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Transforming Growth Factor-β1 Induces Apoptosis through Fas Ligand-independent Activation of the Fas Death Pathway in Human Gastric SNU-620 Carcinoma Cells

Sang Gyun Kim,*† Hyun-Soon Jong,* Tae-You Kim,*‡ Jung Weon Lee,* Noe Kyeong Kim,‡ Seung Hwan Hong,† and Yung-Jue Bang*‡§

*National Research Laboratory for Cancer Epigenetics, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-744, Korea;‡Department of Internal Medicine, Seoul National University College of Medicine, Seoul 110-744, Korea; and †School of Biological Sciences and Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea

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To date, two major apoptotic pathways, the death receptor and the mitochondrial pathway, have been well documented in mammalian cells. However, the involvement of these two apoptotic pathways, particularly the death receptor pathway, in transforming growth factor-β1 (TGF-β1)-induced apoptosis is not well understood. Herein, we report that apoptosis of human gastric SNU-620 carcinoma cells induced by TGF-β1 is caused by the Fas death pathway in a Fas ligand-independent manner, and that the Fas death pathway activated by TGF-β1 is linked to the mitochondrial apoptotic pathway via Bid mediation. We showed that TGF-β1 induced the expression and activation of Fas and the subsequent caspase-8–mediated Bid cleavage. Interestingly, expression of dominant negative FADD and treatment with caspase-8 inhibitor efficiently prevented TGF-β1–induced apoptosis, whereas the treatment with an activating CH11 or a neutralizing ZB4 anti-Fas antibody, recombinant Fas ligand, or Fas-Fc chimera did not affect activation of Fas and the subsequent induction of apoptosis by TGF-β1. We further demonstrated that TGF-β1 also activates the mitochondrial pathway showing Bid-mediated loss of mitochondrial membrane potential and subsequent cytochrome *c* release associated with the activations of caspase-9 and the effector caspases. Moreover, all these apoptotic events induced by TGF-β1 were found to be effectively inhibited by Smad3 knockdown and also completely abrogated by Smad7 expression, suggesting the involvement of the Smad3 pathway upstream of the Fas death pathway by TGF-β1.

INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is the prototype of a widespread and evolutionarily conserved superfamily of cytokines that regulate a broad spectrum of cellular responses, including proliferation, differentiation, and apoptosis (Roberts and Sporn, 1990; Derynck and Feng, 1997; Moustakas *et al*., 2002). TGF-β1 exerts its cellular actions by binding to a heteromeric receptor complex consisting of type I (TβRI) and type II (TβRII) serine/threonine kinase receptor subunits. On ligand binding, TβRII transphosphorylates and thereby activates TβRI, which subsequently phosphorylates receptor-regulated Smad (R-Smad), Smad2, and Smad3. Activated R-Smad then undergoes a conformational change that allows heteromerization with a common partner, Smad4 (Heldin *et al*., 1997; Derynck *et al*., 1998; Massagué and Chen, 2000). These complexes are subsequently translocated into the nucleus and act as TGF-β1–sensitive transcriptional coactivators or corepressors by interacting with a variety of transcription factors (Attisano and Wrana, 2000; Massagué and Wotton, 2000). TGF-β1 responses can also be modulated by the inhibitory Smad, Smad6, and Smad7, which bind to the activated receptors or R-Smad, thereby preventing further propagation of TGF-β1 signaling (Hayashi *et al*., 1997; Imamura *et al*., 1997; Nakao *et al*., 1997).

TGF-β1 has been shown to elicit apoptotic cell death in a variety of cell types. Moreover, TGF-β1–induced apoptosis plays important roles in the selective elimination of damaged or abnormal cells from various normal tissues (Oberhammer *et al*., 1992; Chaouchi *et al*., 1995), resulting in the proper development of a variety of tissues and organs, including the rhombencephalic neural crest (Graham *et al*., 1996), the interdigital fields of the limb (Zou *et al*., 1997), and the mammary gland ductal system (Nguyen and Pollard, 2000). In addition to these significant roles of TGF-β1–induced apoptosis in development, apoptosis by TGF-β1 and the selective elimination of preneoplastic cells may also be involved in TGF-β1–mediated tumor suppression (Gold, 1999; de Caestecker *et al*., 2000). Although TGF-β1–induced apoptosis is a well-documented phenomenon in many different cell types, the biochemical mechanism responsible for mediating this death process is still poorly understood. Some reports have suggested that Bcl-2 family members and caspases, involved in the apoptotic effector system, are activated in cells undergoing TGF-β1–induced apoptosis (Chen and Chang, 1997; Saltzman *et al*., 1998). Recently, it was shown that Daxx, a Fas receptor-associated protein, which

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§ Corresponding author. E-mail address: bangyj@plaza.snu.ac.kr.

mediates the activation of JNK and the apoptotic cell death induced by Fas, physically interacts with TβRII and is involved in mediating TGF-β–induced apoptosis (Perlman *et al*., 2001).

Until recently, two major apoptotic pathways, the death receptor pathway and the mitochondrial apoptotic pathways, are well characterized in mammalian cells (Hengartner, 2000). The death receptor pathway is triggered by members of the death receptor family, such as Fas receptor and tumor necrosis factor receptor (Trauth *et al*., 1989; Itoh *et al*., 1991). The binding of Fas ligand (FasL) to the Fas receptor induces receptor clustering and the formation of a death-inducing signaling complex, which in turn recruits and activates caspase-8, as an initiator caspase, via the adaptor molecule Fas-associated death domain protein (FADD) (Suda *et al*., 1993; Boldin *et al*., 1996; Wallach *et al*., 1999). On the other hand, the mitochondrial pathway is used extensively in response to extracellular cues and internal insults such as DNA damage (Hengartner, 2000). These diverse response pathways converge on mitochondria, often through the activation of a proapoptotic member of the Bcl-2 family. These species are mainly responsible for changes in mitochondria, including the opening of permeability transition pores, a decrease in the mitochondrial membrane potential, and the release of cytochrome *c* into cytoplasm (Adams and Cory, 1998; Green and Reed, 1998). This released cytochrome *c* associates with Apaf-1 and then activates caspase-9 as an initiator caspase (Li *et al*., 1997). Meanwhile, cross-talk and integration between the death receptor and the mitochondrial pathways have also shown that caspase-8 can activate the mitochondrial pathway by cleaving Bid, a proapoptotic member of the Bcl-2 family (Li *et al*., 1998; Yin *et al*., 1999). Ultimately, all of these apoptotic pathways converge at the level of effector caspase activation, e.g., of caspase-3 (Salvesen and Dixit, 1997).

Although two major apoptotic pathways have been well defined in mammalian cells, the involvement of these two apoptotic pathways in TGF-β1–induced apoptosis is still obscure. In particular, the activation of the death receptor pathway by TGF-β1 has not been previously reported. Here, we found that, in human gastric SNU-620 carcinoma cells, the death receptor pathway, more precisely the Fas receptor death pathway, can be activated by TGF-β1 in a ligand-independent manner, and also that this pathway is interconnected with the mitochondrial pathway via caspase-8–mediated Bid cleavage. Furthermore, we found that the Smad3 pathway acts upstream of the Fas-FADD pathway during TGF-β1–induced apoptosis in SNU-620 cells.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies to caspase-3 (H-277), cytochrome *c* (H-104), and Fas (B-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to human caspase-6 (B93–4), -7 (B94–1), -8 (rabbit polyclonal), Bid (rabbit polyclonal), and fluorescein isothiocyanate (FITC)-conjugated antihuman Fas antibody (DX2) were purchased from BD PharMingen (San Diego, CA). Antibodies to neutralizing Fas (ZB4), activating Fas (CH11), and caspase-9 (96-2-22) were purchased from Upstate Biotechnology (Waltham, MA). FasL kit, consisting of a recombinant soluble FasL and its potentiator, was also purchased from Upstate Biotechnology. Recombinant human Fas-Fc chimera was obtained from R&D Systems (Minneapolis, MN), anti-FLAG (M2) from Sigma-Aldrich (St. Louis, MO), and rabbit polyclonal anti-Smad2 and anti-Smad3 from Zymed Laboratories (South San Francisco, CA). The caspase inhibitors, including zVAD-fmk (a pan-caspase inhibitor), zIETD-fmk (caspase-8 inhibitor), zLEHD-fmk (caspase-9 inhibitor), zDEVD-fmk (caspase-3 inhibitor), and zFA-fmk (the negative control), were purchased from Calbiochem (San Diego, CA). The mitochondrial membrane potential probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazoylcarbocyanine iodide (JC-1) was obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich or Calbiochem.

Cell Culture and TGF-β1 Treatment

Human gastric SNU-620 carcinoma cell line (purchased from Korea Cell Line Bank, Seoul, Korea) is described as a cell line established from the malignant ascites of a gastric cancer patient and exhibits anchorage-independent growth as a single cell (Park *et al*., 1997). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 100 U of penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Human recombinant TGF-β1 was rehydrated in a 4 mM HCl, 1 mg/ml bovine serum albumin (BSA) solution at a concentration of 5 μg/ml and used at a final concentration of 5 ng/ml in all experiments.

DNA Analysis by Flow Cytometry

Apoptosis was monitored by measuring hypodiploid DNA content (sub-G1). After various treatments, cells were harvested by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol for 1 h. Fixed cells were then washed with ice-cold PBS and stained with 20 μg/ml propidium iodide (PI; Sigma-Aldrich) containing 10 μg/ml RNase A (Sigma-Aldrich). After incubation in the dark for at least 30 min, the DNA content of cells was determined by a FACSCalibur flow cytometry (FL-2) (BD Biosciences, San Jose, CA). Ten thousand events were counted for each analysis.

Annexin V and Terminal Deoxythymidine Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay

Annexin V and TUNEL assays were used to evaluate apoptotic cell death. Annexin V staining was carried out using an annexin V assay kit (BD PharMingen), and TUNEL assay was performed using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany), as described previously (Kim *et al*., 2001).

Western Analysis

Cells were washed with PBS and then suspended in an extraction buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 μg/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, and 1 mM benzamidine) on ice for 15 min. Lysates were cleared by centrifugation at 10,000 × *g* for 20 min. Equal amounts of cell extracts were resolved on SDS-polyacrylamide denaturing gels, transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and probed with an appropriate primary and horseradish peroxidase-conjugated secondary antibody. Detection was performed using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR was performed to assess Fas and FasL expression in SNU-620 cells. Total cellular RNA was isolated using TriZOL reagent (Molecular Research Center, Cincinnati, OH) at each time point. For RT reactions, 0.5 μg of total RNA was mixed with 0.5 μg of oligo d(T), a 10 mM concentration of each dNTP, 1 μg of RNasin, and RT buffer (50 mM Tris-Cl, pH 8.3, 30 mM KCl, 8 mM $MgCl_2$, and 10 mM dithiothreitol). These mixtures were then incubated at 70°C for 5 min and cooled on ice. After adding 1 U of M-MuLV reverse transcriptase (Invitrogen, Carlsbad, CA) to the mixtures, RT was performed at 37°C for 50 min, and the resulting cDNA was amplified by PCR. Primers for the Fas receptor were sense, 5′-AAG TGA CTG ACA TCA ACT CC-3′; and antisense, 5′-CAC TTC TAA GCC ATG TCC-3′. Primers for the Fas ligand were sense, 5′-ACA TGA GGA ACT CTA AGT ATC C-3′; and antisense, 5′-AAA ATT GAC CAG AGA GAG C-3′. The PCR reactions involved an initial heating at 94°C for 2 min and then 35 cycles of 94°C for 1 min, 55°C (Fas receptor) or 56°C (Fas ligand) for 1 min, and 72°C for 1.5 min. As the quantitative control, β-actin PCR (sense, 5′-CAC TGT GTT GGC GTA CAG GT-3′; antisense, 5′-TCA TCA CCA TTG GCA ATG AG-3′) was also performed for 25 cycles with the same cycle profile as used for the Fas receptor.

Detection of Cell Surface Fas by Flow Cytometry

The expression of cell surface Fas was measured by immunofluorescence flow cytometric analysis. After various treatments, a total of 1×10^6 cells was collected by centrifugation and washed twice with ice-cold PBS containing 1% BSA. Cells were then incubated with 100 μl of fluorescein isothiocyanate (FITC)-conjugated anti-Fas antibody (BD PharMingen) on ice for 40 min. After incubation in the dark, cells were washed twice and resuspended in ice-cold PBS. Immunofluorescence staining of cell surface Fas was analyzed by FACSCalibur flow cytometry (FL-1) by using the CellQuest analysis program (BD Biosciences, San Jose, CA).

Confocal Laser Scanning Microscopy Analysis of Fas Receptor Clustering

After treatment with an activating CH11 anti-Fas antibody or TGF-β1 in the presence or absence of neutralizing ZB4 anti-Fas antibody, Jurkat and SNU-620 cells were harvested and washed twice with ice-cold PBS containing 1% BSA. Collected cells were incubated with 100 μl of FITC-conjugated anti-Fas antibody (BD PharMingen) on ice for 40 min and then washed twice with ice-cold PBS. After staining, the cells were mounted and subjected to confocal laser scanning microscopic analysis (Carl Zeiss, Thornwood, NY). To retard fluorescence fading during laser scanning, 1.4-diazobicyclo-(2,2,2,)-octane (Merck, Darmstadt, Germany) was added to the mounting solution.

Analysis of Cytochrome c Release

To detect mitochondrial cytochrome *c* release into the cytoplasm, cells were harvested at each time point after TGF-β1 treatment, resuspended in isotonic isolation buffer (10 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.6), and collected by centrifugation. The cells were then suspended in hypotonic isolation buffer (10 mM HEPES, 1 mM EDTA, 50 mM sucrose, pH 7.6) and disrupted by passing them through a 27-gauge needle 5–10 times. After adding hypertonic isolation buffer (10 mM HEPES, 1 mM EDTA, 450 mM sucrose, pH 7.6) to balance the buffer's tonicity, cells were centrifuged at $1000 \times g$ for 10 min at 4°C. Supernatant was recovered and centrifuged again at $10{,}000 \times g$. The mitochondrial proteins were recovered in pellet with isotonic isolation buffer and the supernatant was used for the cytosolic protein extracts. After determining the protein concentration of each lysate, the change in the level of cytochrome *c* was measured by Western blotting.

Assessment of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

To measure $\Delta\Psi_m$, 1×10^6 cells treated with TGF-β1 for various times were stained with 5 μg/ml JC-1 (Molecular Probes). This cyanine dye accumulates in the mitochondrial matrix under the influence of $\Delta\Psi_m$ and forms aggregates that have characteristic absorption and emission spectra. After incubation for 30 min at room temperature in the dark, cells were washed twice with ice-cold PBS and then analyzed by a FACSCalibur flow cytometry (BD Biosciences). Fluorescence was induced with an Argon laser (excitation wavelength, 488 nm) and the green fluorescence was collected through 585/42 nm (FL-2) and 530/30 nm (FL-1) bandpass filters. At least, 2×10^4 events were acquired and analyzed using the CellQuest analysis program (BD Biosciences).

Transient Transfection and Cell Death Measurement

For transient transfection, cells were harvested, washed once with serum-free RPMI 1640 medium, and seeded at a density of 2×10^6 cells/35-mm dish in 1.5 ml of RPMI 1640 medium containing 5% fetal bovine serum. Cells were then transfected with 1 μg of pCI (Promega, Madison, WI) or pCI-Fas (kindly provided by Dr. M. Lenardo, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) by using LipofectAMINE 2000 (Invitrogen). In the case of DN-FADD, cells were cotransfected with 0.5 μg of pEGFP-N1 and 0.5–2 μg of either pcDNA3-DN-FADD (kindly provided by Dr. V. Dixit, University of Michigan, Ann Arbor, MI) or pcDNA3. After transfection for 4 h, normal medium was added and cells were further incubated for 12 h before TGF-β1 treatment. After TGF-β1 treatment for 24 or 36 h, cells were harvested, washed twice with ice-cold PBS, and analyzed for the cell death. Apoptosis assessment was performed by detecting active caspase-3 in transfected cells using a phycoerythrin (PE)-conjugated monoclonal anti-active caspse-3 antibody kit (BD PharMingen), according to the manufacturer's recommendations. For the flow cytometric analysis, the green cells (green fluorescent protein [GFP] positive) were first gated using an FL-1 channel, and the fraction of red cells (PE positive) was immediately quantified using an FL-2 channel.

Smad2/3 Knockdown Assay Using Small Interfering RNA (siRNA)

For siRNA treatment, 1.0 μg of control, Smad2, or Smad3 dsRNA oligomers was transfected using LipofectAMINE 2000 (Invitrogen). After transfection for 4 h, normal medium was added and cells were further incubated for 12 h before TGF-β1 treatment. Nonsilencing control siRNA and Smad2/3 siRNA were purchased from Cellogenetics (Gaithersburg, MD).

Adenoviral Infections

Recombinant adenoviruses expressing Smad2, Smad3, Smad4, Smad7, or β-galactosidase were kindly provided by Dr. Kohei Miyazono (The Cancer Institute, Tokyo University, Tokyo, Japan) and were used individually at a multiplicity of infection (MOI) of 50, as described by Fujii *et al*. (1999).

RESULTS

TGF-β1 Induces Caspases-dependent Apoptosis in Human Gastric SNU-620 Carcinoma Cells

To test whether TGF-β1 triggers apoptosis in SNU-620 cells, we first analyzed the cell cycle profile by examining the cellular DNA content. When cells were treated with 5 ng/ml TGF-β1, the population of cells in sub-G1 phase slightly increased until 12 h, and then increased rapidly after 24 h (Figure 1A). To confirm apoptosis, annexin V labeling was also performed to detect phosphatidylserine externalization, a hallmark of apoptosis. In agreement with the result of cell cycle profile, annexin V-positive apoptotic cells were detected and increased time dependently in TGF-β1–treated SNU-620 cells (Figure 1B).

Next, the involvement of caspases activation was examined because caspase are central components of the machinery for apoptosis (Thornberry and Lazebnik, 1998; Earnshaw *et al*., 1999). When caspase-8 and caspase-9, known as general initiator caspases, were investigated by Western blot analyses with their respective antibodies, an active fragment of caspase-8 started to occur 12 h after TGF-β1 treatment. This fragment increased time dependently, whereas the corresponding caspase-9 active fragment occurred 24 h after TGF-β1 treatment (Figure 2A). When effector caspases, caspase-3, caspase-6, and caspase-7, were checked during apoptosis, all of their active fragments began to occur 24 h posttreatment (Figure 2B). These results suggest that activation of these caspases is involved in TGF-β1–induced apoptosis and that caspase-8 may be the first caspase activated by TGF-β1 in SNU-620 cells.

We then examined the effects of various caspase inhibitors on the apoptotic status. Accordingly, cells were analyzed by flow cytometry after PI staining after 48-h incubation with or without TGF-β1 in the absence or in the presence of a specific caspase inhibitor. When TGF-β1 was treated alone, apoptotic cell death was induced by up to 22.6% (versus 4.7% in control cells), and pretreatment with 50 μM zFA-fmk, as a chemical control, did not affect this apoptotic cell death level (Figure 2C). However, in the presence of 50 μM of the pan-caspase inhibitor (zVAD-fmk), TGF-β1–induced apoptosis was completely suppressed (Figure 2C). A similar pattern was also observed with a 50 μM concentration of the caspase-8 inhibitor (zIETD-fmk), caspase-9 inhibitor (zLEHD-fmk), or caspase-3 inhibitor (zDEVD-fmk), although the apoptosis-suppressing effects of these inhibitors were relatively weaker than those of the pan-caspase inhibitor (Figure 2C). These results indicate that TGF-β1-induced apoptosis is dependent on the activation of these caspases.

Activation of the Mitochondrial Pathway by Bid Mediation during Apoptosis

Because TGF-β1 induced the activation of both initiator caspases, i.e., caspase-8 and caspase-9 (Figure 2A), and treatment with their specific inhibitors equally suppressed apoptosis by TGF-β1 in SNU-620 cells (Figure 2C), it is plausible that TGF-β1 activates the mitochondrial apoptotic pathway through caspase-8–mediated Bid cleavage, which results in cytochrome c release and caspase-9 activation. To test this idea, we next checked the status of Bid protein during TGF-β1–induced apoptosis. Although we could not detect the cleaved form of Bid by Western blotting, the level of its native protein abruptly decreased within 24 h of TGF-β1 treatment (Figure 3A), and this was well matched to the appearance of the active caspase-8 fragment (Figure 2A). To assess whether proteolytic cleavage by caspase-8 is really responsible for the truncation of Bid, we investigated the effects of various caspase inhibitors on Bid cleavage. When

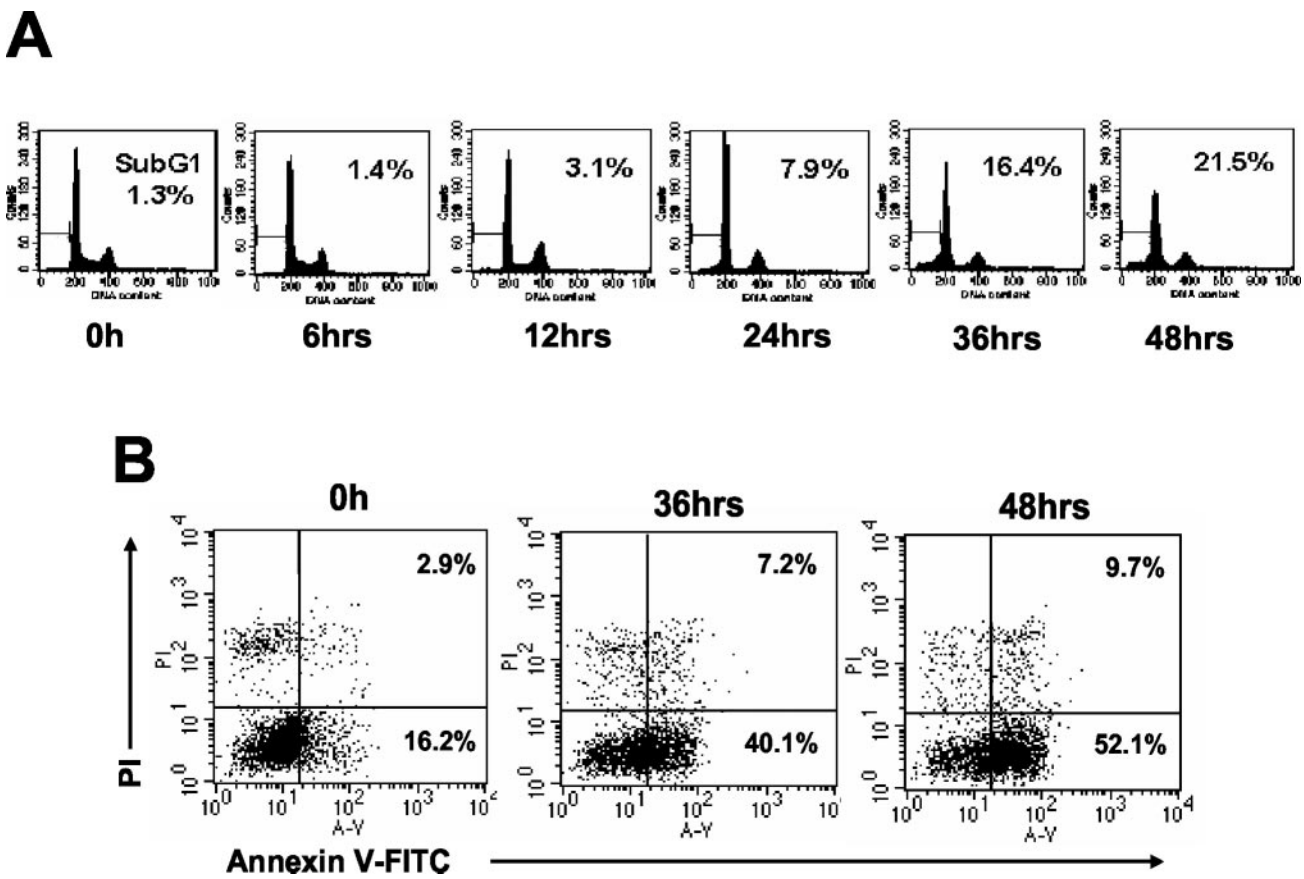

Figure 1. Apoptosis induced by TGF-β1 in SNU-620 cells. (A) The DNA content histograms of TGF-β1–treated cells. At the indicated time points after treatment with 5 ng/ml TGF-β1, cells were fixed with 70% ethanol, stained with PI, and then subjected to flow cytometric analysis. The percentage of sub-G1 phase cells was determined based on the DNA content histograms. Results are typical of at least three individual experiments. (B) The externalization of phosphatidylserine during TGF-β1–induced apoptosis. Control cells or cells treated with 5 ng/ml TGF-β1 for the indicated time periods were stained with annexin V and PI and analyzed by flow cytometry. The percentage of cells in each window is indicated. The percentage of cells in the lower right quadrant dramatically increased in a time-dependent manner.

cells were pretreated with the specific caspase-8 inhibitor zIETD-fmk before TGF-β1 treatment, the decreased level of Bid protein in TGF-β1–treated cells was recovered to near that of the untreated control cells (Figure 3B, lane 4). A similar pattern was observed for the pan-caspase inhibitor zVAD-fmk (Figure 3B, lane 3). However, pretreatment with the specific caspase-9 inhibitor zLEHD-fmk did not prevent the disappearance of native Bid protein (Figure 3B, lane 5). These results indicate that Bid protein is cleaved by caspase-8, which is activated by TGF-β1 treatment, and that the activation of caspase-9 may be a downstream event of this caspase-8–mediated Bid cleavage.

We then examined whether the cleavage of Bid by caspase-8 leads to the activation of the mitochondrial apoptotic pathway. To do this, we sequentially investigated the loss of mitochondrial inner membrane potential ($\Delta\Psi_m$) and the release of cytochrome *c* into cytosol. Loss of $\Delta\Psi_m$ has been shown to be implicated in the execution of apoptosis, because of alterations in permeability transition pores located between the inner and outer membranes (Green and Reed, 1998). When $\Delta\Psi_m$ was measured by staining TGF-β1–treated cells with the $\Delta\Psi_m$ probe, JC-1, the loss of $\Delta\Psi_m$ slowly increased until 12 h after treatment and then abruptly increased after 24 h (Figure 3C), which correlated well with the status of Bid protein (Figure 3, A and C). Concomitantly, mitochondrial cytochrome *c* was released into the cytosol from mitochondria (Figure 3D). These results show that the mitochondrial apoptotic pathway is turned on by the caspase-8–mediated cleavage of Bid during the TGF-β1–induced apoptosis of SNU-620 cells.

Induction of Fas Receptor during TGF-β1–induced Apoptosis

Our current observations suggest that TGF-β1 uses caspase-8 as an initiator caspase to trigger the activation of a downstream caspase cascade and apoptosis in SNU-620 cells. Caspase-8 is known to be principally activated by a death receptor like Fas during the death receptor apoptotic pathway (Wallach *et al.*, 1999). To assess whether the death receptor pathway is also involved in TGF-β1–induced apoptosis, we next checked whether the status of the cell surface Fas receptor, which is a representative member of the death receptor family, is influenced by TGF-β1. Interestingly, immunofluorescent staining and flow cytometric analysis using an anti-Fas monoclonal antibody revealed that the level of the cell surface Fas receptor was dramatically increased in TGF-β1–treated cells compared with the untreated controls (Figure 4A). In addition, RT-PCR and Western blotting for Fas receptor demonstrated that the levels of both Fas mRNA and protein started to increase 12 h after TGF-β1 treatment and that this level was further augmented thereafter (Figure 4, B and C), which might result in a time-dependent increase of the cell surface Fas receptor in SNU-620 cells (our unpublished data). Moreover, these obser-

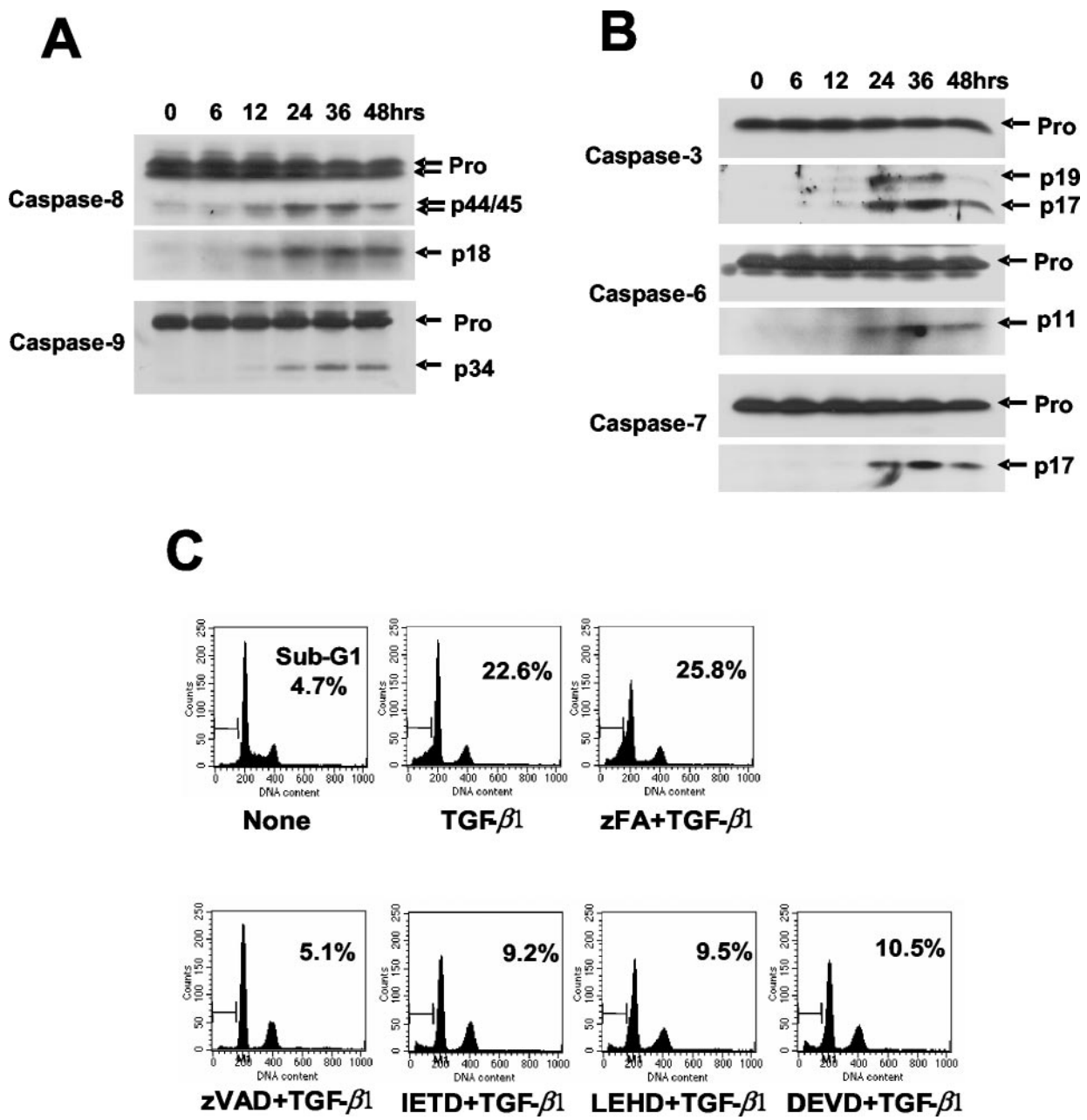

Figure 2. Caspases-dependent apoptosis by TGF-β1 in SNU-620 cells. (A) The activation of initiator caspases during TGF-β1–induced apoptosis. Cells were treated with 5 ng/ml TGF-β1 for the indicated times. Equal amounts of cell extracts were resolved by SDS-PAGE and analyzed by Western blotting with antibodies specific for caspase-8 and caspase-9. The proforms and the cleaved active forms of each caspases were indicated. (B) Activation of effector caspases by TGF-β1. Samples were prepared as described in A and Western blotted with antibodies to caspase-3, -6, and -7, respectively. The proforms and the cleaved active forms of each caspases were indicated. (C) Effects of various caspase inhibitors on TGF-β1–induced apoptosis. Cells were preincubated for 1 h with 50 μM of pan-caspase inhibitor (zVAD-fmk), caspase-8 inhibitor (zIETD-fmk), caspase-9 inhibitor (zLEHD-fmk), caspase-3 inhibitor (zDEVD-fmk), or control peptide (zFA-fmk) before TGF-β1 treatment. Samples were then taken after 48 h, and DNA contents were determined by flow cytometry. The percentages of cells in sub-G1 are indicated.

vations matched the onset of apoptosis (Figures 1A and 4C). Thus, these results suggest that the TGF-β1–induced apoptotic events might be initiated from the induction of the Fas receptor and that this leads to the activation of caspase-8.

Apoptosis by TGF-β1 Is Independent of Fas–FasL Interaction in SNU-620 Cells

Because the main death pathway induced by Fas activation is generally initiated by FasL binding to its receptor, we next examined whether TGF-β1–induced apoptosis involves an interaction between Fas and FasL. To test this, we investigated the effects of the neutralizing ZB4 and activating CH11 anti-Fas antibodies on TGF-β1–induced apoptosis. Previous reports have shown that the CH11 antibody functions like FasL as an agonist and that the ZB4 antibody disturbs Fas–FasL interaction by acting as an antagonist (Yonehara *et al*., 1989; Itoh *et al*., 1991). To confirm the roles of these antibodies, we first experimented with Jurkat cells. When these cells

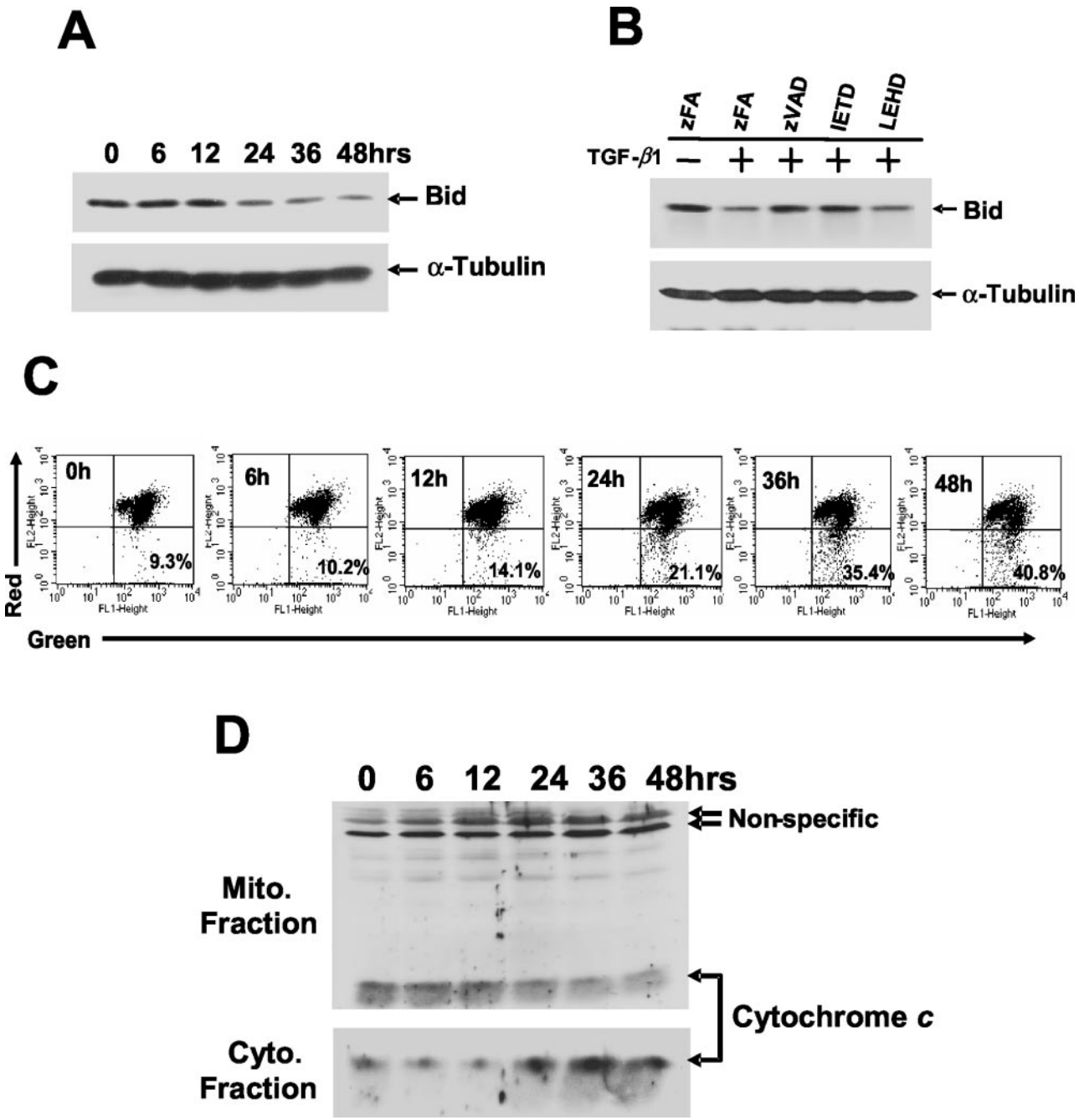

Figure 3. Activation of the mitochondrial pathway by caspase-8–mediated Bid cleavage. (A) Bid cleavage during TGF-β1–induced apoptosis. SNU-620 cells were incubated with 5 ng/ml TGF-β1 for the indicated times. Equal amounts of whole cell extracts were separated by SDS-PAGE, and the level of Bid protein was determined by Western blotting with anti-Bid antibody. The amount of protein loaded in each lane was assessed by stripping and reprobing with an antibody for α-tubulin. (B) Effects of various caspase inhibitors on Bid cleavage. Cells were incubated for 36 h with (+) or without (−) 5 ng/ml TGF-β1 in the presence of 50 μM of various caspase inhibitors or control peptide (zFA-fmk). Whole cell extracts were prepared from each of the treatment groups and the levels of Bid and α-tubulin proteins were determined as described in A. (C) The loss of $\Delta\Psi_m$ during TGF-β1–induced apoptosis. Cells were treated with TGF-β1 for the indicated times and then stained with the mitochondrial membrane potential probe JC-1 (5 μg/ml). The intracellular fluorescence intensity was measured by flow cytometry. The data shown are representative of experiments run more than three times. The percentages of cells with disrupted $\Delta\Psi_m$ are indicated. (D) Release of cytochrome *c* from mitochondria to the cytosol in TGF-β1–treated cells. After incubating cells with 5 ng/ml TGF-β1 for the indicated times, mitochondria were separated from the cytosol, and the level of cytochrome *c* was determined by Western blotting as described in MATERIALS AND METHODS. The amount of loaded protein was confirmed versus a nonspecific protein band.

were treated with CH11 antibody for 36 h, apoptosis was induced by up to 20.5% (versus 1.2% in control cells) in the total population, and this induction was completely inhibited by ZB4 anti-Fas antibody (Figure 5A).

We then examined the effects of these antibodies on SNU-620 cells. When the CH11 antibody was treated alone, it failed to trigger apoptosis in SNU-620 cells (Figure 5B). Comparison of the Fas expression levels between Jurkat and

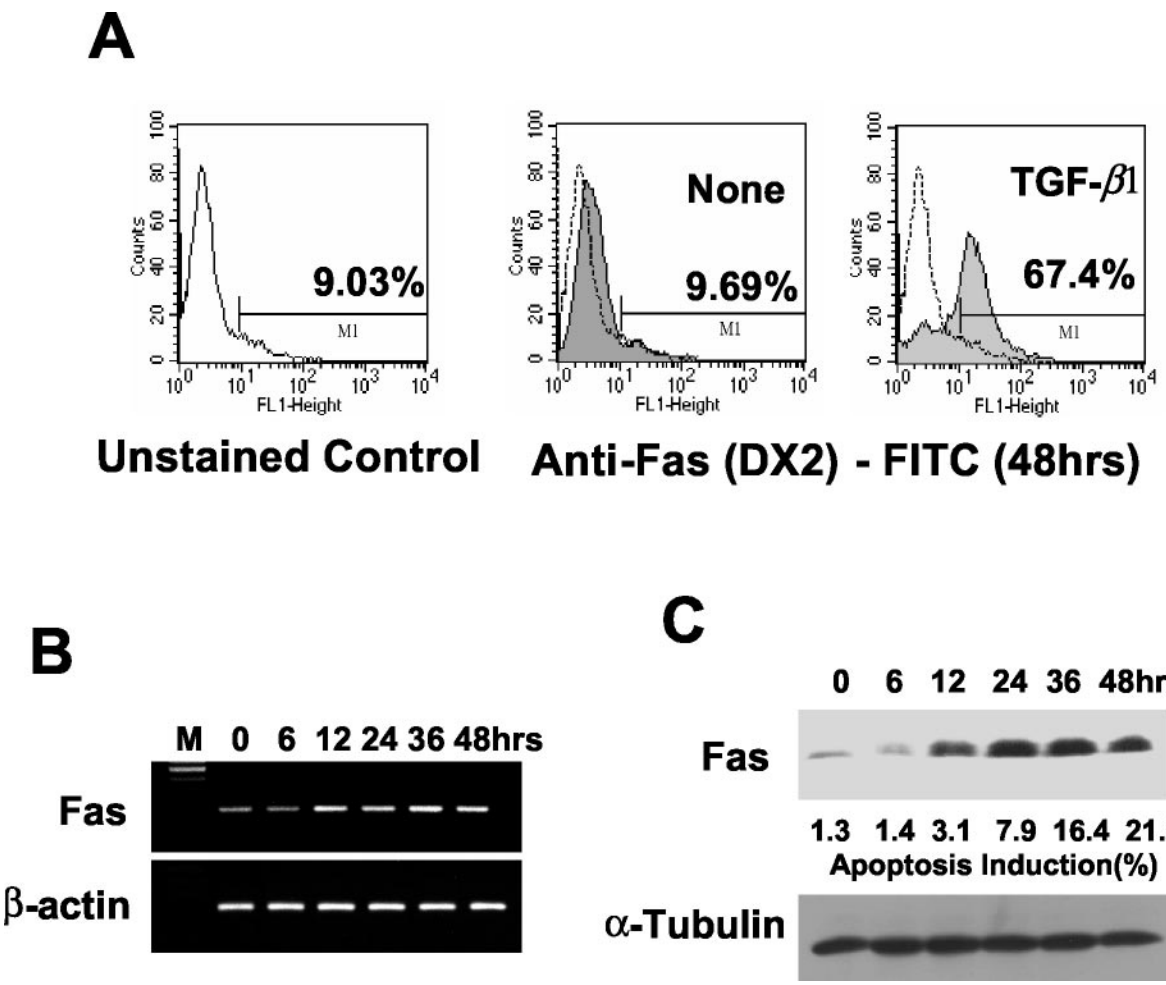

Figure 4. Induction of Fas receptor by TGF-β1 in SNU-620 cells. (A) Evaluation of the cell surface expression of Fas receptor. After incubating cells with or without TGF-β1 (5 ng/ml) for 48 h, cells were stained with FITC-conjugated anti-Fas-specific antibody. The cell surface expression of Fas was analyzed by flow cytometry as described in MATERIALS AND METHODS. The percentages of FITC-positive cells are indicated. (B) Evaluation of Fas mRNA expression by RT-PCR. Total RNA was purified from cells treated with 5 ng/ml TGF-β1 for the indicated times, and the levels of Fas and β-actin mRNA were determined by RT-PCR as described in MATERIALS AND METHODS. Data are representative of three independent experiments. M, DNA size marker. (C) Evaluation of Fas protein expression. Whole cell extracts obtained from cells treated with TGF-β1 for the indicated times were separated by SDS-PAGE. Western blot analysis was carried out using anti-Fas or anti-α-tubulin antibody.

the resting SNU-620 cells revealed that the different responsiveness to CH11 might be caused by the significantly higher level of Fas expression in Jurkat cells than in SNU-620 cells (Figure 5D). However, despite the level of Fas expression increased by TGF-β1 in SNU-620 cells (Figure 4A), cotreatment of CH11 with TGF-β1 for 36 h did not show any enhancing effect on apoptosis by TGF-β1, even at a concentration 20 times higher than that used in Jurkat cells (Figure 5B). In addition, pretreatment with the neutralizing ZB4 antibody before TGF-β1 treatment did not show any blocking effect on TGF-β1-induced apoptosis, even at a concentration 5 times higher than that used in Jurkat cells (Figure 5B). We also used recombinant soluble FasL (sFasL) and soluble Fas-Fc fusion chimera to mimic or block the interaction between Fas and FasL, respectively. However, the TGF-β1–induced apoptosis of SNU-620 cells was not influenced by the presence of sFasL or of Fas-Fc (Figure 5C). These results suggest that the Fas–FasL interaction may not play a central role in TGF-β1–induced apoptosis, which is further supported by another observation, namely, that FasL expression is barely detectable in SNU-620 cells even by RT-PCR (our unpublished data).

FasL-independent Activation of Fas Receptor

Next, we checked whether the activation of the Fas receptor could be triggered by TGF-β1 in a FasL-independent manner in SNU-620 cells. To examine whether Fas receptor clustering, a hallmark of the Fas receptor activation, is induced by TGF-β1, the cell surface distribution of Fas receptor was investigated by confocal immunofluorescence microscopy. In Jurkat cells, treatment with an activating CH11 anti-Fas antibody led to the increased aggregation of Fas receptors on the cell surface, and this aggregation disappeared completely in the presence of a neutralizing ZB4 anti-Fas antibody (Figure 6A). Interestingly, when TGF-β1 was treated to SNU-620 cells, a similarly increased aggregation of Fas receptors was observed on the cell surface versus the untreated control cells (Figure 6B). In addition, this aggregation was not inhibited by pretreatment with a neutralizing ZB4 antibody or Fas-Fc chimera (Figure 6B; our

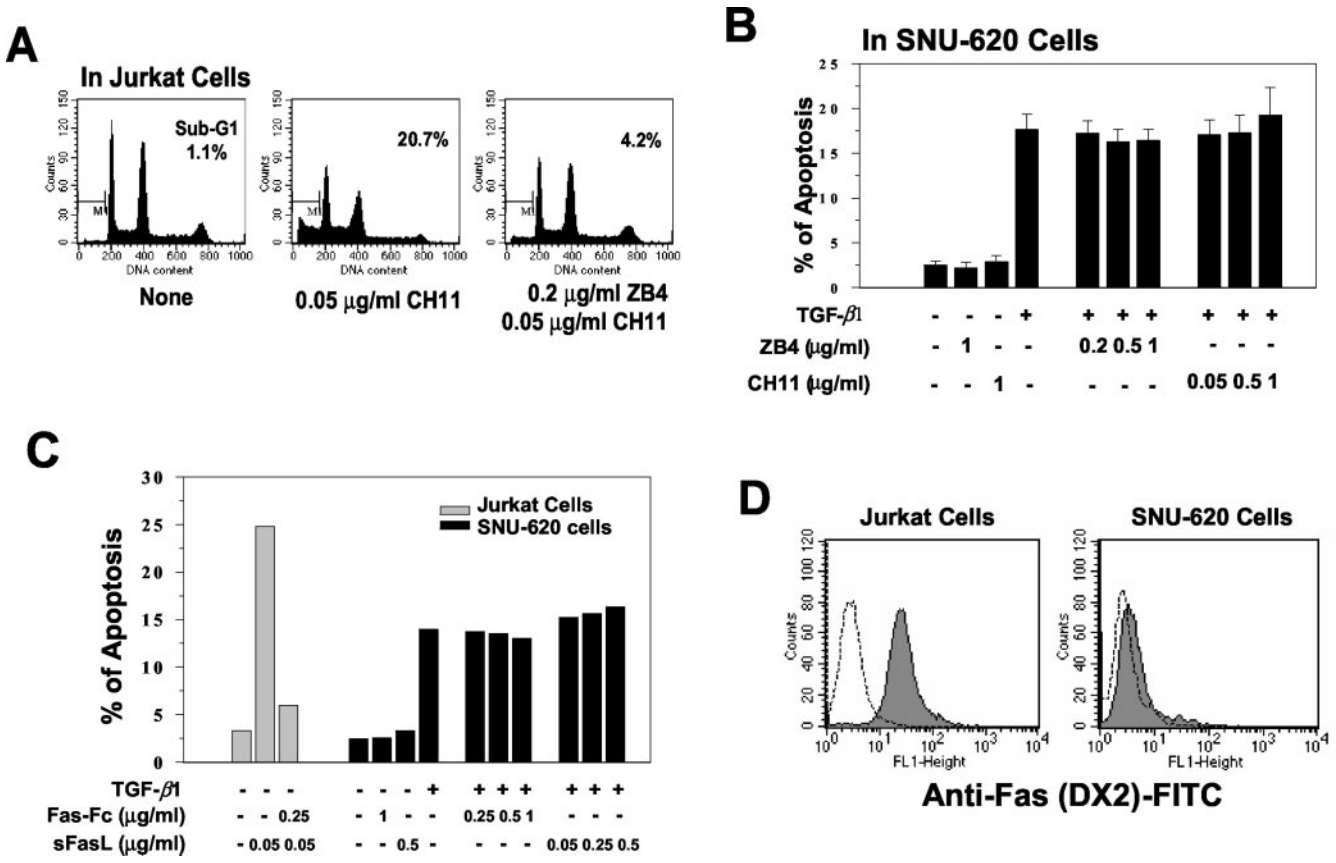

Figure 5. Fas–FasL interaction-independent apoptosis by TGF-β1 in SNU-620 cells. (A) The apoptosis-inducing or blocking effect of CH11 and ZB4 anti-Fas antibody on Jurkat cells. Jurkat cells were stimulated with CH11 antibody (0.05 μg/ml) in the absence or in the presence of ZB4 antibody (0.2 μg/ml) for 36 h. Cells were then stained with PI, and the DNA contents were analyzed by flow cytometry. The percentages of cells with sub-G1 DNA content are indicated. (B) Effects of CH11 and ZB4 antibodies on TGF-β1–induced apoptosis of SNU-620 cells. Untreated (−) or TGF-β1–treated (+) cells were incubated for 36 h in the absence (−) or in the presence (+) of pretreated ZB4 or cotreated CH11 at the indicated concentrations. Apoptotic cell death was measured as described in A. Data are presented as mean ± S.D. of three separate experiments. (C) Effects of recombinant sFasL and Fas-Fc fusion chimera on TGF-β1–induced apoptosis of SNU-620 cells. To confirm the roles of sFasL and Fas-Fc chimera, Jurkat cells (shaded bars) were first tested with sFasL (0.05 μg/ml) in the absence (−) or in the presence (+) of Fas-Fc chimera (0.25 μg/ml) for 36 h as in A. SNU-620 cells (black bars) were also incubated as described in B in the absence (−) or in the presence (+) of cotreated sFasL or pretreated Fas-Fc chimera at the indicated concentrations. Apoptosis was then measured as shown in A. (D) The levels of cell surface Fas expression in Jurkat and SNU-620 cells. Jurkat or SNU-620 cells (1×10^6) were stained with FITC-conjugated anti-Fas-specific antibody, respectively. The cell surface expression of Fas was analyzed by flow cytometry as described in MATERIALS AND METHODS. Each unstained control was presented as a dotted line.

unpublished data), indicating that the FasL-independent activation of the Fas receptor is induced by TGF-β1 in SNU-620 cells.

Fas/FADD-dependent Apoptosis by TGF-β1

To address whether Fas receptor is actually involved in TGF-β1–induced apoptosis, we transiently transfected SNU-620 cells with construct encoding Fas full-length cDNA. The expression of transfected Fas receptor was confirmed by immunofluorescent staining and flow cytometric analysis by using anti-Fas monoclonal antibody (Figure 7A, left). In the absence of TGF-β1 stimuli, expression of Fas receptor itself did not trigger apoptosis in SNU-620 cells (Figure 7B). However, when TGF-β1 was treated for 36 h, TGF-β1–induced apoptosis was enhanced in cells expressing Fas (Figure 7B). In particular, this further increase of TGF-β1–induced apoptosis is well correlated with the increase of cell surface Fas receptor level (Figure 7A, right). These results suggest that, under the TGF-β1 stimuli, the level of Fas receptor is closely linked to the induction of apoptosis by TGF-β1, and also imply that TGF-β1–mediated induction of Fas receptor may be important to trigger apoptosis by TGF-β1 in SNU-620 cells.

Because activated Fas-mediated apoptotic responses involve the recruitment of FADD before the cleavage and activation of caspase-8 (Boldin *et al*., 1995; Boldin *et al*., 1996), we then examined the effects of a dominant negative form of FADD (DN-FADD) on TGF-β1–induced apoptosis by transiently cotransfecting constructs encoding DN-FADD and GFP. To assess the cell death induced by TGF-β1, the population expressing GFP was gated by flow cytometry, and the fraction of apoptotic cells labeled with PE-conjugated anti-active caspase-3 antibody was quantified. Transient transfection of DN-FADD and subsequent stimulation with TGF-β1 for 24 h showed that the TGF-β1–induced activation of caspase-3 was effectively suppressed dose dependently in DN-FADD–transfected cells versus the pcDNA3-transfected controls (Figure 7C). This inhibition was also observed in a time-dependent manner (Figure 7D), suggesting that FADD is necessary for TGF-β1–mediated caspase-3 activation and

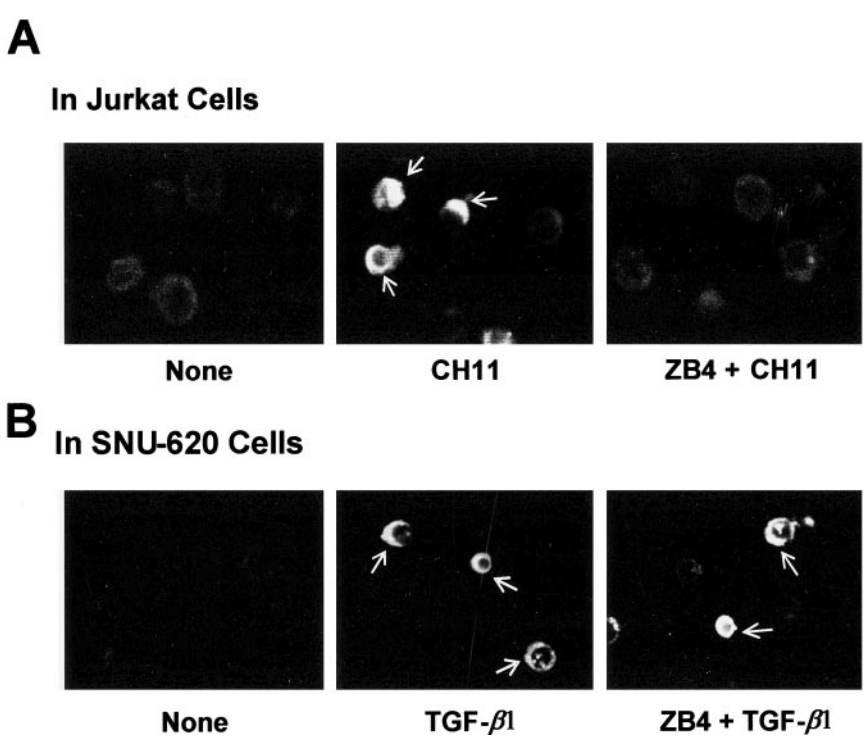

Figure 6. FasL-independent Fas receptor clustering in TGF-β1–treated SNU-620 cells. (A) Effects of CH11 and ZB4 antibody on Fas receptor clustering in Jurkat cells. Cells were left untreated or treated with CH11 (0.05 μg/ml) for 36 h in the absence or in the presence of ZB4 (0.2 μg/ml) and labeled with FITC-conjugated with anti-Fas antibody. Cells were then transferred to slide and analyzed by confocal immunofluorescence microscopy with a 40× objective as described in MATERIALS AND METHODS. (B) Fas receptor clustering by TGF-β1 in SNU-620 cells. After incubating cells for 48 h with or without TGF-β1 (5 ng/ml) in the absence or in the presence of ZB4 (1 μg/ml), cells were analyzed as described in A.

the subsequent induction of apoptosis. Therefore, it is likely that a FasL-independent, Fas/FADD-mediated apoptotic pathway plays a prominent role in the TGF-β1–induced apoptotic cell death of SNU-620 cells.

Anti-Apoptotic Role of Smad7 in the TGF-β1–induced Apoptosis of SNU-620 Cells

Smad7 is classified as an inhibitory Smad, because its interaction with the activated type I receptor prevents phosphorylation of the R-Smads (Heldin *et al*., 1997; Derynck *et al*., 1998; Massagué and Chen, 2000). However, several recent reports have emphasized the proapoptotic role of Smad7 in TGF-β1–induced apoptosis despite the generally known inhibitory function of Smad7 in TGF-β signaling (Landstrom *et al*., 2000; Lallemand *et al*., 2001; Schiffer *et al*., 2001; Edlund *et al*., 2003). To identify the role of Smad7 in our system, we infected SNU-620 cells with adenovirus carrying Smad7 cDNA. The expression of FLAG-tagged Smad7 was confirmed by Western blotting with anti-FLAG antibody (Figure 8A). Without TGF-β1 treatment, infection with adenovirus did not induce apoptotic cell death in SNU-620 cells (Figure 8B). When TGF-β1 was treated, apoptosis was efficiently blocked by the overexpression of Smad7 (Figure 8B). In addition, TGF-β1–mediated induction and activation of Fas receptor, a significant apoptosis-triggering event in our system, were completely suppressed in cells expressing Smad7 (Figure 8, C and D). Moreover, when the effect of Smad7 on the apoptotic events downstream of Fas induction by TGF-β1 were investigated, Smad7 significantly reduced Bid cleavage and caspase-3 activation (Figure 8E). These results suggest that the role of Smad7 is clearly antiapoptotic in SNU-620 cells and that the induction of Fas and the subsequent apoptotic events are restricted to TGF-β1–activated type I receptor, providing the possibility that R-Smads may be involved upstream of TGF-β1–mediated Fas induction and activation.

Smad3-dependent Apoptosis by TGF-β1 in SNU-620 Cells

Next, we assessed whether R-Smads are truly involved in TGF-β1–induced apoptosis. Because Smad2 and Smad3 among R-Smads are known to be restricted to TGF-β1 type I receptor and Smad4 functions as their common partner, we analyzed the role of these Smad proteins in the TGF-β1–induced apoptosis. For this purpose, we first overexpressed these Smad proteins by infecting SNU-620 cells with adenoviruses carrying their cDNAs. The expression of FLAG-tagged Smads was verified by anti-FALG Western blotting (Figure 9A) and in itself did not trigger apoptosis in SNU-620 cells (our unpublished data). When TGF-β1 was treated in cells expressing each Smad, TGF-β1–induced apoptosis was the most efficiently enhanced by the overexpression of Smad3 (Figure 9B). To clear the proapoptotic role of Smad3 in our systems, we then reduced the endogenous levels of Smads by using the siRNA technique in SNU-620 cells (Figure 9C). In relevant to the overexpression data, Smad3 knockdown significantly reduced TGF-β1–induced apoptosis, whereas Smad2 knockdown did not affect apoptosis induced by TGF-β1 (Figure 9D). These results indicate that TGF-β1 induces apoptosis in SNU-620 cells through a Smad3-dependent pathway.

In addition, we further examined whether Smad3 is indeed involved in upstream of TGF-β1–mediated induction of Fas receptor and the subsequent apoptotic events in our systems. As shown in Figure 10A, only Smad3, but not Smad2, knockdown markedly inhibited the induction of Fas by TGF-β1 and accordingly suppressed the downstream Bid cleavage and caspase-3 activation. These results strongly suggest that Smad3 play an essential role in upstream of TGF-β1–dependent Fas induction and the subsequent apoptotic events in SNU-620 cells. However, because TGF-β1–mediated induction of Fas receptor, a critical apoptotic event in SNU-620 cells, completely disappeared in the presence of cyclohexamide (Figure 10B), it seems that the de novo pro-

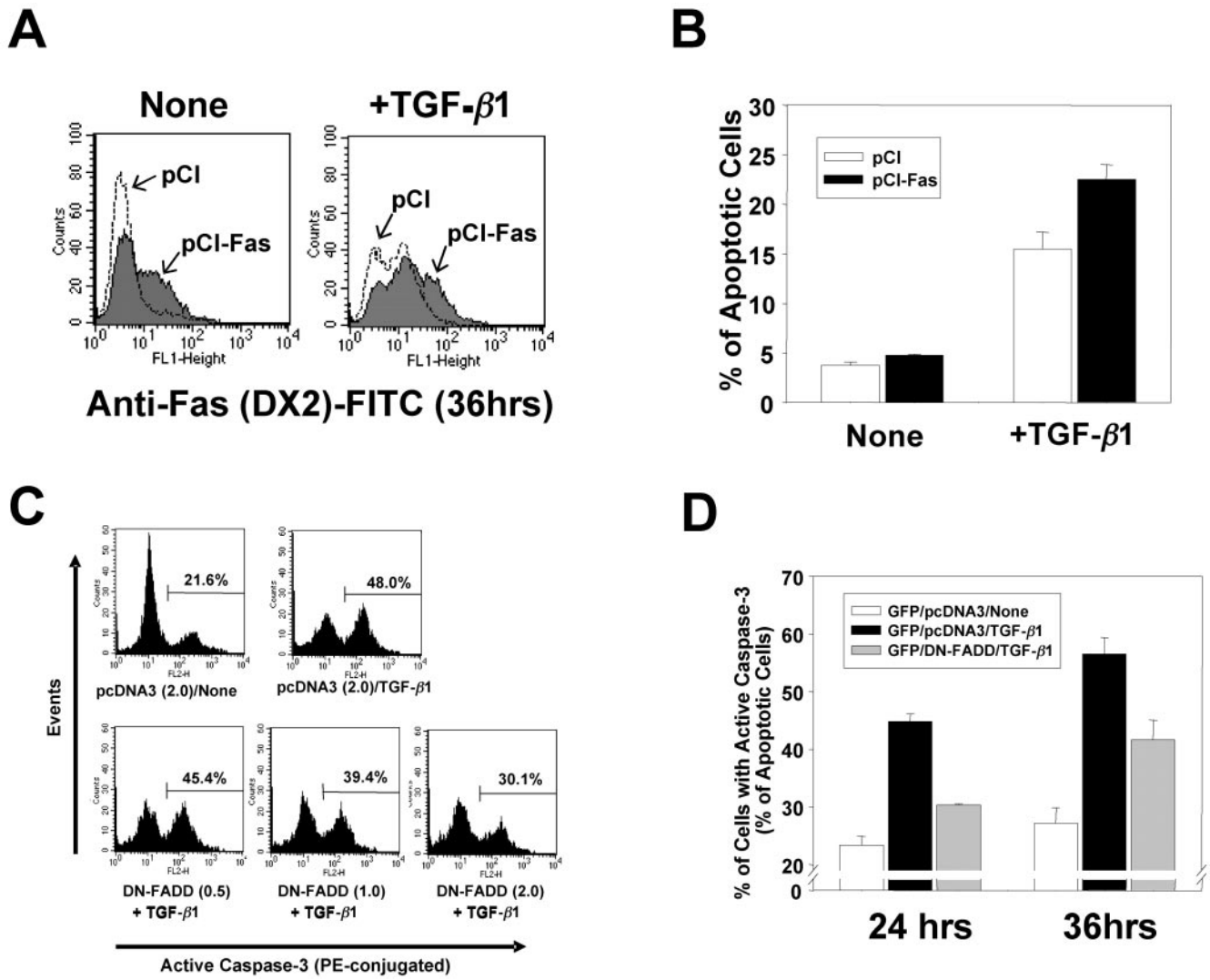

Figure 7. Effects of Fas or DN-FADD expression on the TGF-β1–induced apoptosis in SNU-620 cells. (A) Evaluation of the cell surface Fas expression in Fas-overexpressed cells. Cells were transiently transfected with 1 μg of pCI or pCI-Fas as described in MATERIALS AND METHODS. After incubating cells with or without TGF-β1 (5 ng/ml) for 36 h, pCI- or pCI-Fas–transfected cells were stained with FITC-conjugated anti-Fas-specific antibody, and the levels of cell surface Fas expression were determined as explained in Figure 4A. (B) Effect of Fas expression on TGF-β1–induced apoptosis. Cells were transfected with pCI or pCI-Fas and further incubated in the absence or in the presence of TGF-β1 (5 ng/ml) for 36 h as shown in A. Cells were then stained with PI, and the DNA contents were analyzed by flow cytometry. The percentages of cells with sub-G1 DNA content was measured and graphed. (C) Dose-dependent inhibition of TGF-β1–induced apoptosis by DN-FADD. SNU-620 cells were transiently transfected with pEGFP-N1 (0.5 μg) and either pcDNA3 (2 μg) or pcDNA3-DN-FADD construct (indicated). After incubating transfected cells with or without TGF-β1 (5 ng/ml) for 24 h, cells were fixed and labeled with PE-conjugated anti-active caspase-3 antibody as described in MATERIALS AND METHODS. Apoptotic cell death was quantified by sequentially gating cells with GFP and measuring the fraction of PE-positive cells among GFP-positive cells. The percentages of cells with active caspase-3 are indicated. (D) Time-dependent inhibition of TGF-β1–induced apoptosis by DN-FADD. Cells transiently transfected with pEGFP-N1 (0.5 μg) and either pcDNA3 (2 μg) or pcDNA3-DN-FADD (2 μg) were incubated with (black and shaded bars) or without TGF-β1 (open bars) for 24 or 36 h. Apoptotic cell death was then determined as described in C. Data is presented as means ± S.D. of three independent experiments.

tein synthesis is necessary for Smad3-dependent Fas induction by TGF-β1. This means that, even though Smad3 is principally responsible for the induction of Fas by TGF-β1, its implication in the Fas induction seems not to be the simple direct transcriptional activation of Fas but to be indirect or more complicated, such as involvement of the newly synthesized unknown third party, which notion is also relevant to our results that the time course of Fas induction by TGF-β1 is relatively delayed (Figure 4B) compared with that of $p21^{waf1}$ induction by TGF-β1 in SNU-620 cells. $p21^{waf1}$, a member of the Cdk inhibitors, is known to be one of the direct transcriptional targets of Smad pathway (Pardali *et al.*, 2000), and TGF-β1 started to up-regulate the $p21^{waf1}$ gene expression within 3 h in our systems (our unpublished data).

DISCUSSION

In this study, we focused on characterizing the TGF-β1–induced apoptotic pathway in human gastric SNU-620

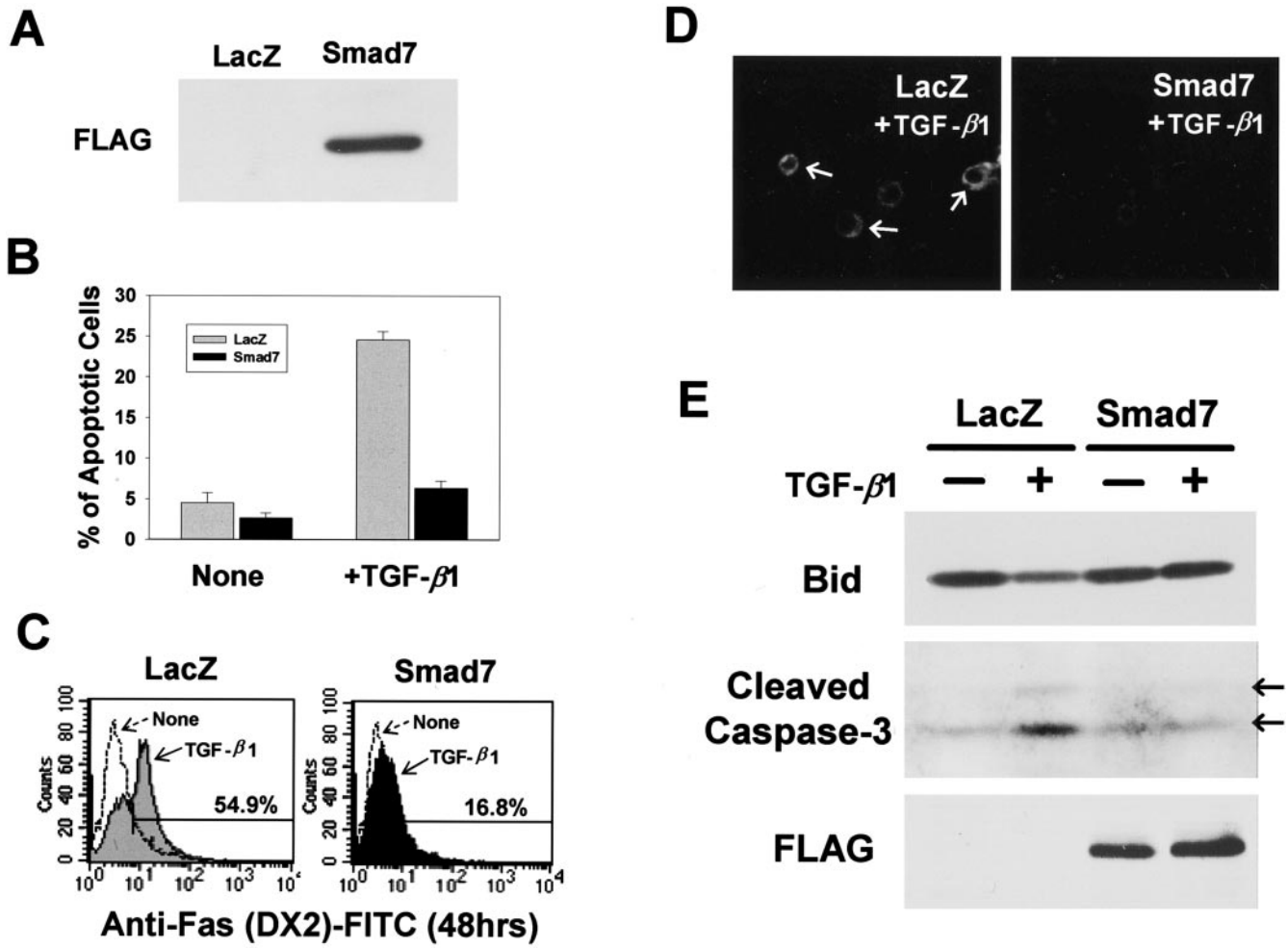

Figure 8. Overexpression of Smad7 antagonizes the TGF-β1–induced apoptosis of SNU-620 cells. (A) Expression of Smad7. SNU-620 cells were infected with adenoviruses carrying β-galactosidase or Smad7 at an MOI of 50 for 24 h. The expression of Smad7 was confirmed by anti-FLAG immunoblotting. (B) Effect of Smad7 on TGF-β1–induced apoptosis. Cells were infected with adenoviruses as shown in A and further incubated in the absence or in the presence of TGF-β1 (5 ng/ml) for 36 h. Apoptotic cell death was measured by TUNEL assay with flow cytometry, and the percentage of TUNEL-positive cells was graphed. Data is presented as the means ± S.D. of three separate experiments. (C) Effect of Smad7 on the cell surface expression of Fas. Cells were infected with adenovirus carrying β-galactosidase or Smad7 for 24 h and then treated with TGF-β1 (5 ng/ml) for 48 h. The cell surface expression of Fas was determined as explained in Figure 4A. (D) Effect of Smad7 on TGF-β1–induced Fas clustering. Cells were infected and cultured as described in C. Fas receptor clustering was then analyzed as explained in Figure 6. (E) Ef-fect of Smad7 on Bid cleavage and caspase-3 activation. Cells were infected with adenoviruses and further incubated as described in B. Cell extracts were prepared and the levels of Bid and cleaved caspase-3 were determined by Western blotting with their specific antibodies.

carcinoma cells and found that the FasL-independent activation of Fas receptor and the subsequent activation of the mitochondrial pathway via Bid mediation is the prominent TGF-β1–induced apoptotic pathway in SNU-620 cells. We also found that the Smad3 pathway plays a major role in upstream of TGF-β1–induced Fas induction and activation. Several lines of evidence support this conclusion. First, TGF-β1 induced the expression of Fas receptor in parallel with apoptosis induction. Second, Fas receptor was clustered for activation by a single treatment with TGF-β1, even in the presence of neutralizing ZB4 anti-Fas antibody or the antagonistic Fas-Fc chimera. Third, the loss of $\Delta\Psi_m$, release of cytochrome *c* into the cytosol, and the activation of caspase-9 and downstream effector caspases were triggered by the caspase-8–mediated cleavage of Bid. Fourth, the expression of Fas sensitizes apoptosis by TGF-β1, whereas DN-FADD overexpression and treatment with each specific caspase inhibitor all effectively blocked TGF-β1–induced apoptosis. Finally, all the apoptotic events induced by TGF-β1 were efficiently inhibited by Smad3 knockdown. These results suggest that TGF-β1 induces apoptosis in SNU-620 cells through a TGF-β1–activated Smad3 pathway and the subsequent ligand-independent Fas receptor apoptotic pathway linked to the mitochondrial pathway.

Until recently, and although TGF-β1 has been shown to activate caspase-8 during apoptosis in hepatoma cells and B cells (Shima *et al*., 1999; Schrantz *et al*., 2001), no link between TGF-β1–induced activation of caspase-8 and the death receptor pathway has been observed. Alternatively, one recent report provided evidence for a caspase-8 activation mechanism, in that TGF-β1–mediated p38 MAPK activation is implicated in caspase-8 cleavage and in the apoptosis of Burkitt's lymphoma BL41 cells (Schrantz *et al*., 2001). Nev-

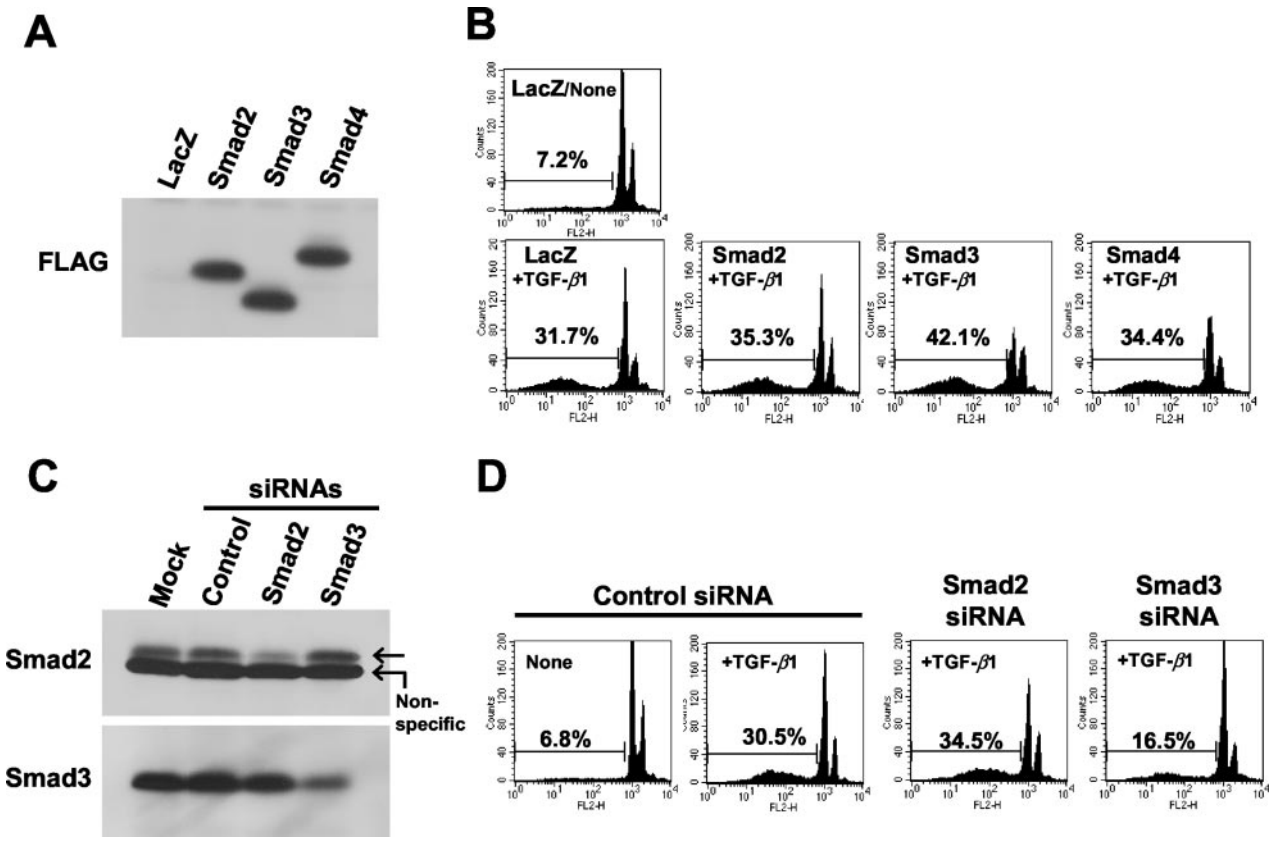

Figure 9. Apoptosis induced by TGF-β1 is dependent on Smad3. (A) Expression of Smads. SNU-620 cells were infected with adenoviruses carrying β-galactosidase, Smad2, Smad3, or Smad4 at an MOI of 50 for 24 h. The expression of Smads was confirmed by anti-FLAG immunoblotting. (B) Effects of Smads on TGF-β1–induced apoptosis. Cells were infected with adenoviruses as shown in A and further incubated in the absence or in the presence of TGF-β1 (5 ng/ml) for 48 h. Adenovirus carrying β-galactosidase (MOI of 50) was used as a control. Data is a representative of three independent experiments. (C) siRNA-mediated depletion of Smad2 or Smad3. Endogenous Smad2 or Smad3 was reduced in SNU-620 cells by using siRNA technique as described in MATERIALS AND METHODS. The depletion of each Smad was confirmed by Western blotting with their specific antibodies. (D) Effects of Smads knockdown on TGF-β1–induced apoptosis. Cells were transfected with nonsilencing control, Smad2 or Smad3 siRNA as described in A and further incubated in the absence or in the presence of TGF-β1 (5 ng/ml) for 48 h. Data are representative of four separate experiments.

ertheless, because caspase-8 activation is known to be principally associated with apoptosis mediated by members of the death receptor family, including Fas receptor (Wallach *et al*., 1999), it remains feasible that caspase-8 can be activated through the death receptor system in TGF-β1–induced apoptosis. Consistent with this notion, our study showed that Fas receptor, a prototypical member of the death receptor family, is induced and activated in concert with caspase-8

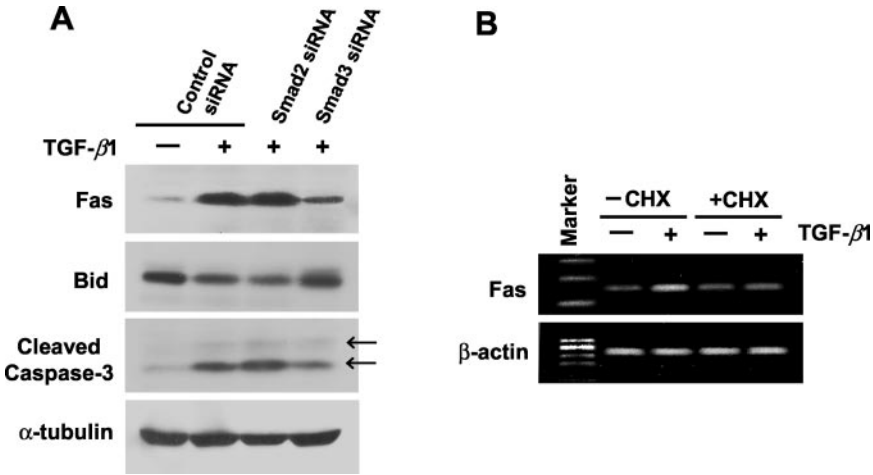

Figure 10. Smad3-dependent apoptotic events by TGF-β1 in SNU-620 cells. (A) Effects of Smads knockdown on the apoptotic events induced by TGF-β1. SNU-620 cells were transfected with each indicated siRNA as described in Figure 9C and further incubated in the absence (−) or in the presence (+) of TGF-β1 (5 ng/ml) for 24 h. Cell extracts were prepared and the levels of Fas, Bid, cleaved caspase-3, and α-tubulin were determined by Western blotting with their specific antibodies. (B) Effect of cycloheximide on the induction of Fas mRNA by TGF-β1. Total RNA was purified from untreated (−) or 5 ng/ml TGF-β1–treated (+) cells for 24 h in the absence (−) or in the presence (+) of cycloheximide (5 μg/ml), and the levels of Fas and β-actin mRNA were determined by RT-PCR as described in MATERIALS AND METHODS.

cleavage and the induction of apoptosis by TGF-β1 in SNU-620 cells. Most significantly, the expression of DN-FADD, which blocks caspase-8 activation due to the lack of death effector domain (Chinnaiyan *et al*., 1995), effectively inhibited caspase-3 activation downstream of caspase-8 and blocked the apoptosis in SNU-620 cells. Thus, the present study suggests that the TGF-β1–mediated activation of the Fas/FADD pathway is implicated in the activation of caspase-8, although evidence for the involvement of a Fas–FasL interaction was not found.

Recent work by others has shown that Fas/FADD-dependent but FasL-independent cell death could be triggered by various apoptotic stimuli, including UV, cisplatin, cyclohexamide, and toxic bile salts, through Fas receptor clustering, leading to the recruitment of FADD and the subsequent activation of caspase-8 (Rehemtulla *et al*., 1997; Faubion *et al*., 1999; Micheau *et al*., 1999; Tang *et al*., 1999). These results are based on the observations that Fas receptor clustering is induced by distinct apoptotic stimuli independently of FasL, and that Fas clustering-induced apoptosis is suppressed by the expression of DN-FADD or antisense FADD. Similarly, in our system, TGF-β1 triggered Fas receptor clustering, as shown by microscopic analysis, and pretreatment with neutralizing ZB4 anti-Fas antibody or antagonistic Fas-Fc chimera did not prevent this clustering as well as TGF-β1–induced apoptosis. Moreover, TGF-β1–induced apoptosis further increased by overexpression of Fas and was efficiently inhibited by both overexpression of DN-FADD and the pretreatment with the specific caspase-8 inhibitor zIETD-fmk. Therefore, it seems that TGF-β1–induced apoptosis might also involve the FasL-independent, more precisely the Fas–FasL interaction-independent, clustering of Fas receptor, which leads to the recruitment of FADD and the subsequent activation of caspase-8 in SNU-620 cells.

Originally, Fas/TNF receptors were thought to exist in monomer status before ligand binding and to be activated by receptor homotrimerization upon ligand binding (Suda *et al*., 1993; Boldin *et al*., 1996; Wallach *et al*., 1999). However, a recent growing body of evidence suggests a new model for Fas/TNF receptor activation. Native Fas/TNF receptors are believed to be closely preassociated as a homotrimer on the resting cell surface membrane. Ligand binding to preassociated receptors may then allow the formation of receptor superclusters, leading to receptor activation (Papoff *et al*., 1999; Chan *et al*., 2000; Siegel *et al*., 2000). This notion is wholly consistent with the current observation that the engagement of Jurkat cells by agonistic CH11 anti-Fas antibody led to a visible aggregation of Fas receptor under the microscope. A similar aggregation of Fas receptor was also observed in TGF-β1–treated SNU-620 cells, suggesting that the Fas receptor may already be trimerized in resting SNU-620 cells and that superclustering is triggered by TGF-β1–mediated stimuli through an unknown ligand-independent mechanism. In particular, TGF-β1–mediated induction of cell surface Fas receptor may be favorable for TGF-β1 stimuli to cause preassociated receptors to form superclusters, which is supported by our results that overexpression of Fas receptor by transient transfection enhanced TGF-β1–induced apoptosis. However, the induction of Fas receptor by TGF-β1 seems to be important, but not the only key step for the subsequent superclustering and activation of Fas receptor, because overexpression of Fas receptor itself did not induce apoptosis in SNU-620 cells. These imply that there is another step induced by TGF-β1, beside the increase of cell surface Fas by TGF-β1, at which TGF-β1 plays a pivotal role in activating the Fas receptor death pathway, FasL independently. Thus, the exact cellular process linking TGF-β1–mediated stimuli and FasL-independent Fas clustering needs to be further examined.

Smad proteins are well known to be key components in TGF-β signaling as TGF-β family receptor substrates capable of transducing signals (Heldin *et al*., 1997; Derynck *et al*., 1998; Massagué and Chen, 2000). Although various observations indicate that Smad proteins are involved in TGF-β1–induced apoptosis (Yanagisawa *et al*., 1998; Yamamura *et al*., 2000), their roles in apoptosis are still ambiguous and controversial. Previous reports have pointed out a distinct Smad each other to emphasize their roles in TGF-β1–induced apoptosis (Yanagisawa *et al*., 1998; Dai *et al*., 1999; Yamamura *et al*., 2000; Kim *et al*., 2002). Moreover, the proapoptotic or antiapoptotic roles of Smad7 have been suggested by other groups (Patil *et al*., 2000; Lallemand *et al*., 2001; Edlund *et al*., 2003). In this study, we identified that the Smad pathway, principally Smad3, is also involved in TGF-β1–induced apoptosis, by acting as an upstream enhancer of TGF-β1–stimulated Fas induction in SNU-620 cells. The present study shows that TGF-β1–induced apoptosis of SNU-620 cells is enhanced by Smad3 overexpression and that this apoptosis is effectively blocked by Smad3 knockdown. In addition, Smad3 knockdown potently suppressed Fas induction and the subsequent apoptotic events induced by TGF-β1, of which events were also completely inhibited by Smad7 overexpression. Accordingly, these indicate the proapoptotic role of Smad3 and the antiapoptotic role of Smad7 in our system.

Together, we suggest that TGF-β1 induces apoptotic cell death in SNU-620 cells through a TGF-β1–activated Smad3-dependent and subsequently an FasL-independent Fas/FADD pathway, which later links with the mitochondrial apoptotic pathway via Bid mediation

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