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TGF-β Inhibits Fas-Mediated Apoptosis of a Follicular Dendritic Cell Line by Down-Regulating the Expression of Fas and Caspase-8: Counteracting Role of TGF-β on TNF Sensitization of Fas-Mediated Apoptosis[1]

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Follicular dendritic cells (FDC) constitute the framework of germinal center (GC) in secondary lymphoid follicles, and the integrity of FDC networks is critically affected by cytokines present in the GC. We have previously shown that TNF promotes Fas-mediated apoptosis of HK cells, an established FDC-like cell line, by up-regulating Fas expression. However, in the developing GC, FDC death is not a hallmark of GC despite the presence of TNF and FasL. In this study, we report that TGF-β inhibits Fas-mediated apoptosis of HK cells by down-regulating the expression of surface Fas and caspase-8. The inhibitory effect of TGF-β can be observed when HK cells were simultaneously treated with TNF and TGF-β, indicating that TGF-β counteracts the effect of TNF in sensitizing cells to Fas-mediated apoptosis. Furthermore, the deprivation of TGF-β by injecting neutralizing TGF-β Abs to the SRBC-immunized mice resulted in the sporadic appearance of FDC undergoing apoptosis in the lymphoid follicles, suggesting that TGF-β functions as a naturally occurring inhibitor that rescues FDCs which are predisposed to apoptosis. Our study documents a novel function of TGF-β in the maintenance of FDC networks. *The Journal of Immunology,* 2005, 174: 6169–6175.

F ollicular dendritic cells (FDC)[3] are found in primary lymphoid follicles, and upon Ag stimulation they mature from FDC precursors and constitute the framework of germinal centers (GC) in secondary lymphoid tissues. Establishment of proper FDC networks is essential for the GC response which is characterized by proliferation and differentiation of thymus-dependent Ag-activated B cells to memory cells or plasma cells. Within the GC, FDCs expand and differentiate forming network structures in the lymphoid follicles (1–3). So far, it is not clear whether FDC network expansion accompanies FDC proliferation or rather it simply represents the occupation of differentiated FDCs, which have a characteristic of elongated cytoplasmic extensions, in the area where massive B cell proliferation occurs. Likewise, the studies published to date do not clearly point out whether FDCs die and if they do, whether it occurs as a consequence of either normal cell turnover or apoptosis caused by a death-inducing factor that exists in the GC microenvironment.

Recently, we have shown that HK cells, an established FDC-like line, proliferate in response to TNF and exhibit a sustained NF-κB activation due to persistent TNF signaling. More importantly, TNF-induced NF-κB activation contributes to the inducible expression of Fas (CD95), thereby rendering the cells more susceptible to Fas-mediated apoptosis (4). This increased susceptibility of Fas killing in the FDC line by TNF seems paradoxical in view of the important role of TNF in the establishment of FDC networks which presumably depends on its mitogenic action on putative FDC precursors (5–8). In addition, FDCs do not seem to undergo apoptosis in developing GC, although FasL was suggested to be expressed in the GC (9–12). Thus, we hypothesized that there exists a factor(s) in the GC environment that counteracts the TNF action and inhibits Fas activation on FDCs. A possible candidate playing such role is the cytokine TGF-β. FDCs in the light zone of the GC have been shown to express TGF-β (13) and TGF-βRII (14). In addition, TGF-β is known as a potent modulator of Fas-mediated apoptosis in various cell types (15–17). In this study, we investigated the effect of TGF-β on Fas-mediated apoptosis of HK cells. Our results indicate that TGF-β counteracts the effect of TNF on HK cell apoptosis by decreasing surface Fas expression as well as intracellular caspase-8 level. The administration of neutralizing anti-TGF-β mAb to immunized mice resulted in a significant increase in FDC undergoing apoptosis in the lymphoid follicles of spleen, suggesting a protective role of TGF-β against FDC apoptosis that can be caused by Fas activation in vivo.

Materials and Methods

Cell cultures and reagents

Cell culture media were purchased from Invitrogen Life Technologies. An established FDC-like line (HK cells) was obtained from Dr. Y. S. Choi (Alton Ochsner Medical Foundation, New Orleans, LA) (18) and grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HK cells with passages 13–17 were used for various experiments. Recombinant soluble FasL (sFasL) used in this study was prepared from culture supernatant of

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[3] Abbreviations used in this paper: FDC, follicular dendritic cell; GC, germinal center; sFasL, soluble Fas ligand; LT-α, lymphotoxin-α; CHX, cycloheximide; cFLIP, cellular FLIP.

stable cell line CHO-K1-sFasL cells that was grown in serum-free medium (CHO-S-SFM II; Invitrogen Life Technologies). The biological activity of sFasL has been previously described (4, 19). In experiments, cells were treated with the sFasL supernatant to the final concentration of 10%. Recombinant human TNF was obtained from the Biotech Research Institute (LG Life Sciences). Human TGF-β1, IL-4, GM-CSF, IFN-γ, and lymphotoxin (LT)-α were purchased from R&D Systems. Cycloheximide (CHX) was from Calbiochem. Rabbit polyclonal Fas (C-29) Ab and monoclonal Bcl-2 Ab were purchased from Santa Cruz Biotechnology. The polyclonal anti-caspase-8 and cellular FLIP (cFLIP) Abs were purchased from BD Pharmingen. Hybridoma-producing TGF-β neutralizing mAb (1D11.16.8) was purchased from the American Type Culture Collection and injected i.p. to nude mice pretreated with Pristane. Ab was purified from ascites using a protein G-agarose column. Bound IgG was eluted with 50 mM glycine-HCl (pH 2.5). Collected fractions were dialyzed against PBS and then sterilized by filtration. The *Limulus* amebocyte lysate assay demonstrated the absence of significant endotoxin contamination in the Ab solution (data not shown).

Western blot analysis

After stimulation, cells were washed with cold PBS, scraped, and resuspended in lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 3 mM benzamidine, 1 mM PMSF, and 100 mM sodium fluoride. Cell lysates were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were electrophoresed through 10% SDS-PAGE. Proteins were transferred to nitrocellucose membrane (Bio-Rad). The blots were blocked in TBST supplemented with 5% skim milk for 1 h, followed by incubation with various primary Abs for 1 h and then with 1/5000 diluted secondary Abs of HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were treated with ECL reagents (Amersham Pharmacia Biotech) and detected by autoradiography.

Measurement of apoptosis (nuclear staining with Hoechst 33258)

HK cells plated in 12-well plates were untreated or pretreated with 20 ng/ml TNF, 10 ng/ml TGF-β, or both for 24–48 h before the sFasL stimuli. Cells given the death-inducing stimuli were then fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 50 ng/ml Hoechst 33258 (Sigma-Aldrich). For quantitation of apoptosis (presented as average ± SEM), cells were scored as apoptotic based on morphological criteria and counted. A minimum of 250 cells was counted for each condition.

Measurement of caspase activity

HK cells treated with sFasL with or without 2 μg/ml CHX for the indicated time periods were lysed in buffer containing 50 mM Tris (pH7.0), 2 mM EDTA, and 1% Triton X-100. Cell lysates were obtained after centrifugation at 15,000 rpm. A total of 10 μg of the cell lysates were incubated with 25 μM caspase substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC; Calbiochem) for 1 h at 37°C in the presence of caspase reaction buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 5 mM DTT, and 0.1% CHAPS. Proteolysis of the fluorescent peptides was measured with excitation at 400 nm and emission at 505 nm using a fluorescence spectrophotometer.

Flow cytometry

HK cells cultured in 100-mm dishes were given stimulation of TNF, TGF-β, or both cytokines for 48 h. Cells were trypsinized, washed with PBS, incubated with 2 μg/ml FITC-conjugated anti-human mouse monoclonal Fas Ab (clone DX2; BD Pharmingen) for 1 h at 4°C, and washed twice with PBS. The cells were then analyzed on the FACSCalibur (BD Biosciences). The expression of surface Fas was calculated as the mean fluorescence intensity with the CellQuest program (BD Biosciences). Negative control cells were incubated with isotype-matched Ab.

TGF-β mAb treatment and immunohistochemistry for apoptotic FDCs

C57BL/6 mice were immunized by injecting 5×10^8 washed SRBC (Medilab Korea) i.p. After 4 wk, secondary immunization was performed by injecting the same number of SRBC. Mice were injected with 1 mg of anti-TGF-β Ab (1D11.16.8) or control Ab, 48 h after the second immunization (n = 3 for each group). Spleens were procured 6 days after the second immunization. For identification of apoptotic cell clusters, frozen splenic sections were stained by the In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer's instructions. In brief, sections were fixed in 2% paraformaldehyde for 20 min followed by incubation with TUNEL reaction mixture. Sections were washed and then

visualized using diaminobenzidine as a color substrate. Serial TUNEL-stained sections were incubated with FDC-M1 (BD Pharmingen), FDC-M2 (Dr. M. Kosco, NovImmune, Geneva, Switzerland), or anti-CD19 Ab (BD Pharmingen) and then with biotinylated anti-rat IgG (Jackson ImmunoResearch Laboratories) for 30 min at 37°C. Subsequently, they were incubated with AP-conjugated streptavidin (BD Pharmingen). Labeled cells were visualized using Fast Red solution (DAKO). Multiple sections were prepared from each mouse, and GCs were identified by FDC-M1 or FDC-M2 Ab. All values were expressed as means ± SEM. The Student *t* test was used to compare the differences between the groups. Values of $p < 0.05$ were regarded as statistically significant.

Results

TGF-β inhibits Fas-mediated apoptosis of HK cells

An established FDC-like line, HK cells are susceptible to Fas killing and their susceptibility is enhanced by TNF treatment (4). To further investigate GC cytokines that can regulate Fas-mediated HK cell death, we tested the effects of IFN-γ, GM-CSF, IL-4, and TGF-β on this apoptotic process. FDCs express cytokine receptors for GM-CSF, IL-4 (13, 14), and cultured FDCs have been reported to be responsive to IFN-γ (20). Because it is well-established that like TNF, LT-α plays an important role in the formation and maintenance of FDC networks (21, 22), we also tested the effect of LT-α on Fas killing of HK cells. Cells were preincubated with cytokines for 24 h, followed by the treatment with sFasL in the presence or absence of CHX. After 18 h, cells were fixed and stained with Hoescht dye for counting apoptotic cells. Fig. 1*A*
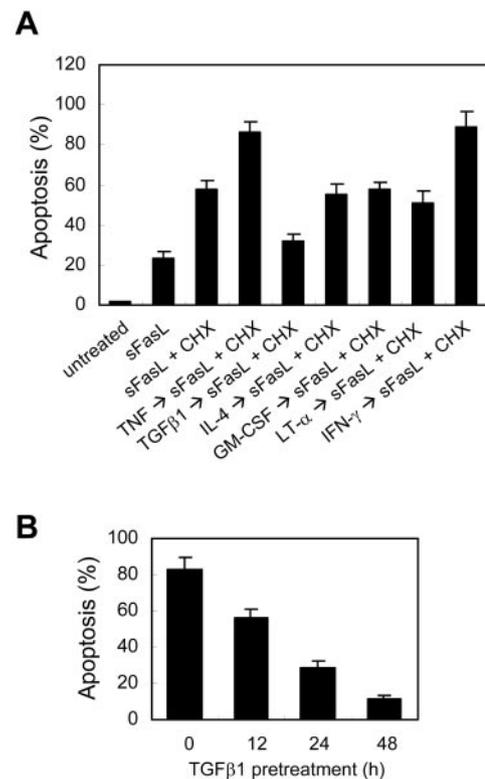

FIGURE 1. TGF-β inhibits Fas-mediated apoptosis in HK cells. *A*, Effect of various cytokines on Fas-mediated apoptosis of HK cells. HK cells pretreated with TNF (20 ng/ml), TGF-β (10 ng/ml), IL-4 (20 ng/ml), GM-CSF (50 ng/ml), LT-α (10 ng/ml), or IFN-γ (100 U/ml) for 24 h were washed twice with PBS, and prepared for treatment with sFasL plus CHX (2 μg/ml) in fresh complete medium. After 18 h exposure of cells to the death-inducing stimuli, cells were fixed, stained with Hoescht dye, and counted for apoptosis. The data are representative of five independent experiments. *B*, HK cells pretreated with TGF-β for the indicated times were treated with sFasL plus CHX (2 μg/ml). After 18 h, cells were stained with Hoescht dye and counted.
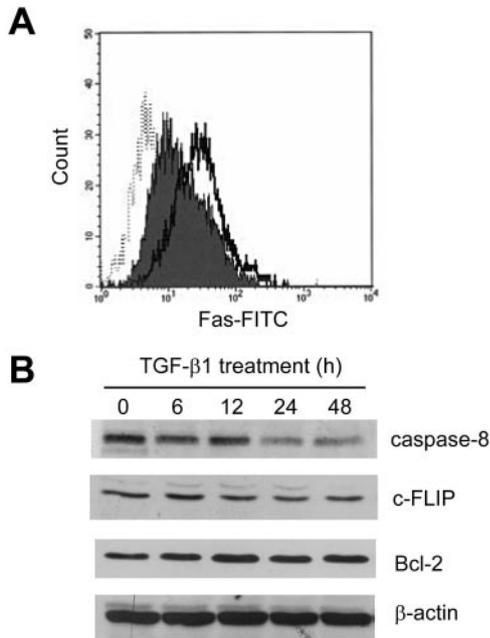
shows that sFasL alone induced 23% of apoptosis in unprimed HK cells, and together with CHX, induced 58% apoptosis. TNF-pretreated cells displayed 86% of apoptosis upon subsequent exposure of sFasL plus CHX, however, in TGF-β-pretreated cells the percentage of apoptotic cells was greatly reduced to 25%. IL-4, GM-CSF, and LT-α had little effect on Fas-mediated HK cell death, while IFN-γ enhanced it. Therefore, TNF and IFN-γ sensitize HK cells to Fas-mediated cell death whereas TGF-β inhibits it. An experiment examining the time required for TGF-β to render HK cells resistant to Fas-mediated apoptosis revealed that 48 h pretreatment of TGF-β almost completely inhibited apoptosis induced by cotreatment of sFasL and CHX (Fig. 1B).

TGF-β down-regulates the expression of surface Fas and caspase-8

In some cell types TGF-β inhibits Fas-mediated apoptosis by down-regulating Fas expression or by up-regulating cFLIP, a molecule known to regulate caspase-8 activation (16, 17). TGF-β was also shown to exhibit its antiapoptotic function by modulating Bcl-2 expression in certain cells (23). To understand the protective function of TGF-β in HK cells, we examined the expression of these proteins after treatment with TGF-β. Flow cytometric analysis for surface Fas expression showed that TGF-β dramatically reduced the basal expression of Fas after 48 h of treatment (Fig. 2A). In addition, Western blot analysis of caspase-8, cFLIP, and Bcl-2 showed that TGF-β treatment led to a significant decrease in caspase-8 expression and a slight, if any, increase in Bcl-2 expression, whereas the level of cFLIP expression remained unchanged (Fig. 2B). Thus, the strong inhibitory effect of TGF-β on Fas-mediated apoptosis of HK cells can be attributed to its ability to down-regulate the expression levels of both Fas and caspase-8.
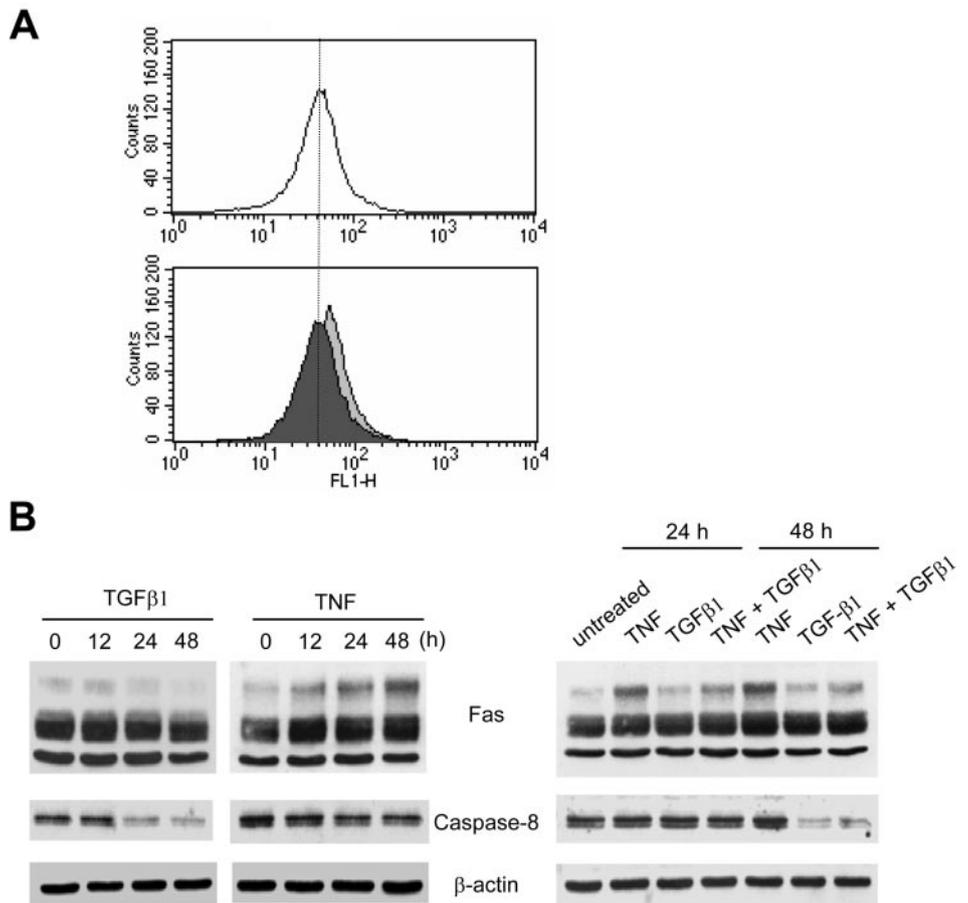

FIGURE 2. TGF-β down-regulates surface Fas expression and caspase-8 level in HK cells. *A*, HK cells were treated with 10 ng/ml TGF-β for 48 h. Cells were then trypsinized for analysis of surface Fas expression by immunofluorescence flow cytometry. Cells treated with TGF-β are shown in the shaded area and untreated cells in the black open line. Cells stained with isotype-matched control Ab are shown in the dotted line. *B*, Total cell lysates of HK cells treated with TGF-β for the indicated times were analyzed by Western blot for caspase-8, c-FLIP, and Bcl-2 expression. Western blot analysis of β-actin was done to show equal loading of protein lysates.

FIGURE 3. TGF-β abrogates the up-regulation of Fas by TNF in HK cells. *A*, HK cells were treated with 10 ng/ml TGF-β in the absence or presence of 20 ng/ml TNF. After 48 h, cells were analyzed for surface Fas expression using FACS. The *upper histogram* is obtained from untreated control. In the *bottom panel*, the histogram shaded with light gray was obtained from cells treated with TNF and the histogram shaded with dark gray was from cells cotreated with TNF and TGF-β. *B,* HK cells were treated with TNF, TGF-β, or both for the indicated periods. Western blot analysis of total cell lysates was performed to examine the level of Fas and caspase-8. Equal loading of protein samples was confirmed by examining β-actin level.

TGF-β counteracts the effect of TNF in sensitizing HK cells to Fas-mediated apoptosis

TNF and TGF-β exhibited opposing effects on Fas-mediated HK cell apoptosis by increasing or decreasing the level of surface Fas expression, respectively. We examined whether TGF-β is able to inhibit TNF-induced Fas up-regulation on HK cells when these two cytokines coexist. Fig. 3A shows that treatment of cells with TNF for 48 h led to the up-regulation of surface Fas, however, in cells exposed to both TNF and TGF-β, the level of surface Fas expression remained unchanged, exhibiting a similar expression level to that of the untreated control, indicating that TGF-β inhibits the up-regulation of Fas by TNF. Western blot analysis was performed to confirm the result of the flow cytometry data (Fig. 3B). Ab raised against the cytoplasmic domain of Fas detected three main forms of Fas. Among these, the increased level of the Fas species with high m.w. has been shown to correlate with the increased susceptibility to Fas-mediated cell death in HK cells (4) as well as thyroid follicular cells (24). TNF increased the level of this high m.w. form of Fas in a time-dependent manner, whereas TGF-β caused the gradual decrease. In HK cells that were given combined treatment of both cytokines, a modest counteracting effect of TGF-β on TNF up-regulation of Fas was observed after 24 h of treatment, which became prominent after 48 h. Western

FIGURE 4. TGF-β counteracts the TNF-sensitizing effect on Fas-mediated apoptosis in HK cells. *A*, HK cells untreated or pretreated with TNF, TGF-β, or both cytokines for 48 h were washed with PBS and given death stimulation of sFasL plus CHX. After 18 h, cells were stained with Hoescht dye and apoptotic cells were counted. Three separate experiments were done and their statistical results were expressed as the mean ± SEM. HK cells given the combined pretreatment of TNF and TGF-β exhibited a significant decrease in susceptibility to Fas-mediated apoptosis compared with the unprimed cells (∗, $p < 0.01$, ANOVA *t* test). *B*, HK cells untreated or pretreated with TNF, TGF-β or both cytokines for 48 h were stimulated with sFasL in the presence of CHX for the various time periods and processed for determining caspase-3 activity. Cell lysates were incubated with the fluorogenic caspase substrate Ac-DEVD-AFC and free AFC was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

blot analysis of caspase-8 using the same lysates revealed that TNF had no effect on caspase-8 level, whereas TGF-β decreased the caspase-8 level after 24 and 48 h of treatment. The effect of TGF-β in reducing the expression level of caspase-8 was not affected by the cotreatment with TNF.

Because the ability of TGF-β to down-regulate surface Fas expression overrode the ability of TNF to up-regulate this protein, we tested whether TGF-β counteracts the effect of TNF in sensitizing HK cells to Fas-mediated apoptosis. We pretreated HK cells with both TNF and TGF-β for 48 h, followed by treatment with sFasL and CHX. After 18 h, the percentage of cells undergoing apoptosis was compared with that of cells treated with each cytokine. As shown in Fig. 4A, in comparison to unprimed cells, cells pretreated with TNF alone showed increased Fas-mediated apoptosis (84 vs 46% apoptosis), whereas pretreatment with TGF-β alone caused total inhibition of cell death. As expected, the percentage of apoptotic cells that were exposed to both cytokines was significantly reduced to 33% compared with that of TNF-pretreated cells, indicating that TGF-β abrogated the sensitization effect of TNF on Fas-mediated apoptosis. To confirm this counteracting effect of TGF-β, we measured the activity of caspase-3, a distal caspase associated with Fas signaling, by using an in vitro fluorogenic substrate DEVD-AFC. The result showed that the time-dependent increase in caspase-3 activity of cells pretreated with both TNF and TGF-β was much reduced compared with TNF-pretreated cells (Fig. 4B).

Down-regulation of caspase-8 by TGF-β contributes to the protection from apoptosis induced by TNF and sFasL

Although HK cells given the combined pretreatment of TNF and TGF-β exhibited significant decrease in susceptibility to Fas-mediated apoptosis than unprimed cells, the level of surface Fas expression still remained at the level similar to that of unprimed control cells (Fig. 3A). This suggests that in addition to Fas down-regulation, the inhibitory effect of TGF-β can also be attributed to the down-regulation of caspase-8. To evaluate the functional significance of caspase-8 down-regulation by TGF-β on the apoptotic process, we analyzed whether TGF-β can inhibit HK cell apoptosis induced by TNF in the presence of CHX because TNF-mediated apoptosis also relies on caspase-8 activation. As shown in Fig. 5, the simultaneous treatment of TNF and CHX induced ∼20% apoptosis of unprimed HK cells. TNF pretreatment for 48 h caused
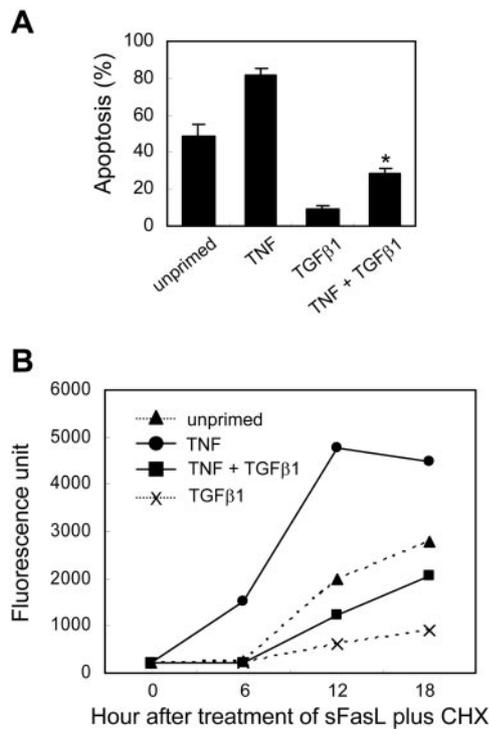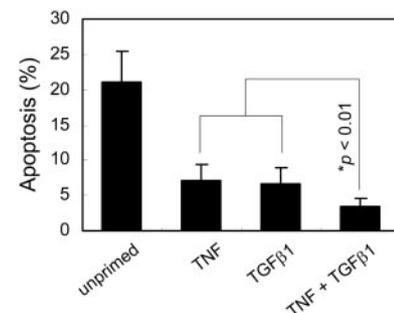

FIGURE 5. Combined treatment of TGF-β and TNF shows additive effect on the protection of HK cells from TNF-induced apoptosis. HK cells untreated or pretreated with TNF, TGF-β, or both cytokines for 48 h were washed with PBS and given death stimulation of TNF plus CHX. After 12 h, cells were stained with Hoescht dye and apoptotic cells were counted. Three separate experiments in triplicate were done and their statistical results were expressed as the mean ± SEM. HK cells given the combined pretreatment of TNF and TGF-β exhibited significant decrease in susceptibility to TNF-induced apoptosis compared with the cells pretreated either cytokine alone (∗, $p < 0.01$, ANOVA *t* test).
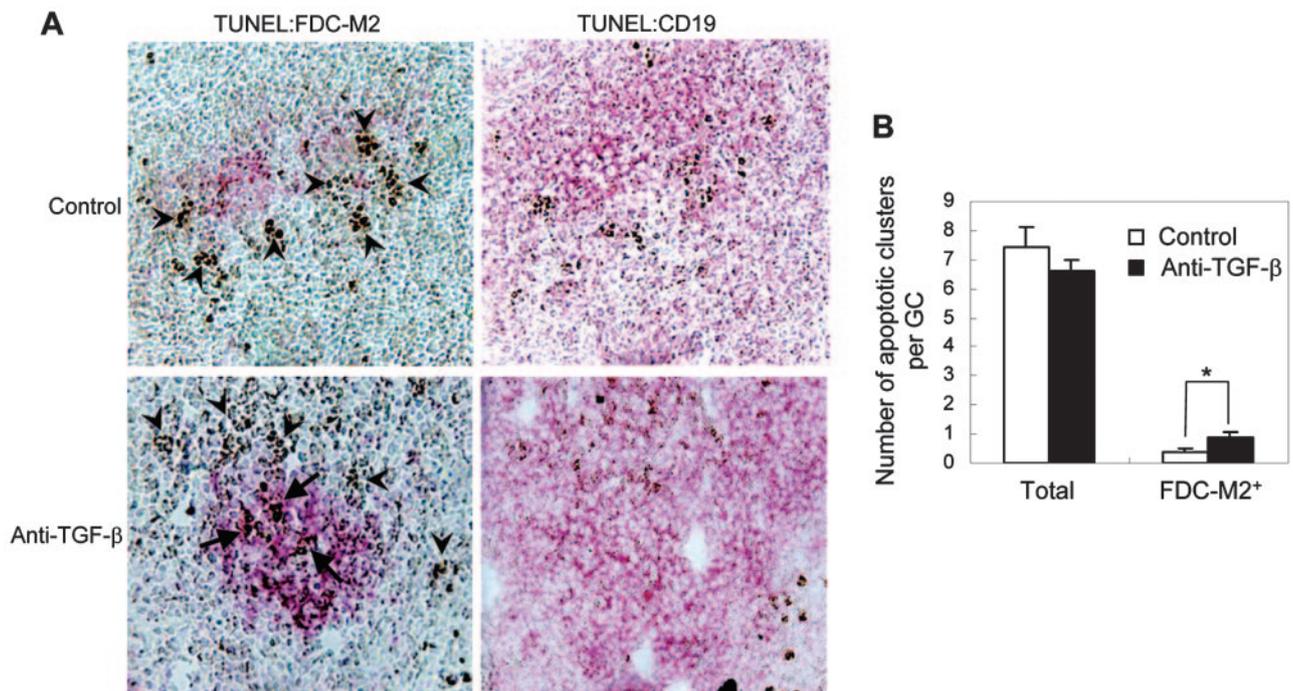
FIGURE 6. Administration of TGF-β neutralizing Ab results in enhanced FDC apoptosis in the GC of immunized mice. *A,* Adjacent splenic sections of mice treated with control or anti-TGF-β mAb were double-stained with TUNEL and FDC-M2 or anti-CD19 Ab. Arrowheads indicate TUNEL⁺FDC-M2⁻ cell clusters, whereas arrows indicate TUNEL⁺FDC-M2⁺ cell clusters. Staining of adjacent sections with anti-CD19 Ab shows that TUNEL⁺FDC-M2⁻ cells are B cells. Original magnification, ×640. *B,* The numbers of total apoptotic cell clusters and apoptotic FDC clusters in the GC were counted from immunohistochemistry slides of splenic tissue sections. The mean numbers of three independent experiments are presented. The anti-TGF-β-treated spleens exhibited more apoptotic FDCs than controls (∗, $p < 0.05$).

protection from apoptosis induced by subsequent cotreatment of TNF and CHX, decreasing apoptotic cells to 7–8%. A similar degree of protection was also observed in cells pretreated with TGF-β for 48 h. In HK cells, we have previously shown that the protective effect of TNF priming is through NF-κB-dependent induction of various antiapoptotic proteins (4). In contrast, the effect of TGF-β priming is through the down-regulation of caspase-8, as shown by the additive effect of TNF and TGF-β on the protection against TNF-induced apoptosis of HK cells, i.e., the combined pretreatment of both cytokines leading to complete protection from apoptosis induced by TNF and CHX (*lane 4,* Fig. 5).

Administration of a neutralizing anti-TGF-β Ab increases cell death of FDCs in the GC of immunized mice

Based on our in vitro data, it can be speculated that in the GC, TNF may render FDC susceptible to Fas activation upon encountering FasL. However, this Fas-mediated FDC death may not occur because of the presence of TGF-β. Thus, we envisioned that the blockade of TGF-β action in the developing GC of immunized mice would result in FDC death, which otherwise would not be easily observed in normal immunized mice. We administrated TGF-β neutralizing mAb (1D11.16.8) into mice after immunization with SRBC, and performed immunohistochemistry of the GC sections with FDC-specific Ab and B cell-specific Ab together with TUNEL staining to identify apoptotic cells (Fig. 6). Because it was technically difficult to count individual apoptotic cells, particularly FDCs with a complicated dendritic network, we enumerated the numbers of apoptotic cell clusters. Although FDC-M1 and FDC-M2 Abs gave rise to similar staining patterns, FDC-M2 Ab produced more discernible results in double staining with TUNEL reagents. The mean numbers of total apoptotic cell clusters in a given GC of the TGF-β mAb mice and control Ab-treated mice

were 6.62 ± 0.36 ($n = 214$) and 7.43 ± 0.71 ($n = 210$), respectively, showing no difference between the two groups ($p > 0.1$). However, a substantial number of apoptotic FDC clusters double-positive for FDC-M2 and TUNEL staining was observed in the GC sections of mice injected with anti-TGF-β mAb, while TUNEL-positive FDC clusters were less frequently detected in mice injected with control Ab, suggesting that some FDCs undergo apoptosis in the absence of a TGF-β signal. In the TGF-β mAb-treated mice, the mean number of apoptotic FDC clusters per GC was 0.89 ± 0.19, whereas in the control Ab-treated mice it was 0.34 ± 0.13, showing a significant increase of apoptotic FDC clusters but not GC B cell clusters in the TGF-β mAb-treated mice ($p < 0.05$).

Discussion

Our previous in vitro study using HK cells suggests that TNF may be involved in the sensitization of FDCs to Fas-mediated apoptosis. However, it is considered that FDCs participating actively in GC reaction may not die by apoptosis or are not turned over, because FDCs bear Ags for many months to years (25). Therefore, we initiated this study to find out why FDCs undergoing apoptosis have not been observed in the GC where the persistent presence of TNF and FasL is suggested. Our present in vitro and in vivo studies suggest that TGF-β, one of the cytokines expressed in the GC by both FDCs and lymphocytes, may function as an inhibitor of Fas-mediated apoptosis on FDCs.

We demonstrated that TGF-β exerts a strong antiapoptotic effect by inhibiting surface Fas as well as caspase-8 expression in HK cells. Importantly, TGF-β counteracts the potentiating effect of TNF on Fas-mediated apoptosis of this cell line. Previous studies have indicated that TGF-β has differential inhibitory mechanisms

on the apoptotic process depending on the cell type. TGF-β reduces surface Fas expression increased by TNF in murine microglial (16) and bone marrow progenitor cells (15). In contrast, TGF-β inhibits apoptosis of human T cells by down-regulating FasL without affecting Fas expression (26). Apart from the differential modulation of surface Fas, TGF-β acts at different stages in the Fas signaling cascade to inhibit the apoptosis process. For example, TGF-β is found to counteract the effect of TNF in sensitizing microglia to Fas-mediated apoptosis by up-regulating cFLIP (17) or by up-regulating Bcl-2 (23). In this regard, we examined the effect of TGF-β on the expression level of FLIP or Bcl-2, however, no apparent change was observed in HK cells. Rather, TGF-β down-regulated caspase-8 expression. Contribution of the down-regulated caspase-8 to the antiapoptotic effect of TGF-β was confirmed by the observation that TGF-β protected HK cells from apoptosis induced by TNF and CHX. In cells in which TGF-β induces apoptosis, TGF-β has been shown to activate caspase-8 (27, 28). In contrast, cells in which TGF-β plays an antiapoptotic role appear to respond by down-regulating caspase-8. In fact, a recent report has shown that in human dermal fibroblasts, TGF-β inhibits Fas-mediated apoptosis partly by decreasing the caspase-8 level (29). It has been shown by our previous study that the reduced caspase-8 expression is responsible for diverting the Fas-mediated apoptotic signal to NF-κB activation in serum-starved human fibroblasts (19). This result, together with the data presented here, suggests that caspase-8 is a target for controlling the Fas-mediated apoptosis pathway.

TGF-β is a strong immunosuppressive cytokine. Studies on the role of TGF-β during GC responses have largely been focused on B cell growth and differentiation. Experiments using human B cell lines, which are phenotypically similar to GC centroblasts, suggest that TGF-β plays a role in both limiting proliferation and stimulating apoptosis of GC B cells (30, 31). It has been reported that TGF-β is expressed in lymphoid follicles by FDCs (13). Primary selection of GC B cells is dependent on signals provided by FDCs. Ags trapped on FDCs provide an effective survival signal to B cells, and TGF-β is known to interrupt this signal, representing a regulatory mechanism for preventing the selection of B cells with a low-affinity BCR (30). However, the effect of TGF-β that is associated with the function of FDCs has not been studied, albeit FDCs express its receptor, TGF-βRII (14). Our in vitro results raise the possibility that TGF-β functions as a naturally occurring inhibitor that rescues FDCs which may be predisposed to apoptosis. The evidence provided in this study supports this hypothesis. In vivo administration of TGF-β neutralizing Ab during Ag exposure resulted in a significant increase in FDCs which were double-stained with FDC-M2 and TUNEL in the GC. In contrast, TUNEL-positive FDCs were hardly detectable in mice injected with the control Ab, suggesting that at least some species of FDC undergo apoptosis in the absence of the TGF-β signal. One question is the source of TGF-β in the murine GC because unlike the human system, the expression of TGF-β has not been demonstrated in murine GC and FDC so far.

In conclusion, our present study demonstrated that TGF-β provides a protective signal to FDCs, which may contribute to the maintenance of intact and functional FDC networks in developing GC. Many in vivo studies have shown that the FDC network degenerates in the resolution stage of normal GC responses or in certain pathological conditions (32–38). Whether the decrease or loss of the active form of TGF-β in local environment of involuting follicles may contribute to the degeneration of the FDC network is a subject of open question. Long-term analysis of TGF-β expression in the secondary lymphoid follicles after induction of GC response, together with detailed experiments studying the ki-

netics, dose response, and duration of the TGF-β neutralizing Ab treatment, will help understand how and when FDCs undergo apoptosis.

Disclosures

The authors have no financial conflict of interest.

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