Focal adhesion kinase (FAK) has an anti-apoptotic role in anchorage-dependent cells via an unknown mechanism. To elucidate the role of FAK in anti-apoptosis, we established several FAK cDNA-transfected HL-60 cell lines and examined whether FAK-transfected cells have resistance to apoptotic stimuli. FAK-transfected HL-60 (HL-60/FAK) cells were highly resistant to apoptosis induced with hydrogen peroxide (1 mM) and etoposide (50 μg/ml) compared with the parental HL-60 cells or the vector-transfected cells, when determined using viability assay, DNA fragmentation, and flow cytometry analysis. Because no proteolytic cleavage of procaspase 3 to mature caspase 3 fragment was observed in HL-60/FAK cells, FAK was presumed to inhibit an upstream signal pathway leading to the activation of caspase 3. HL-60/FAK activated the phosphatidylinositide 3'-OH-kinase-Akt survival pathway and exhibited significant activation of NF-κB with marked induction of inhibitor-of-apoptosis proteins (IAPs: cIAP-1, cIAP-2, XIAP), regardless of the hydrogen peroxide-treated or untreated conditions, whereas no significant IAPs were detected in the parental or vector-transfected HL-60 cells. Apoptotic agents induced higher NF-κB activation in HL-60/FAK cells than in HL-60/Vect cells, and it appeared that sustained NF-κB activation is critical to the anti-apoptotic states in HL-60/FAK cells. Mutagenesis of FAK cDNA revealed that Y397 and Y925, which are involved in the tyrosine-phosphorylation sites, were prerequisites for the anti-apoptotic activity as well as induction of IAPs, and that K454, which is involved in the kinase activity, was also required for the full anti-apoptotic activity of FAK. Taken together, we have demonstrated definitively that FAK-transfected HL-60 cells, otherwise sensitive to apoptosis, become resistant to the apoptotic stimuli. We conclude that FAK activates the phosphatidylinositide 3'-OH-kinase-Akt survival pathway with the concomitant activation of NF-κB and induction of IAPs, which ultimately inhibit apoptosis by inhibiting caspase-3 cascade.

Apoptosis (programmed cell death) contributes to the normal development and tissue remodeling of multicellular organisms (1). The responsible molecules exerting or regulating apoptosis identified so far include the caspase family (2–4), the Bcl-2 family (5–8), caspase-activated DNase (CAD) (9), and inhibitor of CAD (10).

Reactive oxygen species (ROS) are presumed to be important regulators of apoptosis. Production of ROS is found to be stimulated by tumor necrosis factor-α (TNF-α) (11), lipopolysaccharide (12), ceramide (13), growth factor withdrawal (14), human immunodeficiency virus infection (15), or p53-induced apoptosis (16, 17). In contrast, overexpression of thioredoxin (18), manganese superoxide dismutase (19), or Bcl-2 (20, 21) can delay apoptosis. The mechanism of these antioxidant molecules to suppress apoptosis has not fully been elucidated.

Focal adhesion kinase (FAK) has been implicated in the integration of signals from integrins, oncogenes, and neuropeptides (22, 23). FAK has also been shown to play an important role in the cell survival of anchorage-dependent cells (24). Proteolytic cleavage of FAK by caspase-3 has been reported during growth factor deprivation-induced apoptosis in human umbilical vein endothelial cells (25), which implies an association between FAK and apoptosis.

We found that FAK was tyrosine-phosphorylated by oxidative stress before apoptosis occurred (26). Furthermore, protein kinase B/Akt which has been implicated in the pathway of survival signal, was serine-phosphorylated following tyrosine phosphorylation of FAK. This proposed that FAK has an anti-apoptotic role in oxidative stress-induced apoptosis in the human glioblastoma cell line T98G (27). In this paper, we attempted to elucidate the possibility that FAK may also have an anti-apoptotic function in HL-60 cells. We observed that naive HL-60 cells are not resistant to various apoptosis-inducing reagents, whereas FAK-overexpressed cells acquire resistance to oxidative stress, etoposide-induced apoptosis, with a concomitant inhibition of caspase-3 proteases. We further found that the phosphatidylinositide 3'-OH-kinase (PI3-kinase)-Akt survival pathway, NF-κB activation, and increase of inhibitory apoptosis proteins (IAPs) are involved in the FAK-induced resistance to apoptosis. Thus, FAK was found to activate a signal linking NF-κB and IAPs.

Experimental Procedures

Cells and Materials—HL-60 cells (clone C15, donated by Dr. Ackerman, Beth Israel Hospital, Boston, MA) were suspended in RPMI 1640

1 The abbreviations used are: CAD, caspase-activated DNase; FAK, focal adhesion kinase; PI3-kinase, phosphatidylinositide 3'-OH-kinase; PDTC, pyrroldine dithiocarbamate; Ac-YVAD-AMC, N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; ICE, interleukin-1β converting enzyme; FACS, fluorescence-activated cell sorter; IAP, inhibitor-of-apoptosis protein; ROS, reactive oxygen species; TNF, tumor necrosis factor; mAb, monoclonal antibody; RT-PCR, reverse transcription-polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate.
Induction of IAPs by FAK Activation

medium containing 5% fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan). For oxidative stress experiments, growing cells were subcultured at a density of 2 × 10⁶/ml in medium containing 1% fetal bovine serum. Monoclonal anti-hemagglutinin epitope peptide (HA) antibody (mAb 12CA5) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). mAb 1B3 from Sigma Chemical Co. (St. Louis, MO), rabbit anti-Akt and phospho-Akt antibodies from New England Biolabs Inc. (Boston, MA), anti-phosphotyrosine mAb (4G10) and rabbit anti-P13-kinase (p85) antibody from Upstate Biotechnology Inc. (Lake Placid, NY), rabbit goat anti-human caspase-3 (CPP32) antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and horseradish peroxidase-conjugated secondary antibody from DAKO (Denmark). Enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech. Substrates for protease activity, N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC) (caspase-1) and N-acetyl-Asp-Glu-Val-Ala-Asp-4-methylcoumarin (Ac-DEVD-AMC) (caspase-3), were obtained from Peptide Institute, Inc. (Osaka, Japan). Pyrrolidine dithiocarbamate (PDTC) and LY294002 were purchased from Sigma. PD98059 was purchased from Alexis Biochemicals (San Diego, CA).

Transfection of FAK cDNA into HL60 Cells—HA-tagged FAK cDNA and mutated FAK cDNA subcloned into the plasmid pcRCMV were prepared as described elsewhere (28). Each 10 μg of pcRCMV/FAK or pcRCMV control vector was transfected into HL60 cells using electroporation with a Gene Pulsor (Bio-Rad) at 0.55 kV. After incubation with 0.5 mg/ml Geneticin (Life Technologies, Inc.), six clones expressing high FAK activity in the immunoprecipitates were determined as described previously (27).

Analysis of DNA Fragmentation and Cell Viability Assay—A DNA fragmentation assay was performed as described previously (26). In brief, cells were gently lysed for 30 min at 48 °C in a buffer containing 5 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 0.5% Triton X-100. After centrifugation at 15,000 rpm for 15 min, supernatants containing soluble fragmented DNA were collected and treated with RNase (20 μg/ml, Wako Pure Chemicals, Tokyo, Japan), followed by proteinase K (20 μg/ml) digestion. DNA fragments were precipitated in 99% ethanol. Samples were then electrophoresed on a 2% agarose gel, and DNA was visualized by staining with ethidium bromide. Cell viability was determined using trypan blue dye exclusion test. All the HL-60/FAK clones showed essentially similar results with marked resistance, and all the HL-60/Vect clones showed essentially similar results with marked resistance.

Caspase-1 and Caspase-3 Protease Activity—Following phosphate-buffered saline washing, cell lysate was prepared as described by Nicholson et al. (2). The cell lysate (50 μg of protein) was incubated at 37 °C with 50 μM Ac-DEVD-AMC as a caspase-3 substrate for 30 min or Ac-YVAD-AMC as a caspase-1 (interleukin-1β converting enzyme (ICE)) substrate for 60 min. The amounts of released 7-amino-4-methylcoumarin (AMC) were measured with fluorescence spectrofluorometer (Hitachi F-4000, Tokyo, Japan), with excitation at 380 nm and emission at 460 nm. Caspase-1 and -3 activities were expressed as picomoles/min/mg of protein.

Transfection and Luciferase Assays—The NF-κB luciferase reporter gene plasmid (pNF-kB- luciferase) containing four tandem copies of the NF-κB binding sequence of the immunoglobulin light chain kappa gene, was purchased from Promega (Madison, WI). Sato (Chugai Pharmaceutical Co., Japan) provided transient cotransfection of NF-κB luciferase (firefly) reporter gene and luciferase (Renilla; sea pansy) expression vector pRL-TK for normalization of transfection efficiency into cells was performed using the DEAE-dextran method as described previously (29). The cells were cultured in RPMI 1640 containing 5% fetal calf serum for 15 h and treated with or without hydrogen peroxide (0.2 mM) or etoposide (10 μM) for 5 h.

RESULTS

Establishment of HL-60 Cell Lines That Overexpress FAK—To investigate the effect of overexpressed FAK on various stimuli-induced apoptosis, we established six stable transfectants of HL-60 cells that harbor FAK cDNA in the pcRCMV construct and were designated as HL-60/FAK (clones 1–6). Immunoblot analysis revealed significantly higher FAK expression in these HL-60/FAK clones than in the parental HL-60 cells or the control vector transfectants, HL-60/Vect (clones 7–10), in which virtually no or minimal level of FAK was detected (Fig. 1). Endogenous FAK protein in HL-60 could not be detected in unstimulated conditions, but was detected by the stimulation with phorbol 12-myristate 13-acetate (PMA, 10 nM) for 1 day and its expression continued at least till 3 days as shown in Fig. 2. We tested whether the PMA-treated cells were resistant to the hydrogen peroxide-induced apoptosis, i.e. PMA-treated cells for 1–3 days were stimulated with 1 mM hydrogen peroxide, and the apoptotic cells were counted by flow cytometry after propidium iodide staining. As shown in Fig. 2, the amount of apoptosis was 10–25% 4 h after treatment with hydrogen peroxide, which was apparently lower than the 50% observed in untreated HL-60 cells. These results showed that intrinsic FAK has also an anti-apoptotic role. These results also suggest that expression of FAK might correlate with the anti-apoptotic capacity in the HL-60 cell line.

Inhibition of Various Stimuli-induced Apoptosis by FAK Overexpression—To induce apoptosis, cells were treated with hydrogen peroxide (1 mM) or etoposide (50 μM) for various time periods, and cell viability was assessed by trypan blue exclusion test. All the HL-60/FAK clones showed essentially similar results with marked resistance, and all the HL-60/Vect clones showed marked resistance by FAK overexpression by the agents during the 4-h incubation period. Therefore, each representative clone (clones 1 and 7) from HL-60/FAK and HL-60/Vect was used for further studies. HL-60/FAK cells (clone 1) showed marked resistance against these stimuli-induced apoptosis compared with both parental HL-60 and HL-60/Vect (clone 7) (Fig. 3). That is, parental HL-60 and HL-60/Vect cells showed 40–60% cell death 4 h after treatment either with hydrogen peroxide or etoposide, whereas HL-60/FAK cells died only less than 3% by pulse. After preparation of cell extracts, luciferase activities were measured using the Dual-luciferase reporter assay system (Promega) and Lumat LB9501 (Berthold Japan K.K., Tokyo, Japan). Reverse Transcription-Polymerase Chain Reaction of IAPs—RNA was prepared by the method described previously (27). Reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the method of the supplier (Takara Syuzou, Shiga, Japan) using oligo(dT) primers and total RNA (1 μg) for first strand DNA synthesis. Primer sequences were as follows: cIAP-1 sense, 5′-agtgtctgctgtgctgtgtggg-3′; antisense, 5′-atcttccacacggaagatagctt-3′; cIAP-2 sense, 5′-gtctgctgtgtggctctgtg-3′; antisense, 5′-caccttgaaaccacX-IAP sense, 5′-gagacccgggggcgg-3′; antisense, 5′-gcctgggtctcttgatc-3′. PCR was performed at an annealing temperature of 63 °C and 25 amplification cycles. In the case of GAPDH (sense, 5′-gactcgaagaaaaacagcctg-3′; antisense, 5′-tgatgaggggtctcgattt-3′), PCR was performed at an annealing temperature of 55 °C and 25 amplification cycles.

Fig. 1. Western blot analysis of overexpressed FAK. FAK expression in parental HL-60, HL-60/FAK (clones 1–6), and HL-60/Vect cells (clones 7–10) were detected by Western blot analysis. Separation of proteins (50 μg) were achieved on 7.5% SDS-polyacrylamide gel electrophoresis, and the Western blot analysis was performed using anti-FAK mAb (1 μg/ml) as described under “Experimental Procedures.”
these stimuli. As shown in Fig. 4, the absence of DNA fragmentation by these stimuli in HL-60/FAK cells confirmed the anti-apoptotic activity of FAK. FACS analysis also confirmed the above observation. When cells were treated with these apoptotic stimuli for 4 h, a sub-G_{2}/G_{1} apoptotic population in parental HL-60 cells or HL-60/Vect cells increased up to 40–50%, whereas the sub-G_{2}/G_{1} population was only 3–5% in HL-60/FAK cells (data not shown).

Tyrosine Phosphorylation of FAK, Serine Phosphorylation of Akt, and the Association with PI3-kinase in HL-60/FAK Cells—We have previously described that the FAK-PI3-kinase-Akt survival pathway was activated before occurring apoptosis by hydrogen peroxide in a human glioblastoma cell line, T98G (27). To investigate signal transduction of the anti-apoptotic effect of FAK in FAK-transfected cells, we examined tyrosine phosphorylation in these cells. HL-60/FAK and HL-60/Vect cells were incubated with 1 mM hydrogen peroxide for 1 h then lysed and immunoblotted with anti-phosphotyrosine mAb. As shown in Fig. 5A, tyrosine phosphorylation on several protein bands was detected in hydrogen peroxide-treated or non-treated HL-60/FAK cells, showing the activation of these proteins by FAK overexpression regardless of hydrogen peroxide stimulation. The molecular masses of these prominent bands were 120, 52, and 40 kDa, respectively. We identified the band of 120 kDa to be FAK by immunoprecipitation with anti-FAK mAb, followed by blotting with anti-phosphotyrosine mAb (Fig. 5B). From these results, we presumed that the survival pathway (FAK-PI3-kinase-Akt) was activated in HL-60/FAK cells constitutively. Next, we studied the activation of PI3-kinase and Akt, to investigate the link from FAK to PI3-kinase. Cell lysates of HL-60/Vect and HL-60/FAK cells were immunoprecipitated using the anti-FAK mAb, followed by the detection of its associated PI3-kinase, as well as the p85 subunit of PI3-kinase. Without the stimulation of hydrogen peroxide, a significant increase of PI3-kinase activity in HL-60/FAK cells was found in the anti-FAK immunoprecipitates (Fig. 5C). No significant PI3-kinase activity was detected in the HL-60/Vect cells with or without the stimulation of hydrogen peroxide. Immunoblotting of anti-FAK immunoprecipitates with anti-p85 antibody substantially detected the p85 subunit of PI3-kinase (Fig. 5D). These results confirmed that PI3-kinase associates clearly with FAK in HL-60/FAK cells. No significant p85 band was detected in the immunoprecipitates with anti-p85 antibody of HL-60/Vect cells with or without the stimulation of hydrogen peroxide.

In addition, we examined the serine phosphorylation of Akt using anti-phospho-Akt antibody in HL-60/Vect and HL-60/FAK cells. When the cell lysates with or without the stimulation of hydrogen peroxide were subjected to immunoblotting with anti-phospho-Akt or anti-Akt antibodies, the corresponding bands of phospho-Akt were consistently detected in HL-60/FAK cells (data not shown). Tyrosine phosphorylation on several protein bands was detected in hydrogen peroxide-treated or non-treated HL-60/FAK cells, suggesting that enhanced FAK expression blocks apoptosis by inhibiting the increase of caspase-3 activity when induced by these apoptotic stimuli.
Caspase-3 protease is synthesized as a 32-kDa inactive precursor (pro-caspase-3), which is proteolytically cleaved to produce a mature enzyme composed of 17- and 12-kDa subunits (2). To determine at which stages of caspase-3 activation FAK inhibition occurs, we examined the effects on cleavage of the pro-caspase-3 protein in response to these apoptotic inducers. As shown in Fig. 6B, pro-caspase-3 protein disappears concomitantly with apoptosis by hydrogen peroxide treatment in HL-60/Vect cells, indicating that inhibition occurs at the proteolytic cleavage of the pro-caspase-3, which generates active caspase-3 protease fragment. In contrast, pro-caspase-3 protein remains uncleaved in hydrogen peroxide-treated HL-60/FAK cells. Similar results were obtained with etoposide treatment. These results together suggest that FAK suppresses a common upstream step in the pathway leading to the activation of caspase-3 in response to various stimuli rather than directly inhibiting the protease activity.

Overexpressed FAK Induces Sustained NF-κB Activation—It is important to know at which step(s) FAK actually interacts with the apoptosis signal pathway. Therefore, we tested specific inhibitors to locate the inhibition site of the signaling pathway. Addition of 10 μM LY-294002 or 50 μM PDTC, an inhibitor of PI3-kinase or NF-κB, respectively, abrogated the survival effect of FAK partially but not completely (data not shown). The inhibitory effect by LY-294002 and PDTC suggested the involvement of PI3-kinase and NF-κB in the survival pathway used by FAK. Because the importance of PI3-kinase in the anti-apoptotic effect of HL-60/FAK was demonstrated (see Fig. 5), this experiment further confirmed the previous findings.

To further confirm the NF-κB activation in FAK-transfected cells, HL-60/Vect and HL-60/FAK cells were transfected with a luciferase reporter gene that contains putative binding sites for NF-κB (30), followed by the stimulation with hydrogen peroxide or etoposide. A 1.5- and 1.6-fold increase (p < 0.05) in luciferase reporter gene activity was observed in the presence of hydrogen peroxide or etoposide in HL-60/Vect cells compared with unstimulated control cells. In contrast, a 3.2-fold increase (p < 0.005) was observed in HL-60/FAK cells compared with HL-60/Vect cells in unstimulated conditions, indicating that FAK enhances basal NF-κB activity, which was further enhanced with hydrogen peroxide or etoposide treatment (Fig. 7A). The increase of NF-κB activity was inhibited by the addition of 50 μM PDTC or 10 μM LY-294002 down to the unstimulated levels, but still higher than that of HL-60/Vect cells (data not shown), indicating that high basal NF-κB activity in FAK-transfected cells may be related to the anti-apoptotic states of these cells.

Apoptotic Agents and FAK Induce IAP Family Protein Expression—Because NF-κB activation appeared to be critical to the inhibition of hydrogen peroxide- or etoposide-induced apoptosis by FAK, we then explored the expression of survival...
genes, particularly the inhibitory apoptosis protein (IAP) and Bcl-2. The IAP family proteins that block caspase cascade, particularly the proteolytic activation of caspase-3, -7, and -9 (31–33) were reported to be up-regulated followed by the NF-κB activation (34). Therefore, we examined the expression of the IAP family, cIAP-1,2 and XIAP by RT-PCR (Fig. 7B).

Expression of IAPs in HL-60/FAK cells was markedly elevated compared with those in HL-60/Vect cells, and their levels were sustained by hydrogen peroxide or etoposide stimulation. Expression of IAPs in HL-60/Vect cells was only minimally elevated by the above apoptotic stimulation. Expression profiles of the three IAP family proteins were essentially similar. It should be noted that expression of IAPs in HL-60/FAK cells was reduced only partially by the treatment of LY-294002 or PDTC.

As the anti-apoptotic function of the Bcl-2 family proteins involves the inhibition of caspase-3, HL-60/FAK cells may also up-regulate Bcl-2 family proteins. Therefore, we examined the amount of Bcl-2 by immunoblotting, and this indicated that these proteins remained unchanged after hydrogen peroxide or etoposide treatment in HL-60/Vect and HL-60/FAK cells (Fig. 7C).

Anti-apoptotic Effect in Mutated FAK-transfected Cells—To examine further the function of FAK in anti-apoptosis, three mutated FAK cDNA were constructed (35) and tested. One is a kinase-inactive FAK in which the ATP binding site Lys-454 is replaced with Arg (K454R), the second is a mutated form of FAK in which the autophosphorylation site Tyr-397 is replaced with Phe (Y397F), and the third is a mutated FAK of the tyrosine-phosphorylation site Tyr-925 replaced with Phe (Y925F). These FAK mutants were transfected into HL-60 cells, and several clones from each construct were established. The expression level of FAK was confirmed in three lines from the kinase-inactive K454R mutation, six lines with the Y397F mutant, and six lines with the Y925F mutant. The results with the two representative cell lines expressing each of these mutated FAK are shown in Fig. 8A. The anti-apoptotic effect for hydrogen peroxide or etoposide was tested in these cell lines. Because each of the three mutated FAK clones showed similar results, respectively, each representative clone was indicated in Fig. 8, B–D.
NF-κB activation and the expression of IAP proteins, cIAP-1,2 and XIAP, were studied. As shown in Fig. 8, C and D, K454R-transfected cells alone showed a marginal increase of the NF-κB activation and IAPs expression, whereas Y397F- or Y925F-transfected cells did not induce the NF-κB activation and IAPs expression at all.

**DISCUSSION**

In this study, we demonstrated that overexpression of FAK endowed HL-60 cells to protect against apoptosis otherwise induced by two representative apoptosis-inducers, an oxidative stress or an anticancer drug, etoposide. In addition to hydrogen peroxide and etoposide, FAK-overexpressed cells were also found to be resistant to C2-ceramide-induced apoptosis (data not shown).

It has been widely recognized that HL-60 cells do not adhere, but 10 nM PMA-treated cells adhered mildly after 1 day of treatment with PMA and differentiated to macrophage-like cells. HL-60/FAK cells adhered mildly similarly to PMA-treated HL-60 cells (data not shown). It is unknown whether the expression of FAK is prerequisite for or merely concomitant with the differentiation into macrophage form. It should be noted that PMA-treated cells exhibited enhanced FAK expression and acquired the resistance to the hydrogen peroxide-induced apoptosis similar to the HL-60/FAK, strongly suggesting that FAK plays a role in the anti-apoptosis during the differentiation into macrophages. There have been several reports describing the anti-apoptotic roles of FAK in various apoptosis-inducing system. Hungerford et al. (36) reported on anchorage-dependent cells that became apoptotic when cells were microinjected with anti-FAK antibody, or with a peptide corresponding to the portion of the β1-integrin cytoplasmic domain presumed to be required for the β1-integrin-FAK interaction. In another study, Frisch et al. (24) reported that constitutively activated FAK protected MDCK cells from apoptosis consequent to the loss of matrix contact. Furthermore, Xu et al. (37) reported that attenuation of FAK expression leads to apoptosis in some tumor cells. Interestingly, Ilic et al. (38) reported that the extracellular matrix survival signals transduced by FAK suppressed a p53-regulated apoptosis by serum withdrawal in anchorage-dependent cells. Whether FAK has an anti-apoptotic effect on other stress or drug-induced apoptosis has not been explored, and the mechanism of FAK anti-apoptosis has not been elucidated so far. In this study, we showed evidences that demonstrate that FAK has an anti-apoptotic role in the apoptosis induced by oxidative stress as well as etoposide in anchorage-independent HL-60 cells. Etoposide and hydrogen peroxide are known to produce reactive oxygen species (ROS) (39). The conditions used in this study induced apoptosis in parental HL-60 and HL-60/Vect cells during a 4-h incubation period. In contrast, HL-60/FAK cells did not result in apoptosis by these stimuli during the 4-h incubation period. The anti-apoptotic effect by FAK continued at 24 h (data not shown). So far, there has been no reports on the anti-apoptotic role of FAK in ROS-producing stimuli.

Although the mechanism of etoposide-induced apoptosis remains largely unknown, one possible mechanism is that ROS produced by etoposide induce apoptosis. ROS are presumed to destroy mitochondria function and induce apoptosis by activating caspase-3 (19). Caspase-3 is a critical downstream protease in the caspase cascade (40), responsible for the cleavage of important substrates such as poly(adenosine diphosphate ribose) polymerase (41) and inhibitor of caspase-activated deoxyribonuclease (42). The caspase family controls apoptosis by multiple stimuli, including Fas ligand and TNF-α (43, 44). We found HL-60/FAK blocked the caspase-3 activation by hydrogen peroxide or etoposide, which indicated that FAK regulation occurs upstream of caspase-3 activation.

Fas-associated death domain-like ICE inhibitory protein (45) is known as an inhibitor of Fas- and TNF-mediated apoptosis. The anti-apoptotic proteins, CrmA and p35, have also been shown to inhibit apoptosis by directly inhibiting ICE family proteases (3), most likely by functioning as substrates for and as competitive inhibitors of ICE family proteases. In contrast, IAPs bind to caspases and inhibit its activity. Expression of the IAP mRNAs (c-IAP1,2 and XIAP) was minimal in HL-60/Vect cells but remarkable in HL-60/FAK cells during untreated conditions, indicating FAK augmented the expression of these genes. HL-60/FAK showed higher basal NF-κB activation than did HL-60/Vect cells. High constitutive NF-κB activation may be critical to the antiapoptotic states induced by FAK cDNA transfection, which results in sustained expression of IAPs. It is reasonable to link FAK to the IAP, because IAP genes are the NF-κB-regulated genes. We showed here that the FAK-P3-kinase-Akt survival pathway is constitutively activated in HL-60/FAK cells. Recently, it is reported that Akt mediates IκB-kinase phosphorylation following by NF-κB activation (35, 46). We should also take into consideration whether this pathway functions in HL-60/FAK cells.

FAK Tyr-397 is an autophosphorylation site and a high-affinity binding site for Src homology 2 domains of Src family kinases (35). P3-kinase and phospholipase Cγ also interact with this site. FAK Lys-454 is essential for kinase activity. FAK Tyr-925 is a binding site for the Grb2 Src homology 2 domain, and this interaction contributes to integrin-stimulated activation of Ras. Transfection with kinase-inactive FAK (K454R) indicated that the catalytic activity of FAK might be necessary for the full anti-apoptosis effect of FAK. Transfection with FAK mutants (Y397F and Y925F) indicated that tyrosine residues of 397 and 925 were essential for the anti-apoptotic effect. These results suggested that the signals through Src family kinase, P3-kinase and/or Grb2, link to NF-κB activation and induction of IAPs. Further signal analysis of NF-κB activation by FAK should provide more information about the role of FAK in apoptosis. Recently, Chan et al. (47) reported that overexpression of FAK in Madin-Darby canine kidney cells suppressed the UV-induced apoptosis. Furthermore, they compared the anti-apoptotic activity of wild type FAK with those of FAK mutants (D395A, Y397F, p712/715A, and Y925F). These mutants failed to promote cell survival upon UV irradiation. The interaction of FAK with these proteins might lead to survival signals from FAK, proposing that the binding of P3-kinase and p130cas with FAK is required for the anti-apoptotic function of FAK. We demonstrated here that FAK protects against oxidative stress-induced apoptosis in HL-60 cells as well as the glioblastoma cell line T98G (27). In addition, Chan et al. (47) indicated that FAK protects against the UV-induced apoptosis in Madin-Darby canine kidney cells, supporting the notion of the general feature of FAK as a role of anti-apoptosis in various cells. Particularly, the involvement of NF-κB and IAPs and the resulting abrogation of caspase-3 activation was evidenced in the suppression of apoptosis in this study. To our knowledge, the data presented here provide the first line of evidence for the regulation of IAPs through NF-κB by FAK.

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Anti-apoptotic Role of Focal Adhesion Kinase (FAK): INDUCTION OF INHIBITOR-OF-APOPTOSIS PROTEINS AND APOPTOSIS SUPPRESSION BY THE OVEREXPRESSION OF FAK IN A HUMAN LEUKEMIC CELL LINE, HL-60

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