods”). The time course of cell death was dependent on the concentration of TNF-α. Treatment with 10 ng/ml TNF-α resulted in greater than 40% of the L929 cells being apoptotic in 15 h; 1 ng/ml TNF-α required 24–48 h to induce a similar level of L929 cell death (not shown). Serum and growth factor withdrawal induces apoptosis in several cell systems (46, 47), indicating that growth factors have a protective effect against apoptosis. Consistent with this observation was our finding that FGF-2 affected TNF-α-mediated apoptosis (Fig. 1B). Incubation of L929 cells with TNF-α in the presence of FGF-2 significantly reduced the cell death response. A concentration of 0.5 ng/ml FGF-2 was effective at blocking TNF-α-mediated cell death. The protective effect of FGF-2 was not simply due to an increased proliferative response of L929 cells, stresses (27–31).

TNF-α has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines, including the murine fibrosarcoma cell line L929 (32, 33). TNF-α also has been shown to activate p42/p44MAPK in this cell line (34). Recently J NKS were shown to be activated by TNF-α (29, 30, 35), and activation of the J NK pathway correlated with enhanced apoptosis of PC12 cells in response to trophic factor deprivation (36). We have characterized the regulation of MAPKs and J NKS in L929 cells challenged with TNF-α and fibroblast growth factor (FGF-2). We show that TNF-α preferentially activates J NK in L929 cells but that J NK activation is not sufficient to induce apoptosis, because FGF-2 mediates a protective effect against TNF-α-mediated apoptosis without affecting J NK activation. Furthermore, our data indicate that p42/p44MAPK activation is required for FGF-2 suppression of TNF-α-mediated apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture—L929 cells (ATCC CCL1) were maintained in Dulbecco’s modified Eagle’s medium with 5% newborn calf serum and 5% bovine serum albumin. The cells were grown in 10-cm dishes at 37°C in 5% CO2. Cells were made quiescent where indicated by incubation in Dulbecco’s modified Eagle’s medium and 0.1% bovine serum albumin for 24 h. Recombinant murine TNF-α and recombinant human FGF-2 (147 amino acids) were from R&D Systems, Minneapolis, MN. Cells were pretreated where indicated with the MEK-1 inhibitor PD 098059 (10 μM) for 30 min. Cells were then washed three times with 1 ml each of 37°C PBS. The neutral red was extracted with 1.0 ml of 50% ethanol, 50 mM sodium citrate, pH 4.2, and absorbency was measured at 540 nm.

Propidium Iodide Staining—Cells were plated on glass chamber slides (Nunc, Naperville, IL) at a concentration of 0.2–0.6 x 10^5 cells/ml. Ras expression was induced with 5 μg IPTG in Dulbecco’s modified Eagle’s medium with 0.1% bovine calf serum for 8–12 h. Cells were exposed to TNF-α (5 ng/ml) and/or FGF-2 (500 ng/ml) in Dulbecco’s modified Eagle’s medium with 0.1% bovine calf serum for 16 h. The parental LACI expressing cell line (see below) was used as a control. Cells were washed twice in PBS, fixed in acetonemethanol (1:1) at −20°C for 5 min, air-dried, washed twice in PBS, stained with 1 μg/ml propidium iodide in PBS for 20 min, washed in H2O, 100% ethanol, and mounted in 25% glycerol/PBS. Propidium iodide fluorescence was observed using a Nikon inverted microscope equipped with epi-fluorescence and a 580-nm filter. Images were analyzed using IP lab.

Cell Transfections—L929 cells were transfected by CaPO4 (40) with the vector 3’SS (Stratagene, La Jolla, CA), expressing the LACI repressor. Stable clones were selected in 200 μg/ml hygromycin (Calbiochem) and screened for LACI expression by indirect immunofluorescence using rabbit antiserum to LACI (Stratagene, La Jolla, CA) and FITC-donkey anti-rabbit. One clone expressing a high level of nuclear LACI was then transfected with hemagglutinin-tagged inhibitory Asn-17 Ras (41) or activated Val-12 Ras (42–44) into the LACI repressible pPOVS1 vector. Stable clones were selected in 500 μg/ml G418 and screened for inducibility by expression of Ha-Ras by immunoblotting. Induction in 5 μg isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 8–24 h was used to induce Ras expression. Several independent, inducible Asn-17 Ras or Val-12 Ras clones were isolated, and two each were chosen for further analysis.

Immunoblotting—100 μg of cell lysate was fractionated by SDS-PAGE (12.5% acrylamide) and blotted to nitrocellulose in 10 mM CAPS, pH 11, 20% MeOH using a Transphor apparatus ( Hoefer, San Diego, CA) for 1 h at 1A. Blots were blocked in 5% powdered milk in Tris-HCl, pH 7.5, 7.5%, buffered saline. Ras was detected with Y-13259 anti-Ras monoclonal antibody (45) followed by enhanced chemiluminescence (Amer sham Corp.) using horseradish peroxidase-anti-mouse IgG (Bio-Rad).

Quantitation of Data—Phosphorimager analysis of phosphorylated proteins was provided a quantity measurement of specific phosphoproteins. Statistical analysis was performed using the J MP program (SAS Institute Inc., Cary, NC), and the method of Tukey and Kramer was used to determine statistical differences.

RESULTS

FGF-2 Protects L929 from TNF-α-Mediated Apoptosis—TNF-α activates a cell death program resulting in the apoptosis of L929 cells (33). Fig. 1A shows that treatment of L929 cells overnight with TNF-α resulted in substantial cell death using the neutral red assay as a measure of viable cells (see “Materials and Methods”). The time course of cell death was dependent on the concentration of TNF-α. Treatment with 10 ng/ml TNF-α resulted in greater than 40% of the L929 cells being apoptotic in 15 h; 1 ng/ml TNF-α required 24–48 h to induce a similar level of L929 cell death (not shown). Serum and growth factor withdrawal induces apoptosis in several cell systems (46, 47), indicating that growth factors have a protective effect against apoptosis. Consistent with this observation was our finding that FGF-2 affected TNF-α-mediated apoptosis (Fig. 1B). Incubation of L929 cells with TNF-α in the presence of FGF-2 significantly reduced the cell death response. A concentration of 0.5 ng/ml FGF-2 was effective at blocking TNF-α-mediated cell death. The protective effect of FGF-2 was not simply due to an increased proliferative response of L929 cells,
The indicated concentrations of TNFα were used to assess serum starvation. FGF-2 blocks TNF killing of L929 cells. Cells were treated with the indicated concentrations of TNFα for 18 h and were assayed for uptake of neutral red as described under "Materials and Methods." Error bars represent the standard deviation of triplicate determinations. B, FGF-2 blocks TNF killing of L929 cells. Cells were treated with 0.5 and 5.0 ng/ml FGF-2 (shaded bars), or 5.0 ng/ml FGF-2 (hatched bars) and the indicated concentrations of TNFα for 18 h. Cell viability was assessed by neutral red assay. Error bars represent the standard deviation of duplicate determinations. 0.5 and 5.0 ng/ml FGF-2 significantly enhanced neutral red uptake compared with TNFα-treated samples at both 1 and 5 ng/ml TNFα. *, p < 0.005.

FGF-2 receptors possess intrinsic tyrosine kinase activity and are present on L929 cells. Fig. 3 demonstrates that FGF-2 stimulates a robust activation of MAPK in L929 cells. Concentrations of 0.25–0.5 ng/ml FGF-2 gave maximal stimulation of MAPK activity. Fractionation of stimulated cell lysates by MonoQ fast pressure liquid chromatography indicated that both p42 and p44 MAPK were activated by FGF-2 (not shown). Activation of the MAPK pathway by tyrosine kinases involves Ras and the Raf serine-threonine protein kinases. Immuno- blotting demonstrated that both C-Raf and A-Raf are expressed in L929 cells (not shown). Treatment of L929 cells with FGF-2 resulted in the activation of both B-Raf and C-Raf (Fig. 4), measured by their ability to phosphorylate a recombinant kinase-inactive MEK-1 protein (37). MEK-1 is the protein kinase phosphorylated and activated by Raf, which in turn phosphorylates MAPK on both a tyrosine and threonine resulting in MAPK activation (48–51). In contrast, TNFα does not significantly activate either isoform of Raf in L929 cells (Fig. 4).

FGF-2 and TNFα Independently Regulate Cytoplasmic Protein Kinase Cascades—Fig. 5 demonstrates that 10 ng/ml TNFα has only modest stimulatory effects on MAPK activity (C), and 2.5 ng/ml FGF-2 has little or no effect on JNK activity (B). These concentrations of FGF-2 and TNFα give maximal activation of MAPK and JNK, respectively. Co-stimulation of L929 cells with FGF-2, at concentrations that show partial protection against TNFα-mediated killing, did not alter the magnitude of JNK activation in response to TNFα. Similarly, co-stimulation of L929 cells with TNFα at concentrations capable of causing cell death, had little or no effect on FGF-2 stimulation of MAPK activity (C). Thus, in relation to JNK and...
MAPK, TNFα and FGF-2 receptors independently regulate the activity of these two sequential protein kinase pathways in L929 cells.

Inducible Expression of Inhibitory and Activated Ras Influences Apoptosis—Ras activation is required for many of the phenotypic responses resulting from the activation of tyrosine kinases. Signaling by the FGF-2 receptor involves several different effector pathways including Ras activation. To test the involvement of Ras in the FGF-2-promoting response, the Lac Switch-inducible expression system (see "Materials and Methods") was used to control the expression of inhibitory Asn-17 Ras and constitutively activated Val-12 Ras in L929 cells. Fig. 6 shows the functional consequence of expressing inhibitory Asn-17 Ras or activated Val-12 Ras on MAPK and JNK activation in response to FGF-2 and TNFα, respectively. IPTG-regulated expression of the hemagglutinin epitope-tagged Ras mutants (Asn-17 and Val-12 Ras) is shown in D. Expression of Asn-17 Ras significantly blunted FGF-2 stimulation of MAPK (A) but had no effect on TNFα stimulation of JNK (C). It was surprising that Asn-17 Ras was unable to completely block MAPK activation and implies that the level of Asn-17 Ras expression was too low to completely block endogenous Ras or that FGF-2 activates MAPK via a Ras-independent pathway. We found that the clones with the highest inducible levels of Asn-17 Ras expression were most effective at blocking MAPK (Fig. 6, A and D). Therefore, it is likely that the partial block of MAPK activation is due to insufficient expression of Asn-17 Ras, although we cannot rule out a Ras-independent pathway for activation of MAPK by FGF-2. With two independent clones, expression of Val-12 Ras did not constitutively activate the MAPK pathway but did appear to enhance FGF-2 stimulation of MAPK (B). Val-12 Ras expression also had no effect on TNFα stimulation of JNK activity (C). We have previously shown that constitutive activation of MAPK by Val-12 Ras is a cell type-specific phenomenon in that MAPK is constitutively activated in Val-12 Ras expressing NIH 3T3 cells but is not activated by Val-12 Ras in Rat 1a cells (52). Differential regulation of phosphatases that inactivate the MAPK pathway has been proposed to explain this observation (53).

Expression of Asn-17 Ras did not affect TNFα-induced apoptosis of L929 cells (Figs. 7 and 8); Asn-17 Ras did, however, markedly inhibit the ability of FGF-2 to protect cells against TNFα-mediated cell death. These findings indicated that functional Ras signaling is not required for the TNFα-induced apoptotic response but is required for the protective action of FGF-2. Strikingly, constitutively activated Val-12 Ras markedly enhanced TNFα-stimulated apoptosis but had little or no effect on the apoptotic index of L929 cells in the absence of TNFα (Fig. 7). This observation indicates that Val-12 Ras is functional in L929 cells, despite the fact MAPK is not constitutively activated in this cell line and implies that activated Ras likely regulates pathways in addition to MAPK that are involved in apoptosis. Co-stimulation with FGF-2 and TNFα resulted in a diminished apoptotic response relative to TNFα alone in Val-12 Ras-expressing cells, indicating that FGF-2 pathways required for protection against TNFα-stimulated cell death were functional in these cells (Fig. 8). Thus, inhibitory Ras expression prevented FGF-2 protective responses and activated Ras-enhanced TNFα killing. The results suggest multiple Ras-dependent events are involved in controlling apoptosis and the role of Ras signaling can be either positive or negative in regulating the phenotypic response to cytokines such as TNFα.

Inhibition of MEK and MAPK Stimulation Prevents FGF-2 Protection from Apoptosis—The Parke-Davis compound, PD 098059, inhibits the dual specificity protein kinase, MEK-1,
which specifically activates p42/p44MAPK (54). PD 098059 did not inhibit JNK kinase or the activation of JNK (not shown). Pretreatment of L929 cells with PD 098059 inhibited FGF-2 stimulation of MAPK activity (Fig. 9A). The PD 098059 compound had no effect on TNFα-mediated apoptosis but inhibited the protective response to FGF-2 (Fig. 9B). Thus, MEK activation of MAPK is required for FGF-2 protection against TNFα-mediated apoptosis. Interestingly, the phosphatidylinositol 3-kinase inhibitor, wortmannin, did not influence the cell death response to TNFα nor did it inhibit the protective response to FGF-2 (not shown). Treatment of L929 cells with wortmannin had no effect on the ability of FGF-2 to stimulate MAPK activity. Apparently, phosphatidylinositol 3-kinase activity is not required for the action of either TNFα or FGF-2 on the control of the cell death program in L929 cells.

DISCUSSION

TNFα induces apoptosis of L929 cells and FGF-2 is protective against this cell death response. Our results indicate that the activation of JNK in response to TNFα stimulation of L929 cells is not sufficient for the induction of cell death. TNFα maximally stimulates JNK activity in the presence of FGF-2 concentrations that are capable of protecting against cell death. Signals in addition to JNK activation must be involved in the TNFα-mediated death response. The FGF-2 protective response was only partial in that not all the cells were prevented from dying in response to TNFα treatment. This may, in part, be related to cell cycle-dependent signaling by TNFα and FGF-2; the L929 cells used in these studies were asynchronous so we cannot rule out this possibility. Our findings also demonstrate that Ras is involved in integrating responses that

Fig. 6. Effect of dominant-negative Asn-17 Ras or constitutive-active Val-12 Ras on MAPK and JNK activities. A, inhibition of MAPK activation by FGF-2 in Asn-17 Ras expressing L929 cells. Cells were uninduced (−) or induced (+) to express Asn-17 Ras by overnight treatment with 5 mM IPTG. The cells were unstimulated (−) or stimulated (+) for 10 min with 0.5 ng/ml FGF-2. MAPK activity was assayed as in Fig. 1. Error bars represent the standard deviation of triplicate determinations. B, MAPK activity by FGF-2 in Val-12 Ras expressing L929 cell lines. 41.LACI or Val-12 Ras cells were induced with IPTG, stimulated as indicated, and analyzed for MAPK activation. C, Asn-17 Ras or Val-12 Ras expression in L929 cells does not alter TNF stimulation of JNK. The indicated cell lines were uninduced (−) or induced (+) with IPTG and then unstimulated (−) or stimulated (+) with 1 ng/ml TNFα for 10 min. JNK activity was assayed as in Fig. 1. The slight reduction of JNK activity in induced 41.12 Val-12 Ras cells was not reproduced in two other experiments. D, immunoblot of Asn-17 Ras or Val-12 Ras expression. The indicated cell lines were uninduced (−) or induced (+) with IPTG overnight. 100 μg of each cell lysate was fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for Ras expression. Endogenous p21ras and hemagglutinin-tagged Ras is indicated.

Fig. 7. Propidium iodide staining of TNFα-treated L929 cells. Cells were untreated (left panel) or treated with 5 ng/ml TNFα (right panel) overnight. After fixation in MeOH:acetic acid cells were stained with propidium iodide. A and B, L929 41.LACI; C and D, 41.23 Asn-17 Ras; E and F, 41.12 Val-12 Ras. Arrows point to examples of apoptotic cells with condensed nuclei and uniformly stained chromatin.
control apoptosis. Expression of activated or inhibitory Ras influences TNFα killing of L929 cells. The mechanism for enhanced TNFα killing of L929 cells resulting from Val-12 Ras expression is unclear, although it has been observed in C3H mouse fibroblasts as well (55). It may involve an alteration in the expression of specific genes such as c-jun, c-fos, and c-myc which appear to be involved in both growth and apoptotic responses (35, 56–63). In contrast, the effect of inhibitory Asn-17 Ras appears to primarily be the inhibition of MAPK activation in response to FGF-2. This finding is substantiated by the loss of FGF-2 protection against TNFα-mediated apoptosis by the MEK inhibitor PD 098059. Studies using the fungal metabolite, wortmannin, demonstrated that phosphatidylinositol 3-kinase was not involved in FGF-2 protection against apoptosis in L929 cells.

Recently, it was demonstrated using PC12 cells that the JNK pathway was involved in mediating apoptosis in response to serum deprivation and that activation of the MAPK pathway was protective against serum deprivation (36). Phosphatidylinositol 3-kinase activity has also been reported to be necessary to protect PC12 cells from serum deprivation-induced apoptosis (64). Interestingly, the expression of Asn-17 Ras protected PC12 cells from nerve growth factor withdrawal-induced apoptosis (65). The findings indicated that Asn-17 Ras maintained PC12 cells in a quiescent state that allowed them to survive in the absence of trophic factors. Removal of trophic factors from PC12 cells appeared to induce an aberrant proliferative response that resulted in apoptosis. Our findings using Asn-17 Ras expression in L929 cells contrast with those in PC12 cells. TNFα induced apoptosis in growing L929 cells; Asn-17 Ras expression did not affect the apoptotic response, whereas Val-12 Ras expression significantly enhanced apoptosis. Thus, the involvement of Ras-dependent signaling on apoptotic responses of cycling versus quiescent cells may be quite different.

In human B cells, cross-linking of surface IgM stimulated a host of signaling pathways including MAPK but not JNK and resulted in apoptosis (66). CD40, a member of the TNF receptor family, activated JNK while rescuing B cells from anti-IgM-mediated apoptosis (66). Thus, in human B cells MAPK activation is insufficient to protect against apoptosis, and signals including the stimulation of JNK are generated during a protective response. Clearly, the integration of multiple signals appears to be required for apoptosis.

The overlap of signals involved in committing cells to growth or apoptosis is also evident in many transformed cell types. Tumors frequently have a high growth rate but also a high apoptotic index (58, 67). The growth rate is simply greater than the apoptotic rate so that the net result is tumor expansion. In addition, transformed cells frequently have selected mutations and growth factor autocrine loops to inhibit apoptosis. For example, Ras function has been shown to be involved in both transformation and protection against apoptosis in Bcr-Abl-transformed cells (68, 69).

Cumulatively, the results in different cell types indicate it is the integration of multiple signals from cytokines and growth factors that determines the commitment to apoptosis. Similarly, integration of multiple signals and not a single dominant signaling pathway is likely involved in the commitment to growth or differentiation. The requirement for signal integration may allow for specific checkpoints so that cells do not die or grow inappropriately. In this regard, cell systems where specific cytokines or growth factors are added or removed are most relevant in defining the integration of signals controlling growth versus death.

The implication of our findings is that it should be possible to define signal pathways and their integration that control apoptosis in specific cell types. As these findings are further defined, it will be possible to develop strategies to selectively induce a cell type-specific apoptotic response. Development of gene therapy, cytokine, and drug treatments may be possible to selectively promote the death of undesirable cell populations in animals.
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